IDENTIFICATION OF MOTILIN AND GHRELIN AND THEIR ROLES IN REGULATION OF GASTROINTESTINAL MOTILITY IN THE PHEASANT

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CONTENT

ABBREVIATIONS

ACh	Acetylcholine
cDNA	Complementary DNA
C-terminal	COOH-terminal
DNA	Deoxyribonucleic acid
EC_{50}	Concentration for 50% of maximal effect
EM	Erythromycin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GHRP	Growth hormone releasing peptide
GHS	Growth hormone secretagogue
GHS-R1a	Growth hormone secretagogue receptor 1a
GHS-R1b	Growth hormone secretagogue receptor 1b
GI	Gastrointestinal
GOAT	Ghrelin O-acyl-transferase
GPCR	G protein-coupled receptor
GPR38	G protein-coupled receptor 38
Da	Dalton
MAP	Motilin-associated peptide
MLNR	Motilin receptor
MMC	Migrating motor complex
mRNA	Messenger RNA
NPY	Neuropeptide Y
NS	Not significant
N-terminal	NH_2-terminal
PCR	Polymerase chain reaction
Peptide YY	Peptide tyrosine tyrosine
qPCR	Quantitative polymerase chain reaction
RACE	Rapid amplification of cDNA end
ROC	Rhythmic oscillating complex

SEM	Standard error of the mean
TTX	Tetrodotoxin

CHAPTER 1.
INTRODUCTION

1.1. Gastrointestinal motility

Food intake, digestion of foods and absorption of nutrients through the gastrointestinal (GI) wall are fundamental physiological events for living vertebrates. The GI system is the gateway for food entry, and it is well known that GI motility positively influences feeding behavior and contributes to the regulation of energy homeostasis. In general, GI motility in vertebrates is regulated by smooth muscle contractility that is controlled by pacemaker cells, extrinsic parasympathetic and sympathetic neurons, intrinsic enteric sensory and motor neurons located in the submucosal and myenteric plexuses, and some GI hormones [31,141,178]. Hormones are transduction molecules that travel in the bloodstream to transmit biological signals from endocrine cells to target cells. The actions of hormones are mediated by the activation of specific receptors. Many GI hormones including secretin, peptide tyrosine tyrosine (peptide YY), neurotensin, gastrin, gastrin-releasing peptide (GRP), cholecystokinin (CCK), somatostatin, ghrelin (GHRL) and motilin have been identified. GI hormones are produced in specialized gut endocrine or enteroendocrine cells of the GI epithelium, and they act on other digestive organs, associated cells and vagal nerve afferent terminals. The GI tract has functions for control of energy homeostasis through nutrient absorption and excretion, and several gut hormones play functional roles in the regulation of food intake, secretion of other hormones and control of GI motility [10,68,128,163,186].

At the beginning of the 20th century, Boldyreff *et al.* found a pattern of four periodic contraction activities of stomach and small intestine in fasted dogs, with the activity phase lasting about 20 min and the quiet phase lasting about 80 min [12]. This periodic activity was also reported in human [20]. Gastric activity was then divided into four phases: phase I is a quiescent phase with no contraction, phase II is a phase with irregular contractions, phase III, which is the most characteristic of the periodic contraction pattern, is a strong phase regular contractions with the highest frequency, and phase IV is the period of contractions that gradually disappear toward phase I. When the activities (electric or mechanical) of the stomach, duodenum and jejunum were measured simultaneously, phase III activity in the gastric regions migrated towards the small intestine, a phenomenon that is called migrating

motor complex (MMC) and is observed in the interdigestive periods [27]. The MMC in the stomach and upper intestine can be interrupted by eating behavior and motility changes into digestive motility consisting of irregular successive phasic contractions (digestive contractions). The duration of the disrupted MMC was reported that depends on the composition of a meal [39].

The MMC has been thought to flush out and clean up the GI lumen mechanically and chemically to make it ready for receiving the next food and to prevent bacterial overgrowth in the intestinal lumen. Therefore, the MMC is thought to be a housekeeper of the fasting GI tract [68,69,143,170,183]. Recently, it was also proposed that the MMC in the GI tract signals hunger sensation from the periphery to the brain through vagus afferent neurons in humans [167]. Since the MMC plays an important role in pushing food forward, causal relationships between the MMC and GI motility disorders have been discussed. In humans, the absence or weakness of phase III activity has been reported in a variety of GI diseases such as small intestinal bacterial overgrowth, irritable bowel syndrome, functional dyspepsia, and intestinal neuropathy as well as disorders of food intake, obesity, anorexia nervosa and even aging [2,21,38,66,159,182,183].

1.2. Motilin

Brown *et al*. (1971) isolated a polypeptide that stimulates gastric fundic contractility through the purification of a side fraction of porcine intestinal secretin. This polypeptide was named "motilin" because of the original observation that it increased motor activity in gastric pouches [19].

Since 1990, the genes encoding motilin precursors in humans, pigs and rabbits have been isolated. The nucleotide sequences indicate that the 22-amino-acid motilin peptide is synthesized as a precursor of over 100 amino acids. The translated motilin precursor consists of three parts from the N-terminal region: the first part is a hydrophobic signal peptide, the second part represents mature motilin peptide, and the third part represents motilin-associated peptide (MAP) with an unknown function [7,13,157]. In humans, the motilin gene is located in the p21.3 region of chromosome 6 (6p21.3) [56]. The motilin gene spans about 9 kb and motilin mRNA is 0.7 kb encoded by five exons. As shown in Figure 1, exon 1 encodes the 5`-untranslated region (UTR) of the motilin mRNA, exon 2 and exon 3 encode the signal

peptide and the 22-amino-acid motilin peptide, exon 3 and 4 encode the MAP and exon 5 encodes the 3`-UTR [30,157].

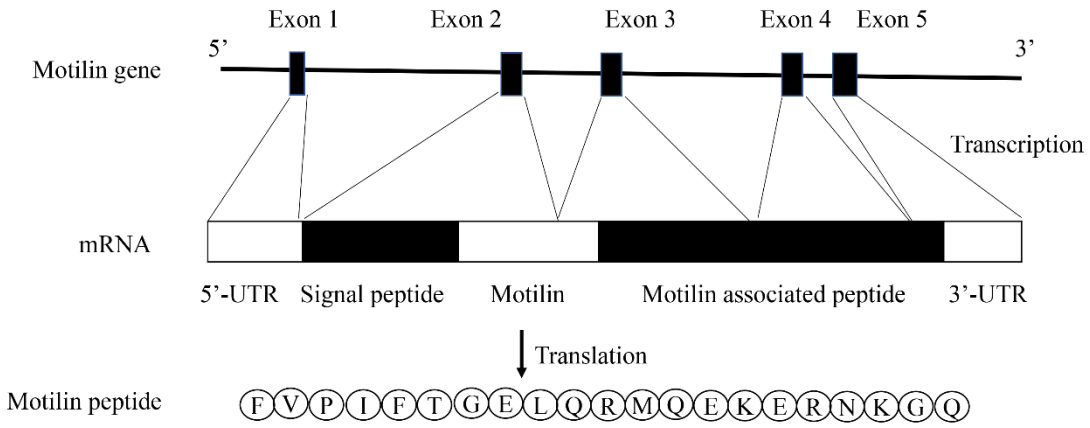

Figure 1. Human motilin gene, the mRNA encoding motilin and mature motilin peptide.

The amino acid sequence of porcine motilin was reported by Brown in 1973. It consists of 22 amino acids with a molecular weight of 2,700 Da [18]. The next year, the sequence was revised with the residue at position 14 being identified as glutamine rather than glutamic acid [154]. It was reported that synthesized motilin shows the same biological activity as that of the purified intestinal substance [113]. Binding and contractility studies with motilin fragments indicated that the N-terminal fragment of 14 amino acid residues amino acids is nearly equipotent as motilin. The N-terminal portion (1-7) constitutes the minimal basic unit of binding and biological activity, while the alpha-helix configuration formed by the C-terminal region (10-22) stabilizes the interaction of N-terminal residues at the active site. The remaining N-terminal region (8-9) is responsible for the binding of N-terminal and C-terminal regions [110]. Motilin has been identified in many species of mammals except for rodents [83]. The N-terminal sequence (FVPIFTHSEL) is relatively conserved in mammals.

Due to the fact that motilin was first discovered as a GI motility-stimulating peptide hormone released from the duodenum, there have been many functional studies on the GI motor-stimulating actions of motilin, especially in mammals. Generally, there are two experimental procedures for investigating the actions of motilin on GI motility. One is an *in vivo* experiment using anesthetized or conscious animals with GI motility being measured as changes in intraluminal pressure, muscle contractility or myoelectric activity. In such

experiments, extrinsic and intrinsic neural networks of the GI tract are intact, and the afferent to efferent autonomic nervous reflex pathways are also active. The other experimental procedure is an *in vitro* study using isolated GI strips obtained from various regions of the GI tract that is isolated from extrinsic innervation connecting to the central nervous system but with enteric neurons in the myenteric and submucosal plexuses being intact, and it is possible to stimulate these neurons electrically. An experiment using isolated dispersed cells is a modification of the above-described *in vitro* experiment in which it is possible to direct actions of bioactive substances on smooth muscle cells. The results from *in vivo* and *in vitro* experiments have their own characteristics. *In vivo* experiments enable investigation of the physiological actions of motilin including direct action (activation of smooth muscles and enteric neurons) and indirect actions (activation of autonomic nerve-mediated reflex and central nervous system) on the GI tract. On the other hand, *in vitro* experiments enable investigation of the local actions of motilin on smooth muscle cells and enteric neurons. It is necessary to examine and to compare the actions of motilin on GI motility in the same species using both *in vivo* and *in vitro* experiments. However, the sizes of experimental animals and different responses to motilin depending on the experimental conditions make it difficult to carry out both *in vivo* and *in vitro* experiments in the same animals.

Motilin is now thought to be a mediator of phase III of the gastric MMC, at least in humans, dogs and house shrew (Suncus). Evidences supporting this notion are as follows. First, motilin applied intravenously caused phase III contractions in dogs, humans and house shrew [70,183]. A developed motilin receptor agonist (erythromycin) also induced phase III contractions in humans [167] and dogs [71]. Second, it was shown that plasma motilin concentration is synchronized with MMC activity, being highest at the beginning of phase III and lowest at phase I [99]. This secretion pattern was also observed in humans and pigs [129,137]. Third, the phase III contraction pattern that was observed in the antrum and duodenum in fasting dogs was abolished by administration of anti-motilin serum that neutralizes endogenous motilin, although it had no effect on phase III contractions of the jejunum and ileum [98,131]. MA2029, a motilin receptor antagonist, was also shown to be effective for decreasing the phase III activity in dogs and house shrew [115,125]. These observations indicate the involvement of motilin in the regulation of phase III of the MMC in humans, dogs and house shrew.

Motilin has also been identified in non-mammalian vertebrates including birds, reptiles and fish [83] (Table 1). The amino acid sequence of chicken motilin is different from that of human motilin in 6 amino acids at positions 4, 7, 8, 9, 10, and 12. Turkey motilin and quail motilin have also been identified and they have the same amino acids sequence as that of chicken motilin except for position 19. In reptiles, the motilin peptide structure in snakes and turtles is different from that in mammals and birds, while alligator motilin has a structure similar to that of mammalian/avian motilin starting with phenylalanine (F). There is no information about motilin in amphibians. Fish also have motilin peptide possessing quite a different amino acid structure from that in other vertebrates (Table 1).

Table 1. Amino acid sequences of mature motilin in vertebrates.

Species	Sequence of amino acids	Size	Accession No.
Human	**FVPIFTYGELQRM--QEKERNK-GQ**	**(22)**	**NP_002409**
Rhesus monkey	FVPIFTYGELQRM--QEKERSK-GQ	(22)	NP_001027979
Cattle	FVPIETYGEVRRM--QEKERYK-GQ	(22)	NP_776363
Horse	FVPIFTYSELQRM--QERERNR-GH	(22)	NC_009163
Rabbit	FVPIFTYSELQRM--QEKERNR-GA	(22)	X63860
Cat	FVPIFTHSELQRI--REKERNK-GQ	(22)	NC_018727
Dog	FVPIFTHSELQKI--REKERNK-GQ	(22)	XP_005627282
House shrew	FMPIFTYGELQKM--QEKEQNK-GQ	(22)	AB325968
Chicken	**FVPFFTQSDIQKM--QEKERNK-GQ**	**(22)**	**NP_001292058**
Turkey	FVPFFTQSDIQKM--QEKERIK-GQ	(22)	XP_010722636
Japanese quail	FVPFFTQSDFQKM--QEKERNK-GQ	(22)	BAU80773
Zebra finch	FMPFFTQSDFQKM--QEKERNKAGA	(23)	XP_004175023
North Island brown kiwi	FLPFFTQSDFRKM--QEKERNK-GQ	(22)	XP_013812966
Band-tailed pigeon	FVPFFTQSDFRKMQLQEKERNKAGQ	(25)	OPJ88883
Chinese alligator	**FLPIFTHSDMQRM--QDRERNK-GQ**	**(22)**	**XP_006025154**
Three-toed box turtle	YLAFFTRSDIERMQLQEKQRNK-AQ	(24)	XP_024054930
Common garter snake	YLAFYSREDFRRM--QEKEKNQ-AQ	(22)	XP_013912065
Western painted turtle	YLAFFTRSDIERM--QEKQRNK-AQ	(22)	XP_005309417
Central bearded dragon	YTALYSWEDFRRM--QERERNQ-AQ	(22)	XP_020650577
Green anole	YTAFFTREDFRKM--QENEKNK-AQ	(22)	XP_008107992
Spotted green pufferfish	**HITFFSPKEMMVLK-QEQEG-----**	**(19)**	**ALD51563**
Three-spined stickleback	HITFFSPKEMMLMK--EREG-----	(18)	ALD51564
Turbot	HITFFSPKEMMLMK--EREG-----	(18)	AWP03197
Japanese medaka	HITFFSPKELLHMRLQEQQE-----	(20)	XP_023810781
Zebrafish	HIAFFSPKEMRELR--EKEG-----	(18)	XP_002665930
Coelacanth	FISFFSPSDMRRM--MEKEKSKAL-	(22)	XP_005995529
Spotted gar	FLSFISPSDMRRM--MEQEKVKAG-	(22)	NC_023181

Amino acid sequences were obtained from the DDBJ/EMBL/GenBank databases. Data were collected by Kitazawa and Kaiya [83].

In 1984, the ultrastructure of motilin-producing enteroendocrine cells, called M cells, was elucidated. The ultrastructure is characterized by relatively small, solid granules with a homogeneous core and a closely applied membrane that is round in humans and round to irregularly shaped in dogs [181]. Immunopositive motilin cells were mainly detected in the digestive tract with high concentrations in the mucosa of the duodenum and the upper jejunum. Beyond the GI tract, motilin mRNA has been found in the pancreas, gallbladder, adrenal gland, kidney and central nervous system including the cortex, cerebellum, hippocampus, amygdala and hypothalamus in mammals as well as in the human brain, thyroid and bone marrow [43,48,52,96].

Besides the key role of motilin in gastric phase III contraction, motilin is also able to influence enzyme secretion in the stomach and pancreas and contraction of the gallbladder and the sphincter of Oddi [72,95,106,169,173]. These phenomena are thought to be highly correlated with GI activity, which can serve as a GI housekeeper through mechanical and chemical cleaning [26,166,183]. Motilin might function in the central nervous system because motilin and its receptor have been detected in the cerebellum and cerebellum Purkinje cells [42,120]. For example, it has been demonstrated that motilin plays a role in the nitric oxide pathway in hippocampal neurons [105]. Motilin seems to be able to control energy metabolism because its administration stimulates lipogenesis and uptake of fatty acids and glucose and promotes energy storage [23,109,149]. Therefore, the cyclic motilin peptide, which is a stabilized form of motilin, is thought to not only participate in gastric phase III contraction but also contribute to other physiological activities [40].

1.3. Motilin receptor

G protein-coupled receptors (GPCRs) for growth hormone secretagogues (GHSs) were identified in human genomic DNA libraries. Among the clones, GPR38 was found to share overall amino acid sequence identity with 52% and 86% for the transmembrane region to the GHS receptor (later named the ghrelin receptor) [64,108]. At that time, GPR38 was classified as an orphan receptor due to the unknown ligand. In 1999, motilin and erythromycin were shown to be the ligand for GPR38, and GPR38 was named the motilin receptor (MLNR) [52,92].

GPR38 (MLNR) is coupled with Gq and phospholipase C signal pathway producing inositol-trisphosphate and diacylglycerol (DAG) [52]. An increase in intercellular Ca^{2+} concentration from intracellular and extracellular origins causes contraction of smooth muscles and regulation of cell membrane ionic channels [45,65,103]. The MLNR has been identified in almost all parts of the GI tract in many species of vertebrates from fish to mammals [123,164,172,188]. The exception is for rodents. It was shown that the MLNR exists as pseudogenes in mice and rats [62,151]. This evolutionary event of loss of the motilin system was speculated to be a result of changes in the function of upper GI tract such as loss of the emetic reflex inducing motilin and MLNR pseudogenes [151].

1.4. Ghrelin

In 1980, Bowers and colleagues reported synthetic opioid peptide derivatives, named of GH-releasing peptides (GHRPs), that induced growth hormone (GH)-release from the anterior pituitary [14,114]. These GHRPs were found to act on GH-releasing pituitary cells [114]. In 1996, the GH secretagogue receptor 1a (GHS-R1a) was cloned as the binding site of GHRPs and GHSs [64]. In 1999, Kojima *et al.* identified an endogenous ligand for GHS-R1a from extract of the rat stomach. This ligand, which is a 28-amino-acid peptide, was named ghrelin. The name originated from "ghre", which is the Proto-Indo-European root of the word "grow" [92].

Ghrelin is encoded by preproghrelin, which also contains a 23-amino-acid peptide obestatin. The human ghrelin gene is located on chromosome 3p25.3 and consists of six exons. The first and part of second exons encode the 5'-UTR. Two transcriptional initiation sites were found in the ghrelin gene [79], resulting in two distinct mRNA transcripts (transcript-A and transcript-B). Transcript-A was found to be mainly responsible for producing ghrelin. Exon 2, 3, 4 and 5 translate the immature ghrelin (preproghrelin) as shown in Figure 2. Preproghrelin undergoes post-translational modification to produce the 28-amino-acid ghrelin peptide. After des-Gln14-ghrelin was identified in the rat stomach extract, it was clarified that two types of ghrelin are translated depending on whether there is an mRNA codon at position 14 for Gln including the ghrelin precursor and des-Gln14-ghrlein precursor, which are exactly the same except for the absence or presence of Gln14 [63,79]. The deletion of Gln14 in des-Gln14-ghrelin is due to the usage of a CAG codon to encode Gln, which results in its

recognition as a splicing signal. Thus, two types of active ghrelin peptide are produced in the rat stomach: ghrelin and des-Gln14-ghrelin [94]. Recently, a C-terminally truncated form of the ghrelin peptide was discovered, named minighrelin, which was encoded by exon 2-deleted variant in humans and mice [156].

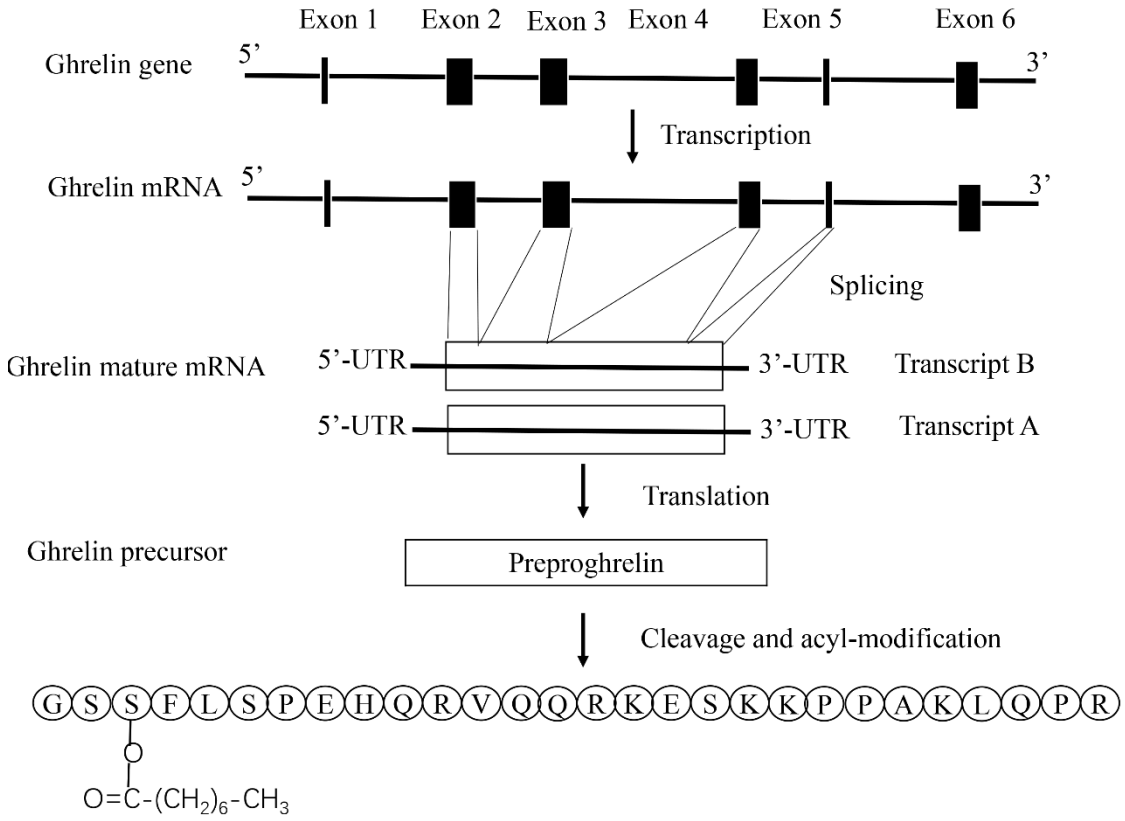

Figure 2. Human ghrelin gene and the mRNA encoding ghrelin. Based on asia.ensembl.org (Transcript ID: ENST00000335542.13).

As shown in Table 2, ghrelin has been identified in almost all vertebrates. The amino acid sequences of ghrelin are well conserved with 10 amino acids beginning from the N-terminal region at positions 13, 15, 16, 17, 19, 20, 21, 24, 25, 27 and 28 being identical in humans, rhesus monkeys, mice, rats, dogs, pigs, sheep and cows (Table 2). The amino acids sequences of ghrelin in avian species such as the chicken, duck, emu, goose, turkey and quail are also well conserved with the N-terminal 7 amino acids being completely identical. Similarity of the N-terminal sequence is also observed in reptiles, amphibians and fish.

Table 2. Amino acid sequences of mature ghrelin in vertebrates.

Species	Sequence of amino acids	Size	Accession No.
Human	**GSSFLSP-EHQ-RVQQRKES-KK-PPAKLQPR---**	**(28)**	**AB029434**
Pig	GSSFLSP-EHQ-KVQQRKES-KK-PAAKLKPR---	(28)	AY028942
Dog	FSSFLSP-EHQ-KLQQRKES-KK-PPAKLQPR---	(28)	AB060700
Golden hamster	GSSFLSP-EHQ-KAQQRKES-KK-PQAKLQPR---	(28)	ACF93721
Rat/mouse	GSSFLSP-EHQ-KAQQRKES-KK-PPAKLQPR---	(28)	NM_021669
Guinea pig	GASFRSP-EHH-SAQQRKES-RK-LPAKIQPR---	(28)	BBB05297
Cow	GSSFLSP-EHQ-KLQ-RKEA-KK-PSGRLKPR---	(27)	NC_037349
Cat	GSSFLSP-EHQ-KVQ-RKES-KK-PPAKLQPR---	(27)	AB089201
Rabbit	GSSFLSP-EHQ-KAQ-RKDA-KK-PPARLQPR---	(27)	XM_002722463
House shrew	GSSFLSP-EHQ-KGP-KKDP-RK-PP-KLQPR---	(26)	AB364508
Opposum	GSSFLSP-EHP-KTQ-RKET-KK-PSVKLQPR---	(27)	XM_001375640
Chicken	**GSSFLSP-TYK-NIQQQKDT-RK-PTARLH-----**	**(26)**	**AB075215**
Japanese quail	GSSFLSP-AYK-NIQQQKNT--RKPAARLH-----	(26)	BAE54265
Turkey	GSSFLSP-AYK-NIQQQKDT-RK-PTARLHPR---	(28)	XM_003210209
Goose	GSSFLSP-EFK-KIQQQNDP-AK-ATAKIH-----	(26)	AY338465
Duck	GSSFLSP-EFK-KIQQQNDP-TK-TTAKIH-----	(26)	EF613551
Emu	GSSFLSP-DYK-KIQQRKDP-RK-PTTKLH-----	(26)	AY338467
Green anole	**GSSFLSP-EQP-KMQQRKVS-QK-SVTKFH-----**	**(26)**	**NW_003338820**
Red-eared slider	GSSFLSP-EYQ-NTQQRKDP-KK-HT-KLN-----	(25)	AB161457
Bullfrog	**GLTFLSPADMQ-KIAER-QS--Q---NKLRHGNMN**	**(28)**	**AB058510**
Tropical clawed frog	GTSFLSP-A-D--LQ--KSSVKR-PPRKLQH-NEH	(27)	NC_030680
Zebrafish	**GTSFLSP-T-Q-KPQGR-----R--PPRVG-----**	**(20)**	**NM_001083872**
Goldfish	GTSFLSP-A-Q-KPQGR-----T--PPRMG-----	(20)	AF454389
Japanese eel	GSSFLSP-S-Q-RPQGK-D---K-KPPRVG-----	(22)	AB062427
Japanese catfish	GSSFLSP-T-Q-KPQNRGD---R-KPPRVG-----	(23)	AB196449
Rainbow trout	GSSFLSP-S-Q-KPQVRQG---KGKPPRVG-----	(24)	AB096919
European seabass	GSSFLSP-S-Q-KPQSR-G---K--SSRVG-----	(21)	DQ665912
Mozambique tilapia	GSSFLSP-S-Q-KPQNKV----K--SSRIG-----	(21)	AB077764
Hammerhead shark	GVSFH-P-RLKEKDDNSSGNSRK---SK-NP----	(25)	AB254128
Stingray	GVSFH-P---QPRSTSKPSA----------	(16)	AB4800033

Amino acid sequences were obtained from the DDBJ/EMBL/GenBank databases. Data were collected by Kitazawa and Kaiya [83].

Ghrelin secretion is characterized by its post-translational modification (acylation) by attaching a fatty acid side-chain to the third position serine residue (Ser3), which is essential for binding and activating GHS-R1a. Acyl modification at the third position Ser3 or Thr3 of ghrelin is conserved in all vertebrates [94]. Ghrelin *O*-acyl-transferase (GOAT) is responsible for ghrelin acylation [59,190]. The non-modified ghrelin peptide was named des-acyl (unacylated) ghrelin, and this peptide lacks activity as ghrelin. However, biological actions of des-acyl ghrelin on cell proliferation, apoptosis, cell metabolism and glucose homeostasis have been reported, although the mechanisms of its actions including the responsible receptor have not been fully clarified [22,41,97].

The discovery of GOAT was a breakthrough for understanding the physiological role of ghrelin because GOAT plays a crucial role in acylation of Ser3, which is essential for binding of ghrelin to GHS-R1a. The following evidence indicated the importance of GOAT. First, ghrelin and GOAT are always co-localized in the same tissues, as found in the stomach and pancreas in humans and in the stomach and intestine in mice [59,101,147]. Second, GOAT is highly conserved in vertebrates as is ghrelin. GOAT activity is observed in humans, rats, mice and zebrafish, and GOAT expression coexisted with octanoylated forms of ghrelin [59]. Finally, octanoyl- and decanoyl-modified forms of ghrelin are completely absent in GOAT-deficient mice [8,59,191,194].

Ghrelin has been isolated from the gastric fundus of dogs, mice, rats and humans, and enteroendocrine X/A-like cells in the mucosal layer were shown to be responsible for producing ghrelin [34,177,187]. Both *n*-octanoyl ghrelin and des-acyl ghrelin coexist in cells [34]. Ghrelin-immnoreactive cells are also found in the duodenum, jejunum, ileum and colon, and the concentration of ghrelin decreases gradually from the duodenum to the colon [34]. The pancreas is also a ghrelin-producing organ [94]. In the central nervous system, ghrelin is found in hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular and arcuate hypothalamic nuclei that are related to regulation of food intake [104]. Ghrelin is also expressed in the kidney, particularly in glomeruli, and *n*-octanoyl ghrelin and des-acyl ghrelin were both found in the mouse kidney [116]. In addition, plasma ghrelin level synchronizes with serum creatinine concentration, suggesting that the kidney is an important organ for ghrelin degradation or clearance [192]. Low concentrations of ghrelin have also been found in the lung, placenta and testes [92,94,175].

Due to the similarity in amino acid sequences of ghrelin and its receptors with motilin and MLNRs (Figure 3) [150], it has been thought that these two peptides originate from the same ancestral gene and form a peptide family [6,60,128]. Ghrelin and motilin are both mainly produced in the gastric and upper small intestinal (duodenum) mucosa, and they have some common functional characteristics such as regulation of GI motility and appetite [40]. Therefore, ghrelin might also be an important GI peptide for regulation of GI motility [94,128].

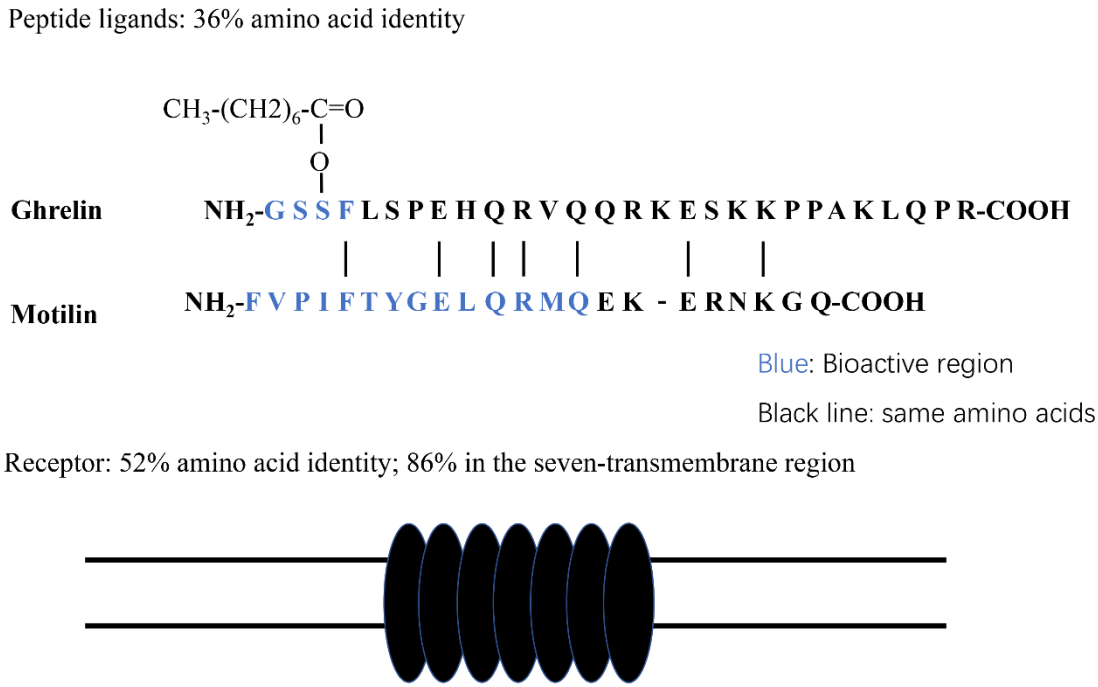

Figure 3. Ghrelin, motilin and their receptors. Difference in the N-terminal amino acids of motilin and ghrelin and difference in the receptor structures resulting in selectivity of activity to the two receptors.

GI motor-stimulating actions of ghrelin and GHS-R1a agonists have been demonstrated in several *in vitro* and *in vivo* studies. The earliest study in anesthetized rats has shown that intravenous injection of ghrelin caused the gastric contraction by activation of cholinergic pathway including in the vagus innervation [107]. Ghrelin caused contraction of rodent gastric fundic circular muscle strips *in vitro* [33]. Taken together, the results of GI contraction studies indicated that ghrelin stimulates GI contractility through action on enteric (motor)

neurons and action through the afferent terminal of the vagus activating vago-vagal reflex pathway [9,44,50,55,80,100]. Expression of GHS-R1a in the enteric neruons and vagal afferent terminals has been demonstrated [33,146]. Ghrelin has been shown to act mainly on receptors expressed in enetric neurons. Direct action of ghrelin on smooth muscle cells in the mammalian GI tract has not been reported, being accordance with the fact that there is no GHS-R1a immunoreactivity in smooth muscle layers [95, 102]. In rats and mice that lack motilin and its receptor, it has been shown that ghrelin induced gastric antral phase III-like contraction of the MMC because ghrelin stimulated the occurrence of phase III activity and a GHS-R1a antagonist inhibited gastric phase III activity [4,54,174]. On the other hand, the MMC in dogs is regulated by motilin but not ghrelin as described in the motilin section (Section 1.2). These phenomena suggest the species-related difference in the involvement of ghrelin or motilin in regulation of the MMC [122].

Many studies have indicated that ghrelin is a multifunctional peptide with various biological activities including activities for GH secretion, food intake, body weight gain and energy metabolism.

1. GH secretion: ghrelin was first identified as an endogenous ligand for GHS-R, which stimulates the release of GH through a different pathway activated by GH-releasing hormone (GHRH). Ghrelin stimulated GH release in both *in vivo* and *in vitro* conditions [5]. Loss of GHS-R1a function might be the main reason for familial short stature [126]. Ghrelin analogs have been evaluated as diagnostic agents for judging GH deficiency as well as clinical drugs to compensate for GH deficiency [118,135]. However, mice with genetic deficiency of GHS-R1a showed no growth abnormality, though the reason for the discrepancy in findings for mice and humans is unclear at present [196].
2. Food intake: ghrelin is able to stimulate food intake and increase body weight gain [117,179]. It was shown that neuropeptide Y (NPY) and agouti-related peptide (AGRP) as well as orexin neurons are essential for the control of feeding behavior and that ghrelin stimulates food intake via increasing NPY and AGRP expression [24,28]. Involvement of NPY and AGRP in the ghrelin-induced increase in food intake was supported by findings that NPY receptor antagonists decreased the responses to ghrelin and that ghrelin failed to stimulate food intake in NPY and AGRP knockout mice [24,28].
3. Adiposity: Enzymes responsible for fat storage were induced by central administration of

ghrelin [176]. In addition, cholesterol levels, particularly the level of high-density lipoprotein (HDL), in plasma were increased by chronic central administration of ghrelin. In ghrelin and GHS-R1a-deficient mice, lower cholesterol levels in plasma than those in wild-type mice were reported [130]. Collectively, the results of studies indicate that the effect of ghrelin on fat storage is processed via central and peripheral signaling pathways of ghrelin.

4. Glucose metabolism: Both ghrelin and GHS-R1a are expressed in pancreatic islets [35,58]. Ghrelin increases plasma glucose level and decreases plasma insulin level [16,17,51]. An inhibitory effect of ghrelin on the release of insulin has been reported in mice [136,138,148]. By blockading the pancreatic-derived ghrelin, insulin secretion was increased [49]. Furthermore, plasma ghrelin level negatively correlated with insulin level; in other words, ghrelin increased with a decrease in insulin secretion [29,53]. The above information suggests that ghrelin secretion could be induced under the negative energy balance and tends to storage the energy in the body.

1.5. Ghrelin receptor

Growth hormone secretagogue-receptor type-1a (GHS-R1a) was discovered as an orphan G protein-coupled receptor in 1996 [64]. Ghrelin was identified as an endogenous ligand for the GHS-R1a by Kojima *et.al* in 1999 [92]. GHS-R1a mRNA is prominently expressed in the arcuate and ventromedial nuclei of the hypothalamus and the hippocampus [11,58]. In peripheral organs, GHS-R1a mRNA has been detected in the heart, lung, liver, kidney, pancreas, stomach, small and large intestines, adipose tissue and immune cells [57,58,61,93]. GHS-R1a couples with Gαq/11, Gαi/o, and Gα12/13 of G proteins and mediates the biological actions of ghrelin [36]. Schwartz *et al.* hypothesized the toggle switch mechanism for GHS-R1a activation by which ligand binding causes receptor conformational change, resulting in elicitation of G-protein function [155]. It has been demonstrated that GHS-R1a shows constitutive activity, indicating that it could be activated even without ghrelin [36]. When GHS-R1a was discovered by Howard *et al.*, another GHS-R molecule was also identified and was named GHS-R1b [64]. GHS-R1b is produced by alternative splicing of the same GHS-R gene and a truncated form with 5-transmembrane (TM) domains of GHS-R1a [64]. The structure of both GHS-Rs are highly conserved across vertebrate species [152].

Unlike GHS-R1a, GHS-R1b cannot stimulate Ca^{2+} release due to the lack of TM domains 6 and 7 [64,92]. However, it has been reported that a low expression level of GHS-R1b promotes GHS-R1a function, while a high expression level of GHS-R1b inhibits signal transduction by causing a negative allosteric effect on GHS-R1a in HEK-293T cells [119].

1.6. Motilin and ghrelin in non-mammals

As shown in Figure 4, the morphology of the GI tract and GI functions in birds are different from those in mammals: 1) the crop in the esophagus in birds is characteristic for the part of food stock, 2) the MMC in birds originates from the duodenum, not the stomach, and it appears in both fed and fasted phases and 3) birds have two functional stomachs including the proventriculus that secretes digestive enzymes for chemical digestion and the gizzard for mechanical digestion. The motilin system (motilin and its receptor) has been identified in chickens, turkeys, Japanese quails and pigeons [3,83,88,188]. In the chicken, mRNA expression of motilin is found in many tissues including the pituitary, brain, thymus, bursa of fabricius, liver, kidney, bone marrow, proventriculus, duodenum, oviduct, ovary and testis, and the highest expression levels are in the proventriculus and middle level in the duodenum, oviduct and testis [188]. *In vitro* functional studies indicated that motilin causes GI contraction via the MLNR in small intestinal smooth muscle, while in the proventriculus, both neural and smooth muscle receptors mediate motilin-induced contraction. On the other hand, no contractile response was observed in muscle strips obtained from crop and colon in chicken [88]. Similar results of the motilin-induced contraction have also been obtained in the quail [3]. In the chicken, Rodriguez-Sinovas *et al*. reported that plasma motilin concentration was high during spontaneous rhythmic oscillating complexes (ROCs) that occurred in the small intestine and that exogenous motilin triggered ROC contractions [140]. ROCs are highly organized myoelectric events consisting of several intestinal spike bursts migrating aborad (from the duodenum to the ileocecorectal junction) followed by groups of orad spike bursts from the end of small intestine to the pylorus. Therefore, it has been suggested that motilin also regulates GI motility in fasting periods in avian species. However, molecular and functional studies have been limited only to the chicken and quail. Studies using other species of birds are necessary to understand the functional roles of motilin peptide, especially its role in the regulation of GI motility, in avian species.

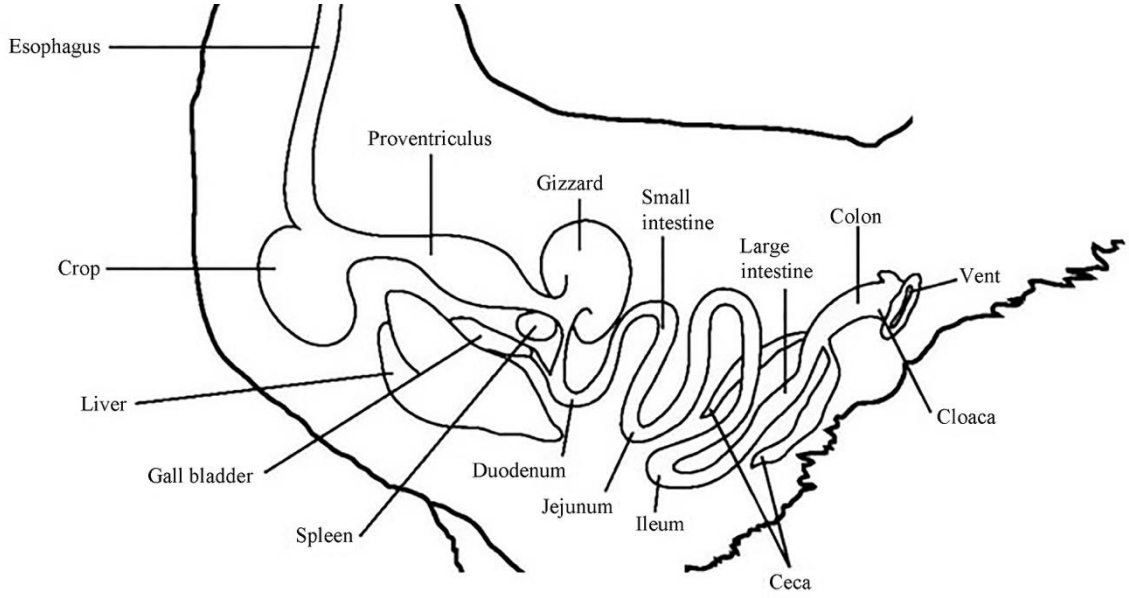

Figure 4. Gastrointestinal tract in birds and its function. The beak gathers food. Subsequently, the food passes to the esophagus, which transports the food and water to the crop. The esophagus contains mucus glands that help to lubricate the passage of food to the crop, where it is stored temporarily. In its passage through the esophagus, the food is softened and undergoes pre-digestion by enzymes. The crop fills up when the bird has eaten enough, and the food passes slowly to the proventriculus, a glandular stomach. Here, foodstuffs are bathed in gastric juices, hydrochloric acid, and digestive enzymes, beginning the process of nutrient breakdown and construction of the food bolus, which then passes to the gizzard. The gizzard, also known as the masticatory organ in birds, accumulates insoluble grains, which are ground by frequent and repeated contractions that exert enormous pressure, breaking the grains down into small particles and mixing them with juices from the proventriculus. From the gizzard, the food passes to the small intestine, an organ that is distinguished histologically by the presence of villi, which completes the digestion of proteins through the secretion of intestinal juices and digestive enzymes; another function is to absorb the nutrients in digested foodstuffs so that they can enter the bloodstream; finally, the small intestine provides peristaltic action that passes undigested materials to the ceca. The small intestine has three sections: the duodenum, the jejunum, and the ileum. The pancreas is the organ that secretes juices enriched with amylases, trypsin, lipases and carboxypeptidases. The liver secretes bile into the

duodenum, which helps break down fats; the bile, though produced in the liver, is stored in the gallbladder. The ileum opens into the ceca, a pair of tubes in which undigested foodstuffs are fermented and which are emptied every 24 h. The water and foodstuffs that are not digested in the small intestine, such as non-starch polysaccharides, are absorbed in the large intestine, a section of the digestive tract that leads from the junction with the ceca, through the colon, and ends in the external opening of the cloaca [25].

In reptiles, the N-terminal region of alligator motilin is close to that of chicken motilin [102], whereas the amino acids of lizard motilin are quite different from those of human and chicken motilin [83]. Concerning contraction study, the effect of motilin on reptile GI motility has not been examined except for in the turtle. Turtle motilin is thought to contract the turtle intestine (Sakata *et al*., Saitama Univ. unpublished data).

In amphibians, the isolated GI tract of the bullfrog responded to human motilin treatment: a small contraction of longitudinal muscle strips of the upper intestine was induced by a relatively high concentration of human motilin (1 μM), while there was no response from the stomach or the middle or lower intestine. Therefore, motilin also caused GI contraction in the frog in a region-dependent manner. The region-dependency is one of the characteristics of responses to motilin in mammals and birds, and this characteristic is also conserved in bullfrogs [86]. In contrast to the bullfrog, the GI tract of the Japanese fire belly newt, a urodele amphibian, failed to respond to human motilin in any GI regions [86], suggesting evolutionary contradictions.

In fish, motilin-like peptide and MLNR genes have been cloned in the zebrafish Takifugu and Medaka [102]. The structures of fish motilin are quite different from those of motilin in other vertebrates. Namely, fish motilin is shorter than mammalian motilin and its C-terminal region is modified by amidication ($-NH_2$). Zebrafish motilin-like peptide caused contraction of the isolated intestinal bulb and the middle or lower intestine of the zebrafish at a very high concentration (10 μM) [124]. The high MLNR mRNA expression level in the brain compared to that in the GI tract might help to explain the low responsiveness of motilin-like peptide in the zebrafish intestine, and it suggests that the motilin system may not be essential for the regulation of intestinal motility in zebrafish [102].

In addition to in mammals, ghrelin has been identified in various avian species including

the turkey, goose, duck, chicken and Japanese quail [83]. Function studies have indicated different effects of ghrelin on GI contraction in avian species. In isolated chicken GI muscle strips, chicken ghrelin caused contraction of the crop, proventriculus and colon, whereas it did not cause any contraction in other parts of the intestine such as the duodenum, jejunum and ileum, where motilin usually causes major contraction [84]. In the Japanese quail, on the other hand, ghrelin does not cause contraction in any GI regions even though GHS-R1a mRNA is abundantly expressed [85]. Studies using other avian species are needed to elucidate the general action of ghrelin in the avian GI tract. Further study is also needed to explain the inconsistency of the lack of a contractile response despite abundant GHS-R1a mRNA expression.

In reptiles, ghrelin peptide has been purified and its sequence has been determined only in the red-eared slider turtle, but there has been no functional reports about its role in GI motility [77].

Ghrelin has been identified in several species of amphibians including the bullfrog and tropical clawed frog [74]. However, bullfrogs, Japanese fire belly newts and rat ghrelin did not cause any contraction in gastric and intestinal muscle strips of the bullfrog and newt, respectively [86]. GHS-R1a mRNA was found to be mainly expressed in the mucosa rather than in the smooth muscle in the bullfrog intestine, and that might explain the ineffectiveness of ghrelin on GI contraction. The expression of receptors in the mucosa may be involved in nutrient absorption and other processes [86]. However, there were species-related difference in the response of ghrelin in frogs. Recently, Xenpous (*Xenopus tropicalis*) stomach strip and upper intestine were found to be sensitive to rat ghrelin causing contraction [193].

Ghrelin has been identified and shown to be mainly expressed in the stomach or intestine in stomach-less fish [83]. Ghrelin failed to stimulate GI contraction in rainbow trout and goldfish indicating that ghrelin might not be involved in the regulation of GI motility in those species [75,82,111]. In zebrafish, however, rat ghrelin has been reported to be able to stimulate contraction of the intestine [124].

1.7. Aims of this study

As mentioned earlier, motilin and ghrelin have been identified in the GI tracts of several avian species including chickens and quails. Although functional studies have been limited to

those two species, it was shown that motilin caused contraction of the GI tract in both species in a region-dependent manner but that the responses to ghrelin differed in chickens (sensitive) and quails (insensitive) in general. Namely, the actions of motilin are the same but those of ghrelin are opposite. Therefore, the aim of the present study was to determine whether 1) motilin is present and causes contraction of the GI tract in a region-related manner in another avian species and 2) ghrelin is present and causes contraction of the GI tract in another avian species. In the present study, the pheasant (*Phasianus colchicus*) was selected as another avian species that belongs to Galliformes as do chickens and quails in order to compare the actions of motilin and ghrelin among closely related species. At first, the primary structures of motilin and ghrelin in the pheasant were determined by a molecular biology technique and then the mechanical effects of motilin and ghrelin and their interaction in isolated GI strips of the pheasant were examined.

CHAPTER 2.
IDENTIFICATION OF PHEASANT MOTILIN AND THE EFFECTS OF MOTILIN ON GASTROINTESTINAL CONTRACTION

2.1. Introduction

Motilin has been identified in the chicken and quail and has been reported to be located in the mucosa of the upper GI tract. The GI motor-stimulating action of motilin has been investigated using isolated GI strips of chickens and Japanese quails *in vitro* [3,37,87,88]. Chicken motilin and human motilin caused contraction of the small intestine of both species by activation of smooth muscle receptors in the intestine. Motilin also caused contraction of the proventriculus, while the crop, gizzard and colon were insensitive to motilin, and the contraction in the proventriculus was smaller than that in the small intestine [3,37,87,88], indicating region-dependent differences in the responsiveness to motilin. But the affinity for chicken motilin was higher than that for human motilin, being different from the results obtained in the rabbit intestine. This suggests that the structure of the MLNR in avian species is different from that in mammals [3,88]. However, as mentioned in Chapter 1, identification of motilin and investigation of its actions on GI contractility have been limited to the chicken and quail as shown in Table 3. Therefore, whether GI contractility-stimulating action of motilin is common among avian species or not is unclear at present. Molecular and functional studies using another species of birds are necessary to answer that question.

Table 3. Comparison of the presence of motilin and MLNR and the GI contractility-stimulating action of motilin in different species of birds.

Species	Motilin	MLNR	GI contractility
Chicken (*Gallus gallus domesticus, Gallus*)	○	○	Contraction
Quail (*Coturnix coturnix, Coturnix*)	○	?	Contraction
Turkey (*Meleagris, Meleagris*)	○	?	?
Pheasant (*Phasianus colchicus, Phasianus*)	?	?	?

(○) The structure has been identified. (?) No report.

At first, the primary structure of motilin was determined, and its distribution in pheasant tissues was determined by molecular biology methods. Second, the effects of three kinds of motilins (human, chicken and pheasant motilins) on the contractility of isolated GI tract regions including the crop, proventriculus, duodenum, jejunum, ileum and colon of the pheasant were examined and compared. The mechanisms of motilin-induced contraction were analyzed using pharmacological reagents (tetrodotoxin, atropine, GM109 and MA2029). The effects of human, chicken and pheasant motilins on isolated longitudinal smooth muscle strips of the rabbit duodenum were also examined and the ranking order of contractile responses was compared with that in the pheasant intestine.

2.2. Materials and Methods

2.2.1. Ethical approval

All experiments were performed in accordance with Institutional Guidelines for Animal Care at Rakuno Gakuen University (VH18D1, VH20A4), Ebetsu, Hokkaido, Japan.

2.2.2. Molecular study

2.2.2.1 Chemicals

The following chemicals were used in the molecular studies: RNAlater solution (Invitrogen by Thermo Fisher Scientific, Lithuania), TRIzol reagent (Molecular Research Center, Inc., OH, USA), ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan), Thunderbird SYBR qPCR Mix (Toyobo Co., Ltd.), 2-propanol (Nacalai Tesque, Inc., Kyoto, Japan), chroroform (Nacalai Tesque, Inc., Kyoto, Japan), ethanol (Nacalai Tesque, Inc., Kyoto, Japan), KOD-Plus (Toyobo Co., Ltd.), nuclease-free water (Promega Co., WI, USA), ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan), DNase digestion (Promega Co.), Prime Script II Reverse Transcriptase (Takara Bio Inc., Shiga, Japan), ExTaq DNA polymerase (Takara Bio Inc.), isoflurane (Pfizer Japan, Tokyo, Japan), proteinase K (20 μg/ml, proteinase K ready-to-use, Dako Japan Inc., Kyoto, Japan), phosphate-buffered saline (PBS) (pH 7.4) (Dainippon-Parma Co., Ltd., Osaka, Japan), blocking solution (protein block serum free, Dako North America Inc., CA, USA), antibody diluent (antibody diluent with background Reducing components, Dako North America Inc.), second antibody solution (labelled polymer, HRP anti-rabbit envision, Dako North America Inc.), isoflurane (Zoetis Inc., Tokyo, Japan).

2.2.2.2. Animals and tissue preparations

Male and female pheasants (30-60 days after hatching, 300 ~ 450 g,) were obtained from a farm (Work Tsukasa) in Iwamizawa City, Hokkaido, Japan. The pheasants were anesthetized with isoflurane in a glass container, stunned, and bled to death. The whole brain, heart, lung, liver, pancreas, esophagus, crop, proventriculus, gizzard, small intestine, caecum and colon were removed for molecular study. Each organ was washed well with 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), and luminal contents of the GI tract were flushed out using ice-cold Krebs solution. The tissues were cut into small pieces with scissors and then stored in RNAlater solution and frozen at -20 °C until used.

2.2.2.3. Cloning of pheasant motilin

Total RNA was extracted from the duodenum of three pheasants by ISOGEN according to the manufacturer's instructions. Trace DNA contamination was removed by DNase digestion, and cDNA was synthesized from 2 μg of DNase-treated total RNA using Prime Script II Reverse Transcriptase and Oligo-dT with an anchor primer 5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTTT VN-3'. Primary 3'-RACE PCR amplification was performed with 1 μl of a template, 100 pmol/μl of primers for a sense 5'-CCGGTTTGCTCCTGGTGTA-3' and antisense 5'-CCAGTGAGCAGAGTGACG-3', and ExTaq DNA polymerase. The reaction conditions were 94 °C for 2 min followed by 40 cycles of 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 for 5 min. The resultant product was subjected to second-round nested PCR. Nested PCR was conducted with 1 μl of the diluted primary PCR product, 100 pmol/μl each of a sense primer 5'-TCAAAGGGCAGAAGAAA TCC-3' and antisense primer 5'-GAGGACTCGAGCTCAAGC-3', and ExTaq DNA polymerase. The reaction conditions were 94 °C for 2 min followed by 40 cycles of 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 for 5 min. For cloning of the 5′ region pheasant of motilin, PCR amplification was performed with 500 ng total RNA, a sense primer 5'-CCGGGTGTGACAAGGAACAAG-3', antisense primer 5'-GCACTGCCATCACGTACACC-3', and ExTaq DNA polymerase. The reaction conditions were 94°C for 2 min followed by 40 cycles of 94 °C for 0.5 min, 50 °C for 0.5 min and 72 °C

for 0.5 min with final extension at 72 °C for 5 min.

Amplification reactions were carried out using a Thermal Cycler (Bio-Rad, Hercules, California, USA). Amplicon size and specificity were confirmed by 2% agarose gel electrophoresis. The PCR product was cloned into pGEM-T Easy vector (Promega Co.) and sequencing was performed by Eurofins Genomics K.K. (Tokyo, Japan).

2.2.2.4. Measurement of motilin mRNA expression in pheasant organs

To investigate the distribution of motilin, the expression levels of motilin mRNA were measured in various organs, including the brain, heart, lung, liver, pancreas, esophagus, crop, proventriculus, small intestine, caecum, gizzard and colon, of three pheasants. Total RNA was extracted from tissues (less than 0.1 g) with TRIzol reagent. cDNA was synthesized from total RNA (500 ng) with a ReverTra Ace qPCR RT Kit. Real-time RT-PCR analysis was performed using a real-time PCR detector (LightCycler 480) with Thunderbird SYBR qPCR Mix. The primer set used for detection of motilin (Accession No. LC469791.1) was: 5'-GCGACATCCAGAAAATGCAGG-3' (Forward) and 5'-GTCCTGTAACACCTCCGTGA-3' (Reverse) (product size, 233 bp), and that used for detection of GAPDH (Accession No. XM_031610235.1) was 5'-CAATGTCTCTGTTGTTGACCTG-3' (Forward) and 5'-ACCATTGAAGTCACAGGAGAC-3' (Reverse) (product size, 151 bp). Amplification conditions were initial incubation at 95 °C for 1 min followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. GAPDH was used as an internal control. Results are presented as $2^{-\Delta Ct}$ (where ΔCt = Ctmotilin - CtGAPDH).

2.2.3 Contraction study

2.2.3.1 Chemicals

The following chemicals were used in contraction experiments: NaCl, KCl, $MgSO_4$, KH_2PO_4, and $CaCl_2$ (Wako Pure Chemical Industries Ltd., Osaka, Japan), $NaHCO_3$ and glucose (Kanto Chemical Co., Inc., Tokyo, Japan), acetylcholine (Wako Pure Chemical Industries Ltd.), medetomidine (Meiji Pharma Co., Ltd., Tokyo, Japan), pentobarbital sodium (Kyoritsu Seiyaku Corp., Tokyo, Japan), erythromycin (U.S. Pharmacopeial Co. Inc., MD, USA), human motilin (Peptide Institute Inc., Osaka, Japan), chicken motilin (Peptide Institute

Inc.), pheasant motilin (Scrum Inc. Tokyo, Japan), tetrodotoxin (Wako Pure Chemical Industries Ltd.), atropine (Sigma-Aldrich, MO, USA), GM109 and MA2029 (donated by Chugai Pharmaceutical Company, Tokyo, Japan), and isoflurane (Zoetis Inc.).

2.2.3.2. Animals and tissue preparations

Male and female pheasants (30-60 days after hatching, 300-450 g, n = 20) were obtained from a farm (Work Tsukasa) in Iwamizawa City, Hokkaido, Japan. The pheasants were anesthetized 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. 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.

Since the rabbit duodenum shows high sensitivity to motilin and has been used to investigate the biological GI contractility stimulating activity of the motilin peptides [1,81], the contractile responses of motilin peptides were also investigated in this study. Japanese white rabbits of both sexes (5 kg) were obtained from Sankyo Lab Service (Sapporo, Japan) and were housed in individual stainlesssteel cages at a regulated temperature (22 ± 2°C) and 60% - 65% relative humidity with a normal 12 to 12-h light/dark cycle. Food and water were available for 24 h every day. The rabbits were sedated with medetomidine (3 mg/kg, s.c.) and deeply anesthetized by pentobarbital sodium (50 mg/kg, i.v.) and then sacrificed by bleeding from the carotid vein. After making a midline incision, the duodenum (next part of the gastric antrum, 10 cm) was dissected out and longitudinal muscle strips were peeled out using fine forceps as previously described [81,91].

2.2.3.3. Effects of motilin on pheasant gastrointestinal contraction

The GI strips of both pheasants and rabbits were suspended vertically in an organ bath (5 mL) containing warmed (37 °C) Krebs solution bubbled with 95% O_2 + 5% CO_2 (pH 7.4) (Figure 5). Mechanical activity in the longitudinal muscle direction was measured with an

isometric force transducer, recorded on a computer, and analyzed using a computer-aided analysis 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 related substances, each muscle strip was subjected to 3 or 4 continuous stimulations with 100 μM acetylcholine (ACh, 15-min intervals) until a reproducible contraction was obtained.

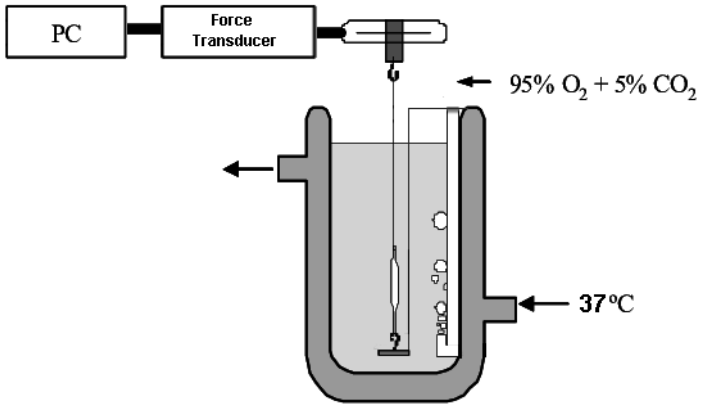

Figure 5. Suspending system used for gastrointestinal strip contraction study. Tissue pieces (10-15 mm in length) were suspended vertically in an organ bath with Krebs solution and 37℃ water was circulated in the outside of the organ bath to mimic body temperature. The upper side was connected to a force transducer device connected with personal computer system (PC) and the lower side was fixed by a curved glass bar. The pH value of the solution was maintained at 7.4 consisting of 95% O_2 + 5% CO_2.

To examine the contractile responses to MLNR agonists in muscle preparations, erythromycin (1 nM - 10 μM), human motilin (0.1 nM - 3 μM), chicken motilin (0.1 nM - 1 μM) or pheasant motilin (0.1 nM - 1 μM) were applied cumulatively in the organ bath for different preparations. Each concentration of motilin was applied after observing the peak contractile amplitude induced by each concentration. ACh caused contraction of all muscle preparations examined in this study, and it was applied cumulatively after reaching the peak response at concentrations from 10 nM to 100 μM. The interval for determining the concentration-response relationships was set at 1 h to prevent desensitization of motilin responses, and the application order of erythromycin, human motilin, chicken motilin,

pheasant motilin or ACh was changed for each preparation.

2.2.3.4 Mechanisms of motilin-induced gastrointestinal contraction

In the isolated GI tract, motilin is thought to cause contraction through direct actions on receptors in smooth muscle or indirect actions on enteric neurons. Cholinergic neurons in the myenteric plexus are stimulated by motilin [15,81,87]. To analyze the mechanisms underlying the motilin-induced contraction, pharmacological properties were examined using a neuron blocker, tetrodotoxin (TTX), and a muscarinic receptor antagonist, atropine.

It has been reported that GM109 and MA2029, MLNR antagonists, decreased motilin-induced responses in the rabbit and dog GI tracts [125,164,171]. In this study, the effects of pretreatment with these antagonists on motilin-induced contractions in the pheasant GI tract were examined.

To confirm the involvement of motilin receptor in the pheasant motilin-induced contraction of the rabbit duodenum, the inhibitory action of GM109 on the responses to motilin was investigated using 100 nM or 1 μM concentration and pA2 values were calculated

2.2.4 Statistical analysis

The experimental data are expressed as means ± SEM of more than three 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). A sigmoid curve fitting procedure (GraphPad Prism6) was used for calculating EC_{50} (concentration causing 50% of the maximum contraction).

2.3. Results

2.3.1. Structure of pheasant motilin

Pheasant motilin cDNA was cloned from mRNA of the duodenum and its nucleotide sequence was determined (Figure 6) (Acc# LC469791.1). The deduced amino acid sequence of mature pheasant motilin was 22 amino acids. Similar to motilin precursors in mammals, an endoproteinase cleavage site was found at Lys23- Lys24 (Figure 6). Mature pheasant motilin

showed high sequence homology with other avian species: turkey (100%), chicken (95.4%) and quail (90.9%). The sequences of the N-terminal region [1–9] (FVPFFTQSD), middle region [11–18] (QKMQEKER) and C-terminal region of pheasant motilin [20–22] (KGQ) were the same as those in other birds such as the turkey, chicken and quail (Figure 7). Pheasant motilin showed moderate homology with mammalian species (68% for human motilin and canine motilin and 64% for house shrew motilin) (Figure 7).

Figure 6. Nucleotide sequence encoding pheasant motilin. The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank databases with the accession No. LC469791.1. Mature motilin peptide is boxed, and a dibasic cleavage site (Lys-Lys) is indicated by bold letters and underline.

```
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
```

Figure 7. Comparison of amino acid sequences of mature motilin in some birds and mammals. Conserved amino acids among all species are indicated by bold. The amino acid sequence of pheasant motilin (LC469791.1) was aligned with the amino acid sequences of human motilin (AAI12315.1), dog motilin (NP_001300735.1), Suncus motilin (BAI66099.1), turkey motilin (XP_010722636.1), chicken motilin (NP_001292058.1) and quail motilin (BAU80773.1).

2.3.2. Motilin mRNA distribution in pheasant organs

Figure 8 shows the relative expression levels of pheasant motilin mRNA in various organs of the pheasant. In the GI system, relative expression levels were high in the duodenum (0.088±0.017), jejunum (0.078±0.030) and ileum (0.046±0.021), moderate in the colon (0.006±0.003), and low in the pancreas (0.00018±0.00001), esophagus (0.00058±0.00005), crop (0.00029±0.00003), proventriculus (0.00201±0.00034), gizzard (0.00079± 0.00009) and caecum (0.00092±0.00033). Outside the GI system, motilin mRNA relative expression levels in the heart (0.00192±0.00137) and lung (0.00264±0.00132) were higher than those in the brain (0.00027±0.00003) and liver (0.00045±0.00005), while expression levels of these organs were much lower than those in the small intestine.

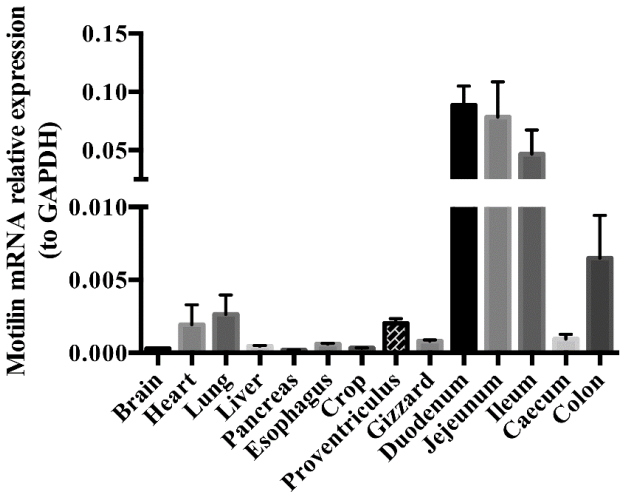

Figure 8. Motilin mRNA expression levels in different organs of the pheasant. Relative expression was calculated using $2^{-\Delta Ct}$ targeting the housekeeping gene GAPDH (Arbitrary unit). Values are means ± S.E.M (n = 3).

2.3.3. Effects of chicken motilin on contraction of the pheasant GI tract

At first, the contractile activity of chicken motilin instead of pheasant motilin on the pheasant GI tract was examined because the structure of pheasant motilin was found to be close to that of chicken motilin. As shown in Figure 9, chicken motilin caused marked concentration-dependent contractions in duodenum, jejunum and ileum preparations. Contraction was evoked at 1-3 nM and reached a maximum at 100-300 nM. The EC_{50} values of the responses and the maximum amplitudes (% to 100 μM ACh-induced contraction) were 20.1 ± 6.8 nM and 79.1 ± 7.9% in the duodenum (n = 8), 30.6 ± 10.8 nM and 73.9 ± 6.0% in the jejunum (n=5), and 26.4 ± 7.6 nM and 88.30 ± 10.3% in the ileum (n=17), respectively (Figure 10). On the other hand, other GI regions such the crop, proventriculus and colon were less sensitive to chicken motilin. The contractile responses in the proventriculus reached a significant level at 300 n M and 1 μM compared with the normal muscle tonus in the absence of chicken motilin, while the muscle tonus in the crop and colon did not reach a significant level even at 1 μM compared with that in the absence of chicken motilin (Figure 10). A comparison of concentration-response curves of chicken motilin in the crop, proventriculus, duodenum, jejunum, ileum and colon is shown in Figure 11. Although it was found a region-dependent difference in the motilin-induced action in the pheasant GI tract, ACh (1 nM

- 100 μM) caused a concentration-dependent contraction in all parts of the GI tract (Figure 12), and the EC_{50} values were comparable among the GI regions examined (EC_{50} values: 760.6 ± 487.3 nM for the crop (n = 4), 533.8 ± 158.0 nM for the proventriculus (n = 7), 396.8 ± 134.2 nM for the duodenum (n = 5), 225.8 ± 62.4 nM for the jejunum (n = 5), 442.2 ± 153.4 nM for the ileum (n = 6) and 343.3 ± 114.8 nM for the colon (n = 6)).

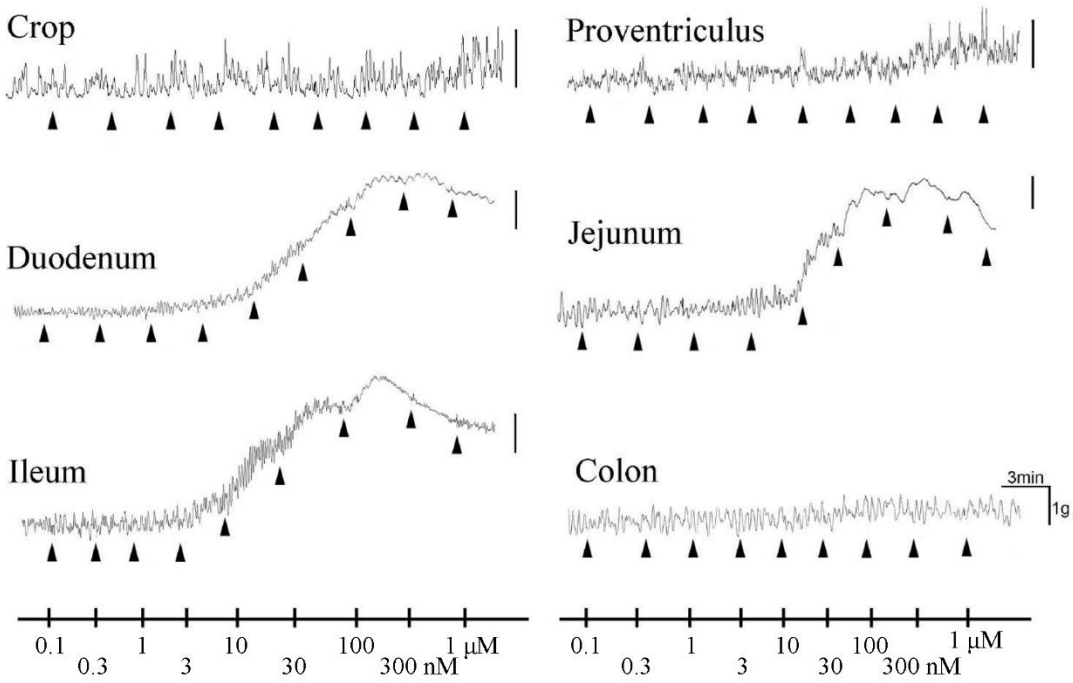

Figure 9. Contractile responses to chicken motilin in different regions of the pheasant GI tract. Representative responses to chicken motilin in isolated strips from the crop, proventriculus, duodenum, jejunum, ileum and colon. Chicken motilin was applied cumulatively (0.1, 0.3, 1, 3, 10, 30, 100, 300 nM and 1 μM). Arrowheads indicate the timing of motilin application.

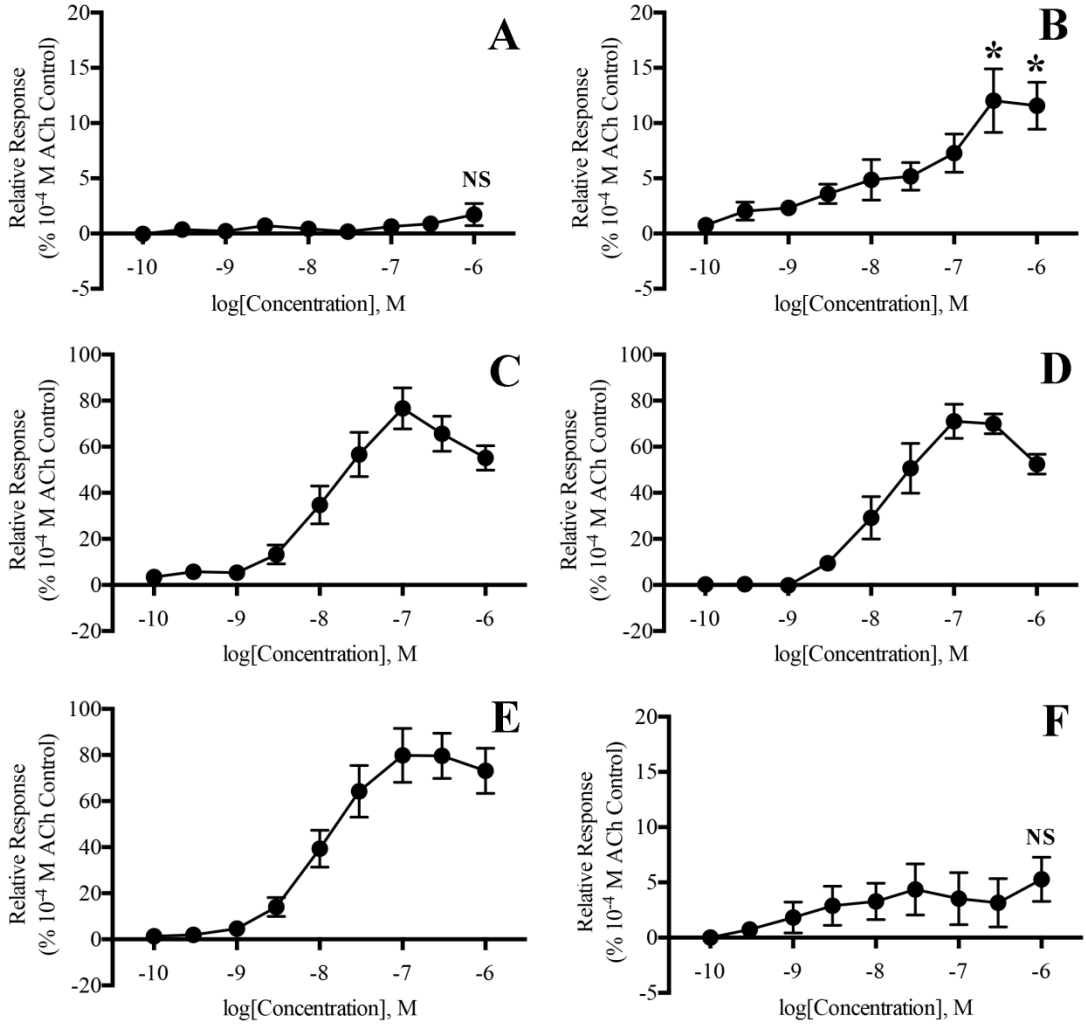

Figure 10. Concentration-response curves for chicken motilin in the crop (A), proventriculus (B), duodenum (C), jejunum (D), ileum (E) and colon (F) of the pheasant GI tract. The amplitude of motilin-induced contractions (y-axis) was normalized by a standard contraction by ACh (100 μM). The x-axis is the concentration of motilin (log M). Values are means ± S.E.M (n = 4-17). In the crop, proventriculus and colon in which sigmoid shape concentration response curves could not be obtained, but significant contractile responses (*) were calculated by one-way ANOVA (Dunnett's test) for comparing smooth muscle tension in the absence of motilin. NS (Not significant).

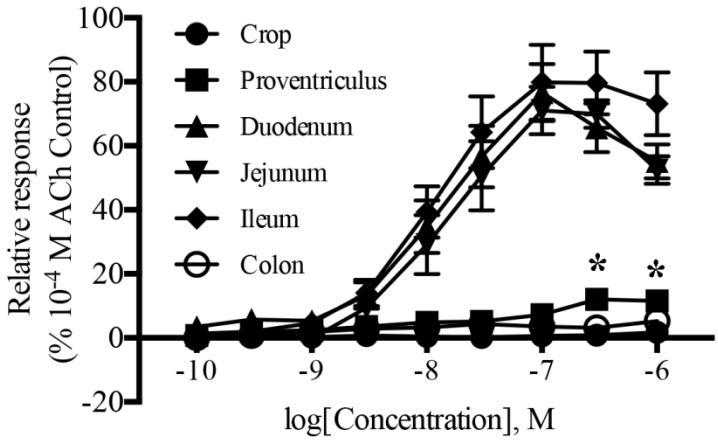

Figure 11. Comparison of concentration-response curves for chicken motilin in the crop (●), proventriculus (■), duodenum (▲), jejunum (▾), ileum (◆) and colon (○) of the peasant. The amplitude of motilin-induced contractions (y-axis) was normalized by a standard contraction by ACh (100 μM). The x-axis is the concentration of motilin (log M). Values are means ± S.E.M (n = 4-17). Among less sensitive GI regions (crop, proventriculus and colon) to motilin, the increase of muscle tonus in the proventriculus was significant compared to that the absence of chicken motilin (*, $p < 0.05$), whereas the responses of 1 μM chicken motilin in the crop and colon were not significant compared to that in the absence of motilin by one-way ANOVA (Dunnett's test) for comparing relative contractions.

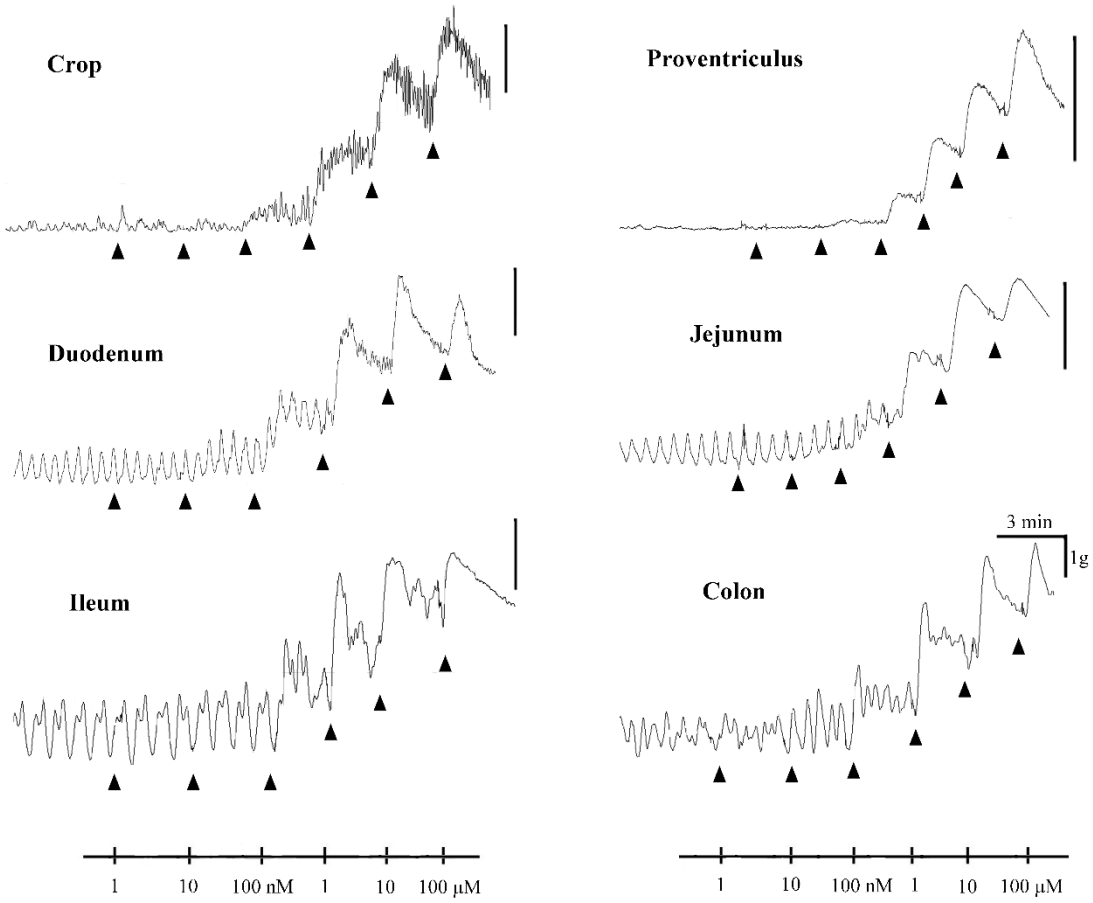

Figure 12. Contractile responses to ACh in different regions of the pheasant GI tract. Representative mechanical responses to ACh in the crop, proventriculus, duodenum, jejunum, ileum and colon. ACh was applied cumulatively (1, 10, 100 nM, 1, 10 and 100 μM). Arrowheads indicate the timing of application.

2.3.4. Effects of human motilin and erythromycin on contraction of pheasant gastrointestinal tract

Human motilin also caused contraction in small intestinal preparations of the pheasant (Figure 13). The maximum responses to human motilin were comparable to those to chicken motilin in three different intestinal regions (duodenum, jejunum and ileum), but the EC_{50} values of human motilin were significantly higher than those of chicken motilin (276.4 ± 22.7 nM for the duodenum (n=4), 227.5 ± 63.1 nM for the jejunum (n=6) and 117.3 ± 32.8 nM for the ileum (n = 7)). Human motilin-induced responses were smaller than those of chicken motilin in the proventriculus, and they were not significant even at 3 μM. As was observed for

chicken motilin, human motilin did not cause contraction in the crop and colon (data not shown).

Erythromycin (EM), known as an MLNR agonist in rabbit and dog GI tracts [71,127], did not cause any contractions in the pheasant proventriculus, duodenum, jejunum and ileum at doses of 1 nM - 10 μM (Figure 13).

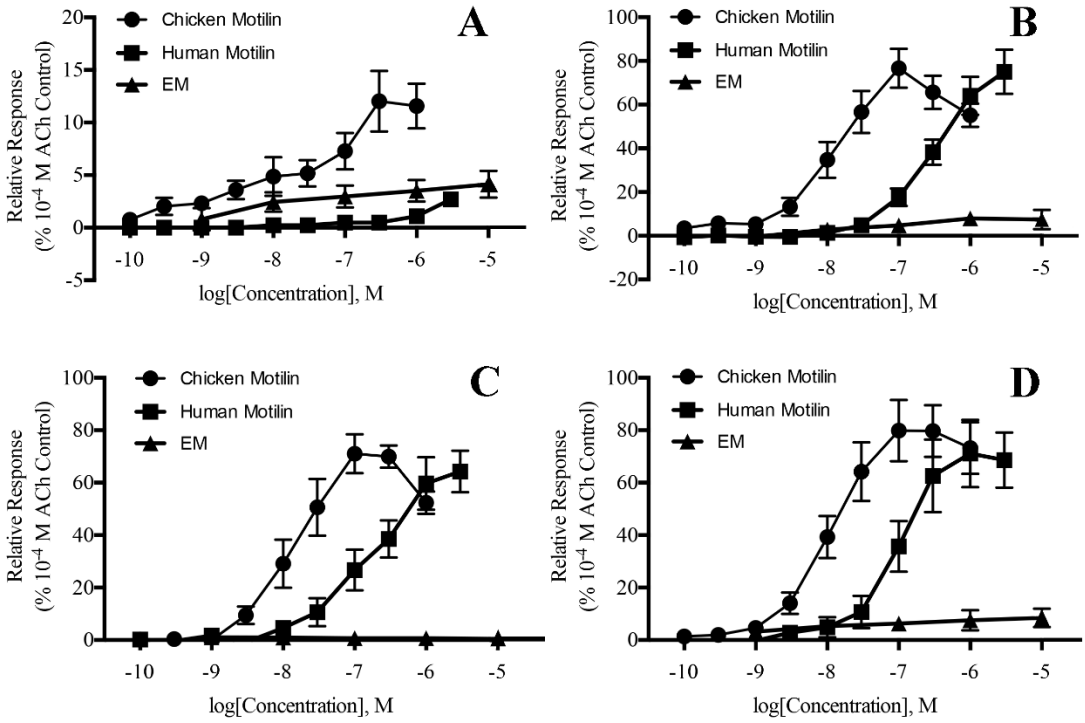

Figure 13. Comparison of contractile responses to chicken motilin, human motilin and erythromycin (EM) in the proventriculus and small intestine of the pheasant. The symbols indicate concentration-response curves for chicken motilin (●), human motilin (■) and erythromycin (EM, ▲) in the proventriculus (A), duodenum (B), jejunum (C) and ileum (D). The amplitude of contractile responses (y-axis) was normalized by a standard contraction induced by ACh (100 μM). The x-axes are concentrations of motilins or erythromycin (log M). Values are means ± S.E.M (n = 4-17).

2.3.5. Effects of synthetized pheasant motilin on contraction of the pheasant gastrointestinal tract

Pheasant motilin, the structure of which was determined in this study, was synthetized and its effects on the isolated GI tract of the pheasant were examined. Pheasant motilin (0.1

nM-1 μM) caused contraction in the proventriculus and small intestinal preparations (duodenum, jejunum and ileum) but did not cause any responses in the crop and colon (Figure 14). The maximum responses and EC_{50} values to pheasant motilin in the small intestine (80.9 ± 65.7 nM for the duodenum (n=7), 18.4 ± 6.3 nM for the jejunum (n=6) and 27.3 ± 14.5 nM for the ileum (n = 6)) were comparable to those to chicken motilin. In the crop, proventriculus and colon, the responses to pheasant motilin were very small, similar to the responses to chicken motilin.

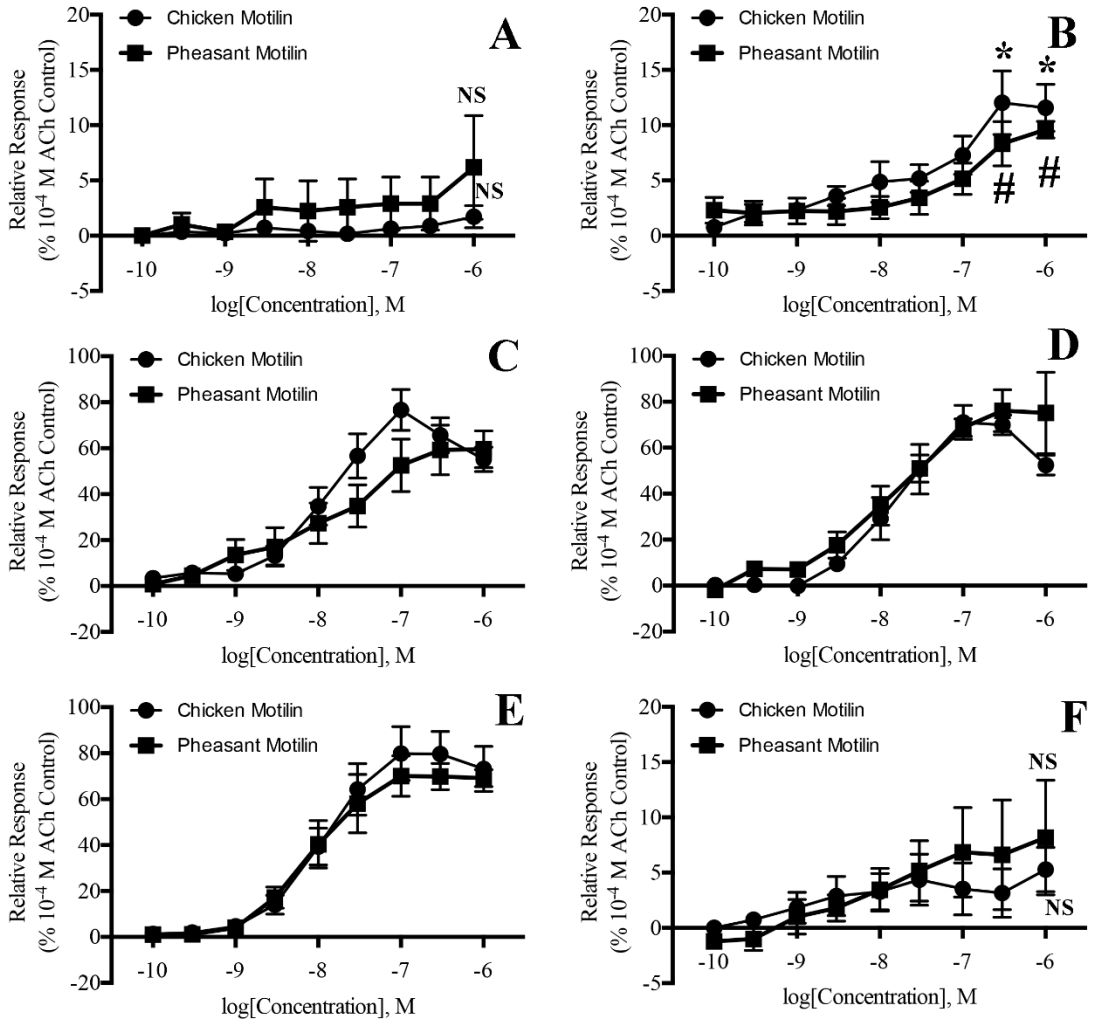

Figure 14. Contractile responses to pheasant motilin in the pheasant GI tract. The symbols indicate the concentration-response curves for pheasant motilin (●) and chicken motilin (■) in the crop (A), proventriculus (B), duodenum (C), jejunum (D), ileum (E) and colon (F). The amplitude of contractile responses (y-axis) was normalized by a standard contraction by ACh (100 μM). The x-axes are concentrations of motilin (log M). Values are means ± S.E.M (n =

3-17). Same as chicken motilin, among less sensitive GI regions (crop, proventriculus and colon) to motilin, the increase of muscle tonus in the proventriculus was significant compared to that with absence of pheasant motilin (*, #, $p < 0.05$), whereas the responses of 1 μM pheasant motilin in the crop and colon were not significant (NS) compared to that in the absence of motilin by one-way ANOVA (Dunnett's test) for comparing relative contractions.

2.3.6. Effects of motilin peptides and erythromycin on contraction of rabbit duodenum

The rabbit duodenum is highly sensitive to motilin and has been used for measurement of motilin-like activities of substances. Human motilin, chicken motilin, pheasant motilin (0.1 nM -1 μM) and erythromycin (1 nM - 10 μM) caused concentration-dependent contractions in the rabbit duodenum. The relative amplitudes of the maximum contractions were almost the same for human, chicken and pheasant motilin treatments (104.2 ± 6.2% for human motilin (n=6)), 100.7 ± 6.8% for chicken motilin (n=6), and 86.8 ± 6.9 for pheasant motilin (n=6)), while the relative amplitude was slightly lower for EM treatment (83.2 ± 2.7%, n=6) (Figure15). However, the affinities (EC_{50} values) of motilin peptides and erythromycin were different. The EC_{50} values were 4.2 ± 1.8 nM for human motilin (n=6), 23.0 ± 8.7 nM for chicken motilin (n=6), 22.0±10.8 for pheasant motilin (n=6) and 1.1 ± 0.2 μM for EM (n=6). The ranking order of the contraction was human motilin > pheasant motilin = chicken motilin > erythromycin. To confirm that the actions of motilin peptides are elicited by activation of the MLNR, the effect of pretreatment with GM109 (100 nM and 1 μM) on the concentration-response curve of motilin was examined. GM109 (100 nM and 1 μM) caused a rightward shift of the concentration response curves of chicken motilin and pheasant motilin. The EC_{50} values for chicken motilin in the presence of 100 nM and 1 μM GM109 were 30.8 ± 3.5 nM (n=6) and 190.5 ± 15.5 nM, respectively, and those for pheasant motilin were 31.8 ± 4.6 (n=6) and 167.4 ± 21.2 (n=4), respectively (Figure 16). Estimated pA2 values for GM109 were 7.2 ± 0.3 (n=6) for chicken motilin and 7.6 ± 0.2 (n = 6) for pheasant motilin. The contraction induced by EM (1 nM - 10 μM) was fully inhibited by pretreatment with GM109 (1 μM) (Figure 16).

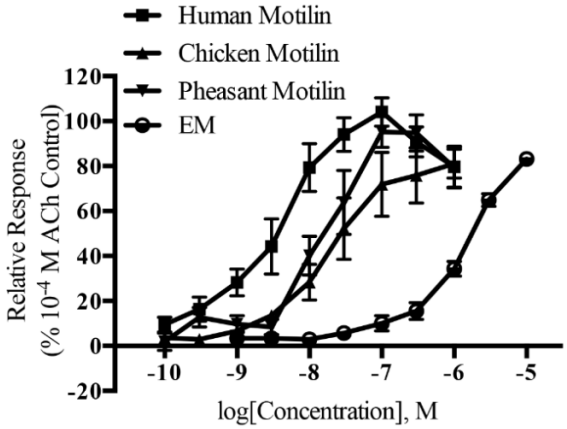

Figure 15. Contractile responses to human motilin, chicken motilin, pheasant motilin and erythromycin in the longitudinal muscle of rabbit duodenum. The symbols indicate the concentration-response curves for human motilin (■), chicken motilin (▲), pheasant motilin (▾) and EM (○) in the rabbit duodenum. The amplitude of contractile responses (y-axis) was normalized by a standard contraction by ACh (100 μM). The x-axes are concentrations (log M). Values are means ± S.E.M (n = 4-6).

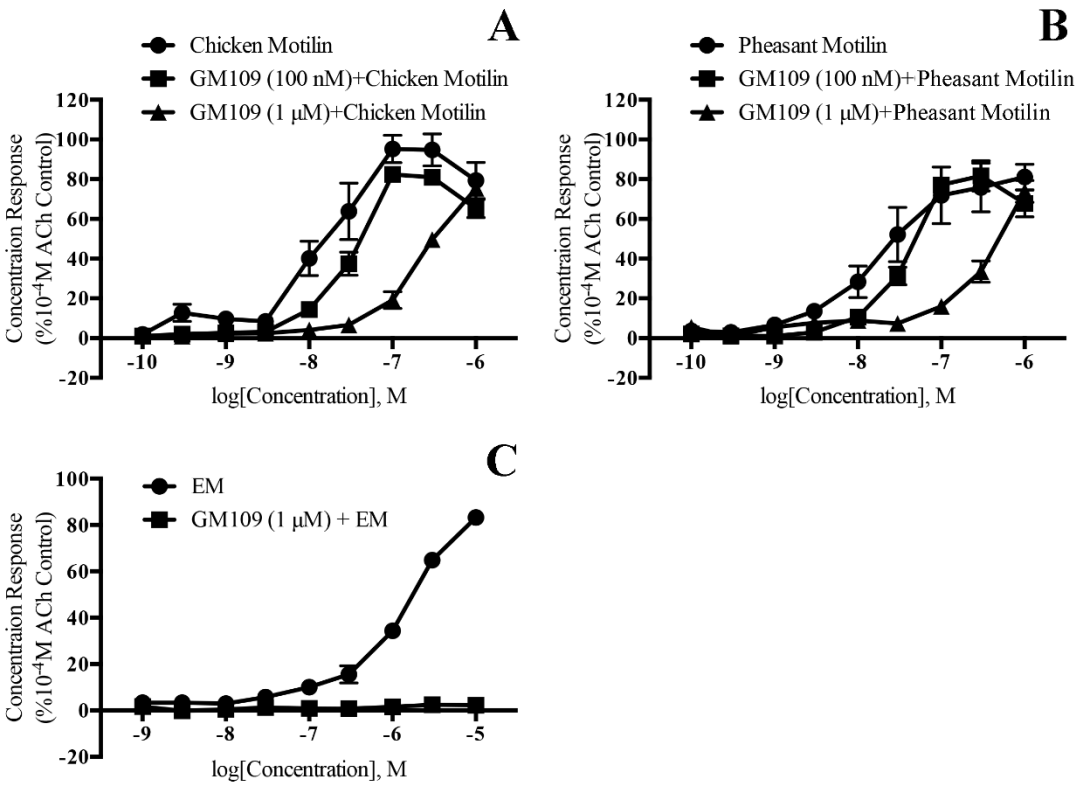

Figure 16. Effects of GM109 on contractile responses to chicken motilin, pheasant motilin and EM in the longitudinal muscle of rabbit duodenum. (A) Chicken motilin in the absence

(control, ●) and presence of GM109 (100 nM, ■) and (1 μM, ▲) in the duodenum of the rabbit. (B) Pheasant motilin in the absence (control, ●) and presence of GM109 (1 μM, ■) in the duodenum of the rabbit. (C) EM in the absence (control, ●) and presence of GM109 (1 μM, ■) in the duodenum of the rabbit. The amplitude of contractile responses (y-axis) was normalized by a standard contraction by ACh (100 μM). The x-axes are concentrations of motilin (log M) applied. Values are means ± S.E.M (n =4-6).

2.3.7. Mechanisms of motilin-induced contraction in pheasant gastrointestinal tract

To characterize chicken and pheasant motilin-induced contractions in the pheasant GI tract, the effects of GM109 and MA2029 [164,171] on the concentration-response curve for each motilin were examined. Pretreatment with GM109 (1 μM) did not have any effects on GI motility and did not change the concentration response curves for chicken motilin and pheasant motilin in the proventriculus and ileum (Figure 17). The EC_{50} value and maximum contraction of chicken motilin in the presence of GM109 were 35.6 ± 11.9 nM (n = 11) and 92.3 ± 9.7%, respectively (n = 11). For pheasant motilin, EC_{50} was 72.0 ± 26.0 nM (n = 6) and the maximum contraction was 84.9 ± 5.9% (n = 6) in the presence of GM109 (1□μM). Pretreatment with MA2029 (1 μM) also did not have any effects on the concentration response curves for chicken motilin and pheasant motilin in the ileum. For chicken motilin in the ileum, EC_{50} and the maximum response were 14.3 ± 8.3 nM (n = 4) and 92.6 ± 5.5% (n = 4), respectively, in the presence of MA2029 (1 μM). For pheasant motilin in the ileum, EC_{50} and the maximum response were 22.1 ± 5.2 nM (n = 4) and 106.0 ± 9.7% (n = 4), respectively, in the presence of MA2029 (1 μM) (Figure 17).

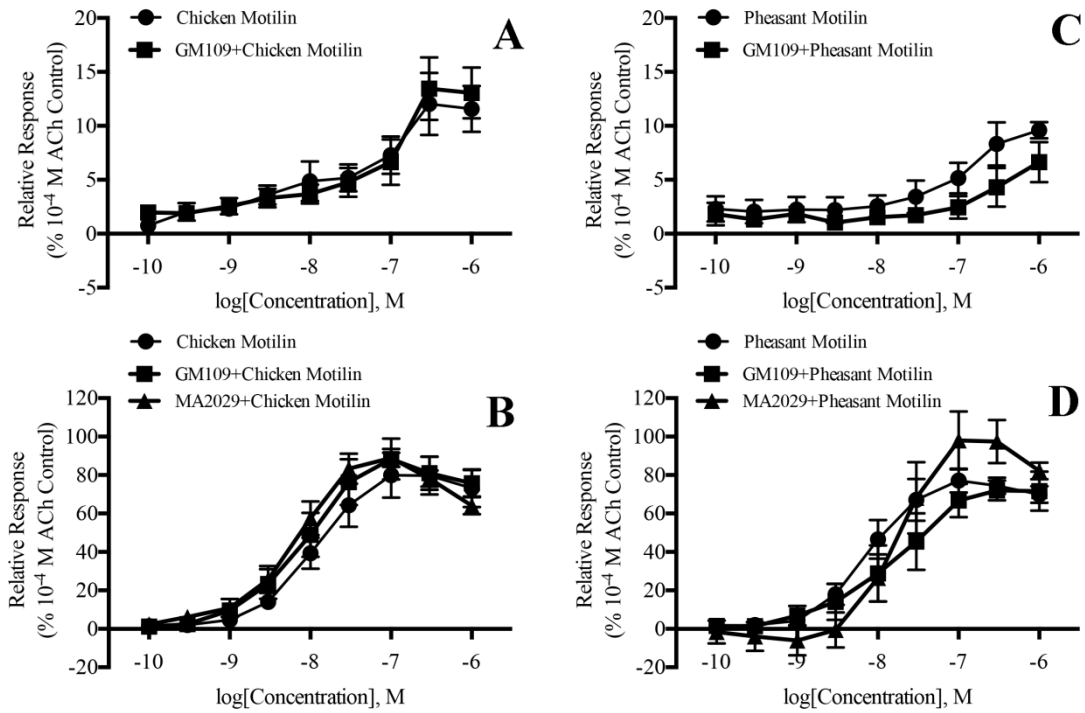

Figure 17. Effects of mammalian MLNR antagonists on contractile responses to chicken motilin and pheasant motilin in the proventriculus and ileum of the pheasant. (A) Chicken motilin in the absence (control, ●) and presence of GM109 (1 μM, ■) in the proventriculus. (B) Chicken motilin in the absence (control, ●) and presence of GM109 (1 μM, ■) or MA2029 (1 μM, ▲) in the ileum. (C) Pheasant motilin in the absence (control, ●) and presence of GM109 (1 μM, ■) in the proventriculus. (D) Pheasant motilin in the absence (control, ●) and presence of GM109 (1 μM, ■) or MA2029 (1 μM, ▲) in the ileum. The amplitude of contractile responses (y-axis) was normalized by a standard contraction by ACh (100 μM). The x-axes are concentrations of motilin (log M) applied. Values are means ± S.E.M (n = 4-17).

In *in vitro* experiments, action sites of motilin are thought to be located in the smooth muscle cells and enteric neurons [83]. The effects of TTX and atropine on the responses to chicken motilin and pheasant motilin were investigated to clarify the mechanisms of the contraction. As shown in Figure 18, motilin-induced contraction in the ileum was not affected by pretreatment with TTX (1 μM). For chicken motilin, the EC_{50} value (33.0 ± 12.8 nM, n = 10) and the maximum contractile amplitude (73.1 ± 6.9%, n = 10) were almost the same as those of the control. The contractile responses to chicken motilin in the duodenum and jejunum were also not decreased by pretreatment with TTX (EC_{50} and maximum contraction:

duodenum, 53.1 ± 15.7 nM and 69.60 ± 14.6%, respectively, n = 9; jejunum, 46.1 ± 20.4 nM and 58.3 ± 10.4%, respectively, n = 10). For pheasant motilin-induced responses in the ileum, EC_{50} (16.9 ± 4.5 nM) and maximum contraction (89.2 ± 3.3%, n = 4) were comparable with those of the control responses. On the other hand, motilin-induced responses in the proventriculus were decreased by TTX pretreatment (Figure 18). The relative amplitude of contraction at 1 μM of chicken motilin (3.3 ± 1.0%, n = 14) was significantly smaller than that of the control value (13.6 ± 2.9%, n = 14). The relative amplitude of contraction at 1 μM of pheasant motilin (1.8 ± 2.6%, n =4) was also significantly smaller than that of the control value (8.2 ± 0.6%, n = 8)

Atropine is used to investigate the involvement of cholinergic neurons in the motilin-induced responses. The effects of atropine on 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) (Figure 18). The pheasant motilin-induced response in the ileum was not decreased by atropine treatment (EC_{50}: 62.7 ± 48.2 nM, maximum contraction: 84.4 ± 9.7%, n=3), whereas atropine significantly decreased the contraction induced by pheasant motilin in the proventriculus. The relative amplitude of 1 μM pheasant motilin-induced contraction was only 3.7 ± 2.0% (n = 6)

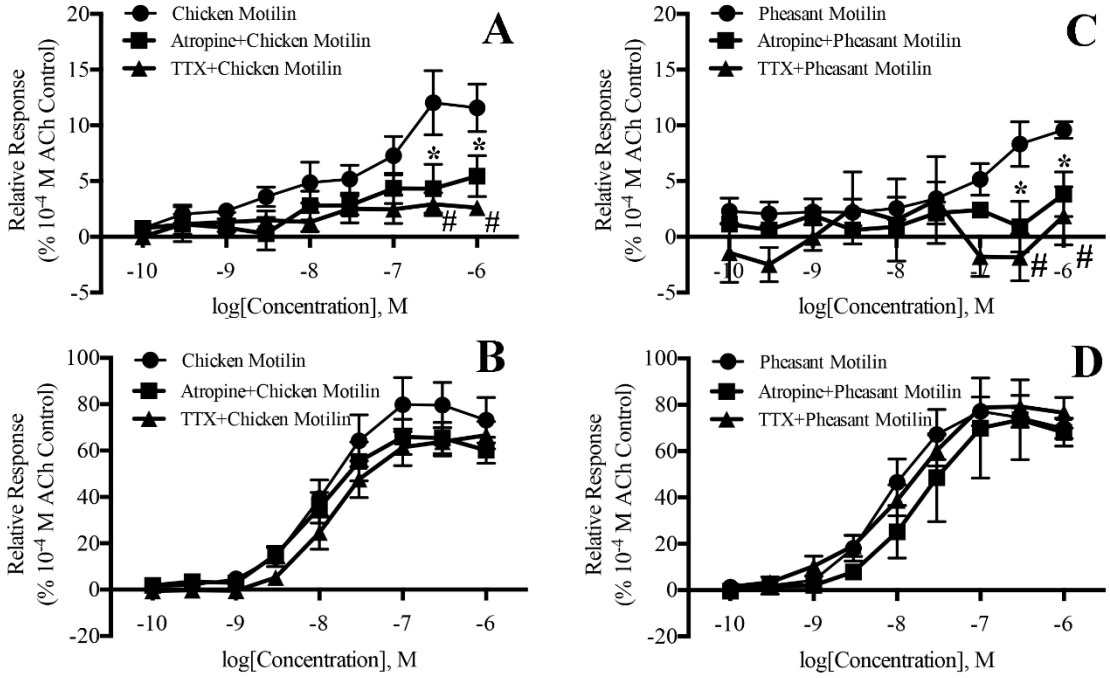

Figure 18. Effects of TTX and atropine on contractile responses to chicken motilin and pheasant motilin in the proventriculus and ileum of the pheasant. The symbols indicate concentration-response curves for chicken motilin (A: proventriculus, B: ileum) and pheasant motilin (C: proventriculus, D: ileum) in the absence (control, ●) and presence of tetrodotoxin (1 μM, ▲) or atropine (1 μM, ■). The amplitude of contractile responses (y-axis) was normalized by a standard contraction by ACh (1 μM). The x-axes are concentrations of chicken or pheasant motilin (log M). Values are means ± S.E.M (n = 4-17). *, # $P < 0.05$; compared with corresponding control responses to chicken motilin or pheasant motilin.

2.4. Discussion

The deduced mature sequence of pheasant motilin determined in this study was FVPFF TQSDI QKMQE KERIK GQ. It was exactly the same as that of turkey motilin, and there was difference of only one amino acid from that of chicken motilin or quail motilin. In mammals, the N-terminal part of the amino acid sequence is quite important and it was found to be able to affect full agonistic activity when examined using cell lines that overexpressed the MLNR GPR38 [132]. The first eight amino acids (FVPFFTQS), which comprise an important N-terminal structure for the activity of motilin, were identical among avian species (pheasant, chicken, turkey and quail). However, the N-terminal amino acids are not exactly the same

among humans, rhesus monkeys, cattle, horses, cats, dogs and the other mammals [68,83,180]. From the viewpoint of whole amino acid sequences of mammalian motilins, the structure of human motilin is the same as that of porcine motilin, but canine motilin is different in five amino acids, and house shrew motilin and rabbit motilin are different in three and four amino acids in 22 amino acids, respectively, from those of human motilin [68,83,180]. In avian species, chicken motilin is different from turkey, quail and pheasant motilins in 1 position of 22 amino acids [68,83,180]. Although further experiments using different species of birds available (pigeon, ostrich and duck) are needed, it is speculated that the species difference in motilin structure might be quite small in avian species. On the other side, a marked species-related variation of motilin structure exists in mammals. Mammals are the final stage of evolution and there are wide variations in foods and related functions of GI tracts. Variation of motilin structure in mammals might reflect these variations.

Motilin-immunopositive cells are localized as open-type endocrine cells scattered in the mucosa of the upper intestine (duodenum) in humans [160], monkeys, dogs [134], cats [46] and rabbits [153]. Although motilin-immunopositive cells were widely found from the gastric antrum to the distal colon in rabbits, the numbers of immunopositive cells were largest in the duodenum, moderate in the jejunum and small in the gastric antrum, ileum, caecum, proximal colon and distal colon [153]. Immunohistochemical studies also demonstrated that motilin-immunopositive cells were localized in the duodenum but not in the proventriculus and gizzard of chickens [37] and quails [3]. In a preliminary examination using a human motilin antibody (This antibody could detect quail motilin in a study by Apu *et al.* [3].), it was failed to detect motilin-immunopositive cells in the pheasant (data not shown). In the previous studies, Smith *et al.* (1981) could not detect immunopositive cells using an anti-rabbit motilin antibody, but Vogel and Brown (1990), Smith *et al.* (1981) and Sakai *et al.* (1994) demonstrated the distribution of motilin-immunopositive cells in the rat GI tract [144,161,184]. However, recent genome-wide analysis has revealed that mice and rats lack genes for motilin and its receptor [62,151], meaning false positive results with the antiserum. Taken together, these findings strongly suggest that the specificity and identity of the antiserum might be important for drawing a conclusion from an immunohistochemical study regarding the presence or absence of immunopositive substances. Therefore, immunohistochemical study should be done using a specific motilin antiserum after

identification of the species-specific endogenous form of motilin in the designated species. If an anti-pheasant motilin antibody is made, it might be possible to stain immunopositive cells in the pheasant GI tract.

The results of the molecular biological study showed high expression levels of motilin mRNA in the small intestine, including the duodenum, jejunum and ileum, of the pheasant. The heterogeneous presence of motilin in the GI tract is the same as that reported in other birds (chickens and quails) and mammals (humans, dogs, cats and rabbits) although the expression level of motilin in the duodenum was higher than the expression levels in the jejunum and ileum [3,37,46,133,153,160]. Therefore, as in mammals and other birds, motilin would be located in the mucosa of the upper small intestine of the pheasant, although direct evidence such as immunohistochemical detection was not obtained in this study by unmatched motilin antiserum.

In mammals, motilin is the present in upper GI tract, but species differences were found in the responsiveness to motilin on the contractility of the isolated GI tract in response to motilin. Motilin caused contraction of human, rabbit and house shrew GI strips, while it was ineffective in dog and pig GI tracts [83]. Motilin is also present in birds, and its GI contractility-stimulating actions have been investigated in chickens and quails [3,84,85,88]. In the present study using pheasants, a different species of birds, human motilin, chicken motilin and identified pheasant motilin caused contraction of the pheasant GI tract, and a species-related difference in responses of the GI tract to motilin was not observed among chickens, quails and pheasants, although further studies using different avian species are needed.

In the contraction study, the small intestines were much more sensitive to motilin than were the proventriculus, crop and colon. No significant difference in responsiveness to motilin was found among the duodenum, jejunum and ileum in the pheasant. Previous studies in chickens and quails have already demonstrated similar GI region-dependency of the magnitude of motilin–induced contractions [3,84,88]. Therefore, GI region-related differences in the responsiveness to motilin are common in avian GI tracts, and the differences might be due to the heterogeneous expression level of MLNR mRNA shown in chickens [90]. These results suggest that the small intestine is the main target organ of motilin in birds in physiological conditions. In fact, motilin has been shown to be a mediator of ROC in the

chicken small intestine observed in fasting periods [140]. In addition, although the responsiveness of the chicken proventriculus to motilin decreased with aging, that of the ileum did not change significantly with aging and the expression of MLNR mRNA in the ileum was the highest among GI regions [90].

De Clercq *et al*. (1996) showed the presence of an appropriate amount of motilin in the chicken colon [37]. In this study, the expression level of motilin mRNA in the colon was higher that the expression levels in other GI regions such as the crop, esophagus and caecum. A relatively high expression level of motilin mRNA in the colon might be a characteristic of the distribution of motilin in avian GI tract. However, motilin was not able to cause contraction of the colon in the pheasant and chicken, suggesting that motilin might not regulate colonic motility but might have a function for absorption and secretion in the colon [87].

Among motilin peptides and erythromycin, pheasant motilin and chicken motilin were more potent than human motilin. Interestingly, erythromycin, an MLNR agonist in mammals [127,144], did not cause any contraction even at 10 μM. On the other hand, the results in the rabbit duodenum were quite different from those in the pheasant ileum. Three motilin peptides and erythromycin caused almost the same amplitudes of maximum contractions, suggesting that these compounds act on the rabbit MLNR as full agonists. The ranking order of affinity in the rabbit duodenum was human motilin > chicken motilin = pheasant motilin > erythromycin, which is quite different from that in the pheasant ileum. The different sensitivities of the rabbit duodenum and pheasant ileum to MLNR agonists might be due to the difference in the structure of the pheasant MLNR from that in rabbits. This notion is also supported by the results of a functional study using MLNR antagonists. GM109 and MA2029, both antagonists, were quite ineffective of decreasing the responses to chicken motilin and pheasant motilin in the pheasant intestine, but they decreased the responses to chicken motilin and pheasant motilin in the rabbit duodenum. The pA2 value (affinity) of GM109 for the rabbit motilin receptor has been reported to be 7.3 when human motilin was used as an agonist [171]. The pA2 values of GM109 were 7.2 for chicken motilin and 7.6 for pheasant motilin in this study. pA2 values similar to that of human motilin suggest that chicken motilin and pheasant motilin cause contraction of the rabbit duodenum through activation of motilin receptors. In the rabbit duodenum, GM109 pretreatment fully blocked the contractile effect of

EM, confirming that GM109 is able to inhibit activation of the MLNR. The insensitivity of erythromycin (agonist) and GM109 (antagonist) in the pheasant was that same as that reported in the chicken GI tract [88]. Homology of the chicken MLNR with human and rabbit MLNRs has been reported to be 59% and 65%, respectively [188]. On the other hand, the homologies of mammalian MLNRs to the human MLNR are considerably high (rabbit: 84%, house shrew: 76%, dog: 71%) [32,123,165]. These results suggest that the structural difference of pheasant MLNR affects the affinity of the MLNR agonists and antagonists. Cloning of the pheasant MLNR could demonstrate the difference of the structure of MLNR.

The mechanisms of motilin-induced contraction were characterized by using atropine and TTX. Motilin-induced contractions in the small intestine were not attenuated by atropine or TTX, but those in the proventriculus were decreased by each blocker. These results indicate that motilin activates the MLNR in smooth muscle cells of the small intestine and the neural (cholinergic) MLNR in the proventriculus. The same region-related different contraction mechanisms have been already demonstrated in chickens and quails [3,88]. Taken together, the results for three bird species indicate that region-dependent different contractile mechanisms of motilin are a common characteristic in avian GI tracts.

The results for the pheasant GI tract indicated that motilin causes GI region-dependent contraction and that the mechanisms of contraction are different in regions of the GI tract, such as the proventriculus (neural origin) and small intestine (smooth muscle origin), as has been observed in other birds examined so far. Species-related difference in the responses to motilin reported in mammals (sensitive or insensitive to motilin) [83] was not observed in the avian species. Therefore, motilin might be a regulator of GI motility, especially in the small intestine, of avian species. However, the three species of birds (chicken, quail and pheasant) examined so far belong to the close families, and further examination using a quite different genus is required to draw a conclusion regarding the universality of the actions of motilin in the bird GI tract. Experiments conducted in conscious animals indicate that motilin increases the release of 5-hydroxytryptamine (5-HT) and 5-HT stimulates the vago-vagal reflex pathway through activation of 5-HT3 receptors on the afferent terminals of vagus nerves. Consequently, stimulation of vagus efferent neurons stimulates neurons in the myenteric plexus causing contractions [67]. Therefore, to understand functional roles of motilin in regulation of GI motility, *in vivo* contraction experiments and simultaneous sampling of blood

to measure motilin are needed in future.

CHAPTER 3.
IDENTIFICATION OF PHEASANT GHRELIN AND ITS EFFECTS ON THE GASTROINTESTINAL CONTRACTION

3.1. Introduction

Ghrelin has been identified in the gastric mucosa of mammals and non-mammals, and it has been shown to be a gut peptide with multiple functions including regulation of GH release, glucose homeostasis, food intake, endocrine and exocrine pancreatic functions, cardiac function and regulation of GI motility [92,94,152]. Since ghrelin shows structural homology with motilin, GHS-R1a also shows structural homology with motilin receptor, they are thought to be derived from the same ancestor gene [6,128]. Therefore, the actions of ghrelin on GI motility have also been focused on. Stimulatory action of ghrelin on GI motility has been investigated in humans and some experimental animals including rodents [44,54,80,168]. In mice and rats, ghrelin stimulates GI contractility both *in vitro* and *in vivo* experimental conditions, and the ghrelin system is thought to act as a substitute for the motilin system, especially in regulation of GI motility, such as initiation of the MMC [4,54,195], where motilin and the MLNR have been found to be pseudogenes in these species [73,151].

The mechanisms of ghrelin-induced GI contractility-stimulating actions are different depending on the species, experimental methods (*in vitro* or *in vivo*) and GI regions. *In vivo* experiments in conscious rats and house shrew 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 [54,112]. Expression of GHS-R1a in vagal afferent nerve terminals and enteric neurons has been demonstrated [146]. *In vitro* experiment in rats showed that ghrelin alone did not cause any mechanical responses in non-electrically stimulated preparations but potentiated electrical stimulation-induced contraction through activation of neural GHS-R1a in mice [44,80], as has been demonstrated by an immunohistochemcal study [33]. At present, smooth muscle receptors like MLNR have not been reported to be involved in the ghrelin-induced actions in mammals. In contrast, chicken ghrelin caused contraction of the chicken crop through activation of smooth muscle receptors [84]. Smooth muscle GHS-R1a has only been found in chickens, suggesting that chickens are suitable animals for analysis of ghrelin actions in GI motility [89]. However,

ghrelin did not cause any changes in contractility of the GI tract of Japanese quails despite clear expression of *GHS-R1a* mRNA (Table 4) [3,85]. Therefore, actions of ghrelin in the avian GI tract are species-dependent and different from those of motilin. Further studies using different species are needed to determine what the general actions of ghrelin in avian GI tracts are.

Table 4. Comparison of the presence of ghrelin and GHS-R1a and the GI contractility-stimulating action of ghrelin in different species of birds.

Species	Ghrelin	GHS-R1a	GI contractility
Chicken (*Gallus gallus domesticus,Gallus*)	○	○	Contraction
Quail (*Coturnix coturnix,Coturnix*)	○	○	No effect
Turkey (*Meleagris, Meleagris*)	○	○	?
Pheasant (*Phasianus colchicus, Phasianus*)	?	?	?

(○) The structure has been identified. (?) No report.

In some mammals, such as dogs and house shrew, that express both ghrelin and motilin and their receptors in the GI tract, interaction of the two peptides in the regulation of GI motility has been reported. Ghrelin itself did not produce any contractile responses in the house shrew stomach, but it caused obvious contraction in the presence of a low concentration of motilin (a concentration that does not induce contraction), and in the presence of ghrelin, motilin-induced contraction was also potentiated both in *in vitro* and *in vivo* experimental conditions [115]. On the other hand, ghrelin inhibited motilin-induced gastric contraction through GHS-R1a in conscious dogs [121]. It was shown that ghrelin decreased motilin release and that motilin decreased ghrelin release in dogs fasting periods. Therefore, phase III activity of the MMC was decreased by infusion of ghrelin. A plasma ghrelin peak was observed in phase I and ghrelin concentration was minimum during the phase III period in dogs [121]. These findings suggest that ghrelin is a regulator of motilin release and is able to regulate the cyclic change of motilin in dogs, although the mechanisms of the cyclic change of ghrelin are not clear at present. Expression of both ghrelin and motilin has also been demonstrated in chickens and quail. There was no interaction of ghrelin and motilin in the quail intestine [3]. However, study of ghrelin and motilin interaction in GI motility has been

limited to the quail, and a comparative study using another avian species is necessary.

In this study, at first, the ghrelin gene sequence and the distribution of ghrelin mRNA in several organs of the pheasant were determined. Second, in a study on GI tract contraction, actions of rat, quail and chicken ghrelins on contractility of isolated pheasant GI tracts were examined. Finally, interaction of ghrelin and motilin in the pheasant GI tract was examined. Namely, the effect of pretreatment with ghrelin on motilin-induced contraction was examined. The effect of a low concentration of motilin on the ghrelin-induced contraction was also examined.

3.2. Materials and methods

3.2.1. Ethical approval

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

3.2.2. Chemicals

The following chemicals were used in the experiments: RNAlater solution (Invitrogen by Thermo Fisher Scientific, Lithuania), TRI reagent (Molecular Research Center, OH, USA), ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd.), Thunderbird SYBR qPCR Mix (Toyobo Co., Ltd.), 2-propanol (Nacalai Tesque, Inc., Kyoto, Japan), chroroform (Nacalai Tesque, Inc.), ethanol (Nacalai Tesque, Inc.), KOD-Plus (Toyobo Co., Ltd.), nuclease-free water (Promega Co.), ISOGEN (Nippon Gene Co., Ltd.), DNase digestion (Promega Co.), prime Script II Reverse Transcriptase (Takara Bio Inc.), ExTaq DNA polymerase (Takara Bio Inc.), isoflurane (Pfizer Japan), proteinase K (20 μg/ml, Proteinase K ready-to-use, Dako Japan Inc.), phosphate-buffered saline (PBS) (pH 7.4) (Dainippon-Parma Co., Ltd., Osaka, Japan), blocking solution (Protein Block serum free, Dako North America Inc.), antibody diluent (Antibody Diluent with Background Reducing components, Dako North America Inc.), second antibody solution (Labelled Polymer, HRP Anti-rabbit Envision, Dako North America Inc.), NaCl, KCl, $MgSO_4$, KH_2PO_4, $CaCl_2$ and tetrodotoxin (Wako Pure Chemical Industries Ltd.), $NaHCO_3$ and glucose (Kanto Chemical Co., Inc.), acetylcholine (Wako Pure Chemical Industries Ltd.), erythromycin (U.S. Pharmacopeial Co. Inc.), rat ghrelin (Peptide Institute Inc.), chicken ghrelin and quail ghrelin (synthesized by Peptide Institute Inc.), atropine

(Sigma-Aldrich), isoflurane (Zoetis Inc.), Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany), ExTaq DNA polymerase (Takara Bio Inc.), and malinol medium (Muto Pure Chemicals Co., Ltd., Tokyo, Japan).

3.2.3. Animals and tissue preparations

Male and female pheasants (*Phasianus colchicus versicolor,* ~30 days after hatching, 300 ~ 450 g) were obtained from a farm (Work Tsukasa) in Iwamizawa City, Hokkaido, Japan. The pheasants were anesthetized with isoflurane, stunned, and bled to death. The brain, heart, lung, liver, pancreas, esophagus, crop, proventriculus, small intestine, caecum, gizzard 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 tissues were used for immunohistochemical and contraction studies. Tissues were also cut into small pieces and stored in RNAlater solution for molecular studies.

3.2.4. Cloning of pheasant ghrelin

Pheasant ghrelin gene was determined by 3'- and 5'-RACE PCRs. For 3'- RACE PCR, total RNA (1 μg) from the proventriculus was transcribed with GeneRacer 3' Oligo-dT Primer using a Transcriptor High Fidelity cDNA Synthesis Kit (final volume of 20 μl). Primary 3'-RACE PCR was performed with 2 μl of a template, 100 pmol/μl of degenerated primers for a common sequence of ghrelin (GSSFLSP-dg-s1, 5'-GGN TCN AGY TTY TTR TCN CCN-3', GSSFLSP-dg-s2, 5'-GGN TCN AGY TTY TTR AGY CCN-3', GSSFLSP-dg-s3, 5'-GGN TCN AGY TTY CTN TCN CCN-3', GSSFLSP-dg-s4, 5'-GGN TCN AGY TTY CTN AGY CCN-3'), 3'-primer and ExTaq DNA polymerase. The reaction conditions were 94 °C for 2 min followed by 35 cycles of 94 °C for 0.5 min, 53 °C for 0.5 min and 72 °C for 1 min with final extension at 72 °C for 3 min. The amplified product was purified by the Wizard PCR Preps DNA Purification System (Promega Co.), and the resultant product was subjected to second-round nested PCR. Nested PCR was conducted with another 100 pmol/μl of a degenerated sense primer designed by a common sequence of avian ghrelin (KijiGRL-dg-s1, 5'-GAA TWT AAA AAM ATA CAG CAA CAA-3') combined with degenerated anti-sense primers (KijiGRL-dg-AS1 [5'-AGT TTC TTT AGC ATT KTC TTY-3'] and dg-AS2 [5'-KTC

TTY RAG AAT GTC CTG TAG- 3']) or a 3'-nested primer, template after purification by PCR preps and ExTaq DNA polymerase under conditions similar to those for the primary PCR with only modification of the annealing temperature to 57 °C. The obtained product was sub-cloned into the pCRII-TOPO vector (Life Technologies Japan), and the nucleotide sequence was determined according to the protocol of the BigDye^{TM} 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 the 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 0.5min 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.

3.2.5. Measurement of ghrelin mRNA expression in pheasant organs

We measured mRNA expression levels of ghrelin in different organs including the brain, heart, lung, liver, pancreas, esophagus, crop, proventriculus, small intestine, caecum, gizzard and colon in three pheasants. Total RNA was extracted from tissues (less than 0.1g) with TRIzol (Molecular Research Center, Inc., OH, USA). cDNA was synthesized from total RNA (500 ng) with a ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd.). Real-time RT-PCR analysis was performed using a real-time PCR detector (LightCycler480: NIPPON Genetics, Tokyo, Japan) with Thunderbird qPCR mix containing SYBR Green (Toyobo Co., Ltd.). The primer set used for detection of the ghrelin (Accession No. XM_031605285.1) was 5'-CTAGGAATTCTCCTTCTCAGGATC-3' (Forward) and 5'-AGAATGTCCTGTAGCATCTTCTCC-3' (Reverse) (product size, 293 bp). Amplification conditions were initial incubation at 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. GAPDH was used as an internal control. The primers set used for

detection of GAPDH (Accession No. XM_031610235.1) was 5'-CAATGTCTCTGTTGTTGACCTG-3' (Forward) and 5'-ACCATTGAAGTCACAGGAGAC-3' (Reverse) (product size, 151 bp). Results are presented as arbitrary units of $2^{-\Delta Ct}$ (where ΔCt = Ctghrelin - CtGAPDH).

3.2.6. Immunohistochemistry of ghrelin in pheasant gastrointestinal tract

Pheasants were euthanized by exsanguination via the abdominal aorta under deep anesthesia with isoflurane. The digestive canal including the esophagus, crop, proventriculus, 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, and mounted on gelatin-coated (super-frost) glass slides. For immunohistochemical detection of ghrelin-immunopositive (ip) cells, the sections were deparaffinized with xylene and dehydrated with ethanol. After immersion in deionized water, proteinase K (20 μg/ml, Proteinase K ready-to-use, Dako Japan Inc.) 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) was 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 medium. The sections were viewed under a light microscope (FSX100, OLYMPUS, Tokyo, Japan).

3.2.7. Measurement of effects of ghrelin on pheasant gastrointestinal contraction

Effects of ghrelin on contractility of the pheasant GI tract were examined using the same method as that described in Chapter 2 (Section 2.2.3.2). In brief, the crop and proventriculus were cut open, and muscle strips in the longitudinal muscle direction (1 mm in width and 10-15 mm in length) were prepared. 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. Each muscle strip was suspended vertically in an organ bath containing warmed Krebs solution bubbled with 95% O_2 and 5% CO_2.

Mechanical activity in the longitudinal muscle direction was measured with an isometric force transducer, recorded on a computer, and analyzed using a computer-aided analysis 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 ghrelin, each muscle strip was subjected to 3 or 4 continuous stimulations with 100 μM ACh (15-min intervals) until a reproducible contraction was obtained.

In the experiment, 1 μM ghrelin was applied to an organ bath to examine the effectiveness of ghrelin as described in a previous report [84]. If this concentration of ghrelin caused contraction, concentration-response relationships were established by applying different concentrations of ghrelin.

Interaction of ghrelin and motilin for contractility of the pheasant GI tract was investigated as follows. First, in the presence of ghrelin (1 μM), chicken motilin was added cumulatively to establish concentration-response relationships and compared with the condition without ghrelin (control). Second, in the presence of different concentrations of motilin that did not cause contraction alone, ghrelin (1 μM) was applied and the responses induced by ghrelin were compared with the control without motilin.

3.2.8. Statistical analysis

The experimental data are expressed as means ± SEM of more than three experiments. The significance of differences between the values was determined at $P < 0.05$ using Student's t-test (paired) for single comparisons or ANOVA followed by Dunnett's test for multiple comparisons by GraphPad Prism6 (GraphPad Software Inc., CA, USA).

3.3. Results

3.3.1. Structure of pheasant ghrelin

Pheasant ghrelin cDNA was cloned from mRNA of the proventriculus, and its nucleotide sequence was determined (Figure 19) (Acc# LC459605). The deduced amino acid sequence of mature ghrelin was 26 amino acids (GSSFLSPAYKNIQQQKDTRKPTGRLH). Two amino acids at positions 8 and 23 in pheasant ghrelin were different from those in chicken ghrelin, and three amino acids at positions 17, 22 and 23 were different from those in Japanese quail ghrelin. Turkey ghrelin is a 28-amino-acid peptide, and within the sequence [1–26], only one amino acid at position 23 was different from that of pheasant ghrelin (Figure 20).

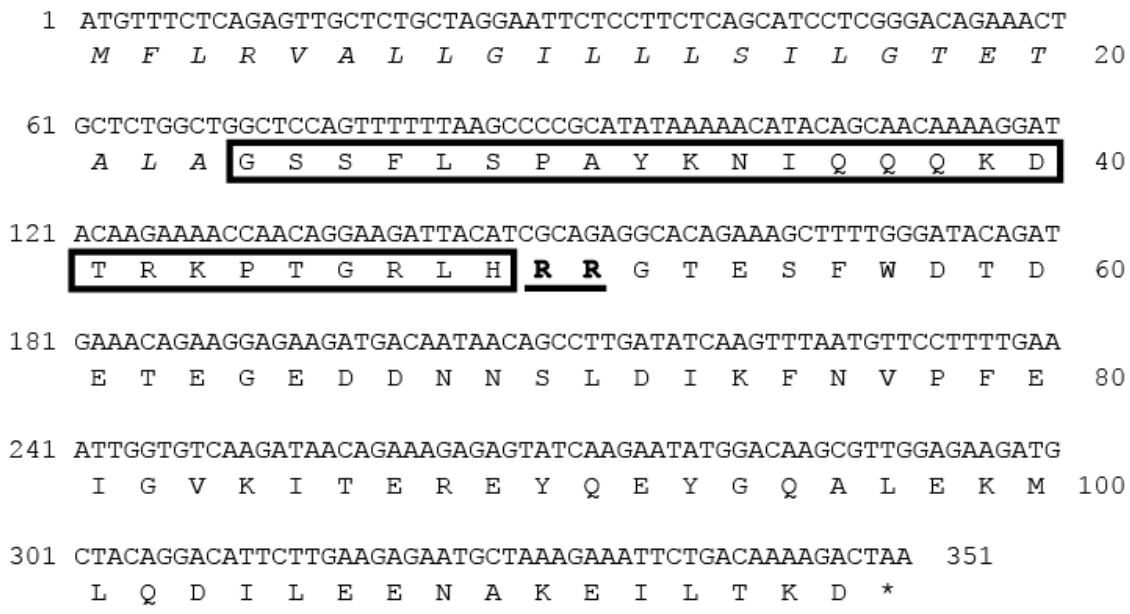

Figure 19. Pheasant ghrelin structure. Nucleotide sequence encoding the pheasant ghrelin precursor. The nucleotide sequence has been deposited in the DDBJ/ EMBL/GenBank databases with the accession no. LC459605. Mature ghrelin peptide is boxed, and a dibasic cleavage site (Arg-Arg) is indicated by bold letters and underline.

```
Pheasant          1 GSSFLSPAYKNIQQQKDTRKPTGRLH-- 26
Chicken           1 GSSFLSPTYKNIQQQKDTRKPTARLH-- 26
Duck              1 GSSFLSPERKKIQQQNDPTKTTAKIH-- 26
Emu               1 GSSFLSPDYKKIQQRKDPRKPTTKLH-- 26
Goose             1 GSSFLSPEFKKIQQQNDPAKATAKIH-- 26
Japanese quail    1 GSSFLSPAYKNIQQQKNTRKPAARLH-- 26
```

Figure 20. Comparison of amino acid sequences of mature ghrelin in some birds. Conserved amino acids among all species are indicated by bold. The amino acid sequence of ghrelin was aligned with those of the chicken (AB075215), duck (AY338466), emu (AY338467), goose (AY338465), Japanese quail (AB244056) and turkey (AY333783).

3.3.2. Ghrelin mRNA distribution in pheasant organs

Figure 21 shows the distribution of pheasant ghrelin mRNA in various organs of the pheasant. In the GI tract, the relative expression level was very high in the proventriculus (0.167±0.044) and was almost negligible in other regions of the GI tract, including the esophagus, crop, gizzard, duodenum, jejunum, ileum, caecum, colon and rectum. The relative expression levels in the heart (0.00544±0.00308), lung (0.00400±0.00285) and pancreas (0.00161±0.00055) were higher than those in other organs but were less than 3% of the expression level in the proventriculus.

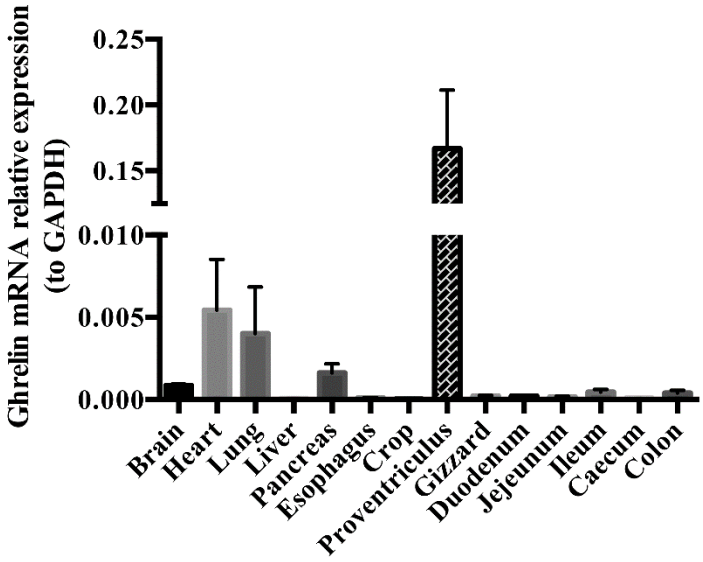

Figure 21. Ghrelin mRNA expression in different organs of the pheasant. Relative expression was calculated using $2^{-\Delta Ct}$ targeting on the housekeeping gene GPADH (Arbitrary unit). Values are means ± S.E.M (n = 3).

3.3.3. Immunohistochemistry for ghrelin in pheasant gastrointestinal tract

Three antibodies were used for detection of ghrelin-immunopositive cells in the pheasant GI tract. The antibody for octanoyl ghrelin failed to stain any cells. But antibodies for decanoyl ghrelin and unacylated ghrelin were able to stain scattered ghrelin-containing cells in the mucosa of the proventriculus. The number of decanoyl ghrelin-immunopositive cells was comparable to the number of ghrelin-immunopositive cells detected by the unacylated ghrelin antibody (5-6 cells/160 mm^2) (Figure 22). A few ghrelin-immunopositive cells were detected in the mucosa of the duodenum by the unacylated ghrelin antibody but not by the other antibodies (Figure 22).

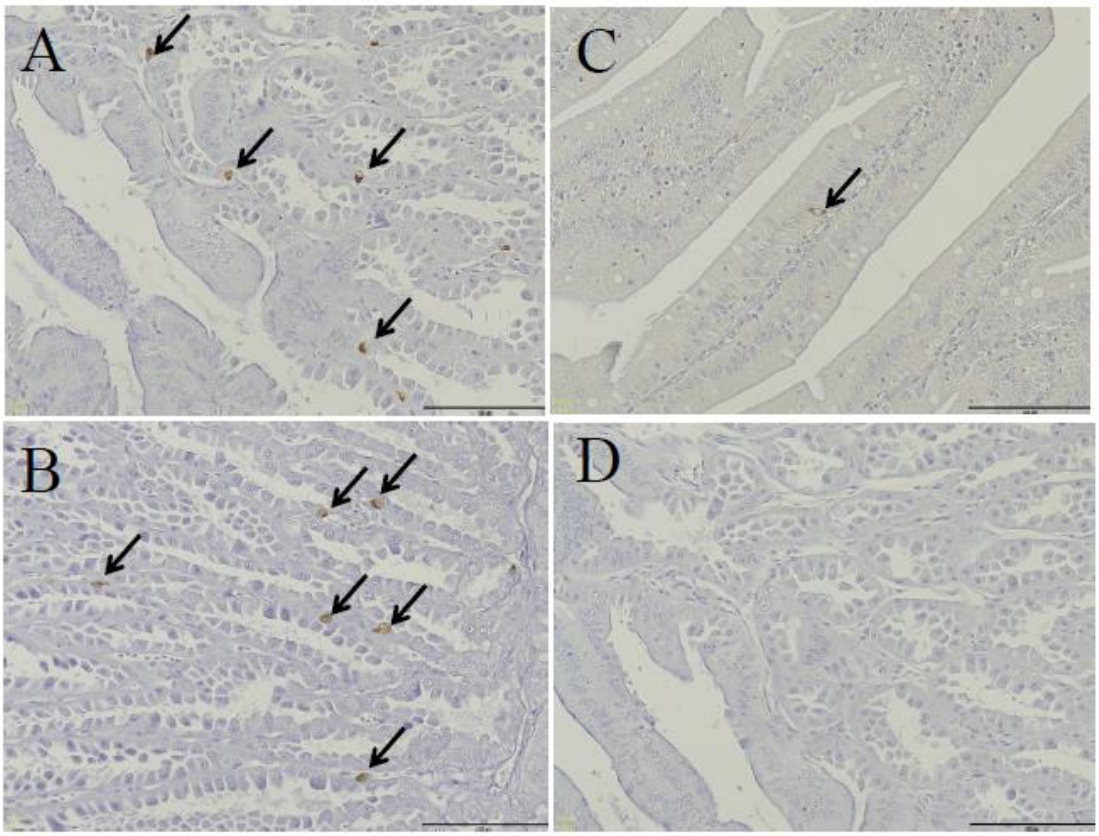

Figure. 22. Ghrelin-immunopositive cells in the proventriculus and duodenum of the pheasant. A: Proventriculus. Arrows indicate immunopositive cells stained by antiserum that recognizes unacylated ghrelin. B: Proventriculus. Arrows indicate immunopositive cells stained by antiserum that recognizes decanoylated ghrelin. C: Duodenum. Arrows indicate immunopositive cells stained by antiserum that recognizes unacylated ghrelin. D: Proventriculus stained by normal rabbit serum (negative control). Horizontal bars indicate 100 μm.

3.3.4. Effects of ghrelin on pheasant gastrointestinal contraction

Figure 23 shows typical mechanical responses to three kinds of ghrelin in the pheasant GI tract. Rat, chicken and quail ghrelins (1 μM) did not cause any contractility changes in the crop, proventriculus, ileum and colon. The relative changes in muscle tonus (contraction by 100 μM ACh=100%) 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), 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%

and 1.9 ± 1.3% in the colon (n = 4), respectively. There were no significant differences in muscle tension in the absence and presence of each ghrelin.

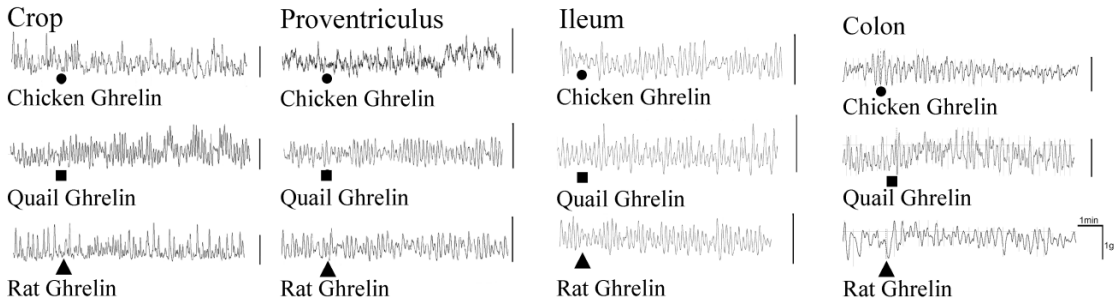

Figure 23. Representative effects of rat, quail and chicken ghrelin on spontaneous contractility of the crop, proventriculus, ileum and colon isolated from pheasants. Each ghrelin at 1 μM was applied at the mark (●, ■, ▲) and effects were observed for 5 min.

3.3.5. Effects of pretreatment with ghrelin on motilin-induced contraction in pheasant gastrointestinal tract.

The effects of pretreatment with chicken ghrelin on responses to chicken motilin in the crop, proventriculus and ileum were examined. Concentration-response curves of chicken motilin did not change in the presence of 1 μM chicken ghrelin in the isolated crop, proventriculus and ileum (Figure 24). Pretreatment with 1 μM quail ghrelin also did not change the responses to chicken motilin in the proventriculus and ileum (not shown).

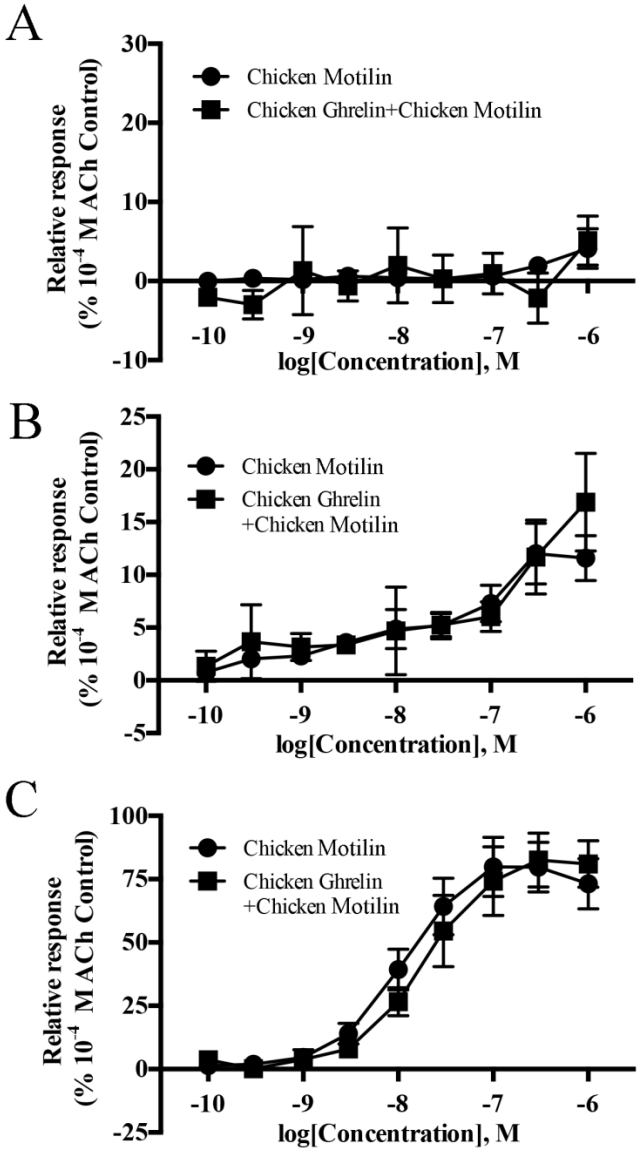

Figure 24. Effects of pretreatment with 1 μM chicken ghrelin on chicken motilin-induced responses in the crop, proventriculus and ileum. Concentration-response curves for chicken motilin were constructed in the crop (A), proventriculus (B) and ileum (C) in the absence (control, ●) and presence of chicken ghrelin (1 μM, ■). The amplitude of contractile responses (y-axis) was normalized by a standard contraction by 100 μM ACh. The x-axes are concentrations of chicken motilin (log M). Values are means ± S.E.M (n = 4-6).

3.3.6. Effects of pretreatment with motilin on ghrelin-induced responses in pheasant gastrointestinal tract.

We also examined the effects of pretreatment with low concentrations of chicken motilin (0.3 nM, 3 nM and 10 nM) on ghrelin-induced responses in the crop and proventriculus. These concentrations of chicken motilin did not cause any contraction changes in the preparations, and pretreatment with chicken motilin (0.3 nM, 3 nM and 10 nM) did not affect the contraction response to 1 μM chicken ghrelin (Figure 25). Chicken ghrelin (1 μM) did not cause any contraction of the ileum in the presence of three chicken motilin concentrations. In the crop, proventriculus and ileum pretreated with three chicken motilin concentrations, quail

ghrelin (1 μM) did not cause any contraction (data not shown).

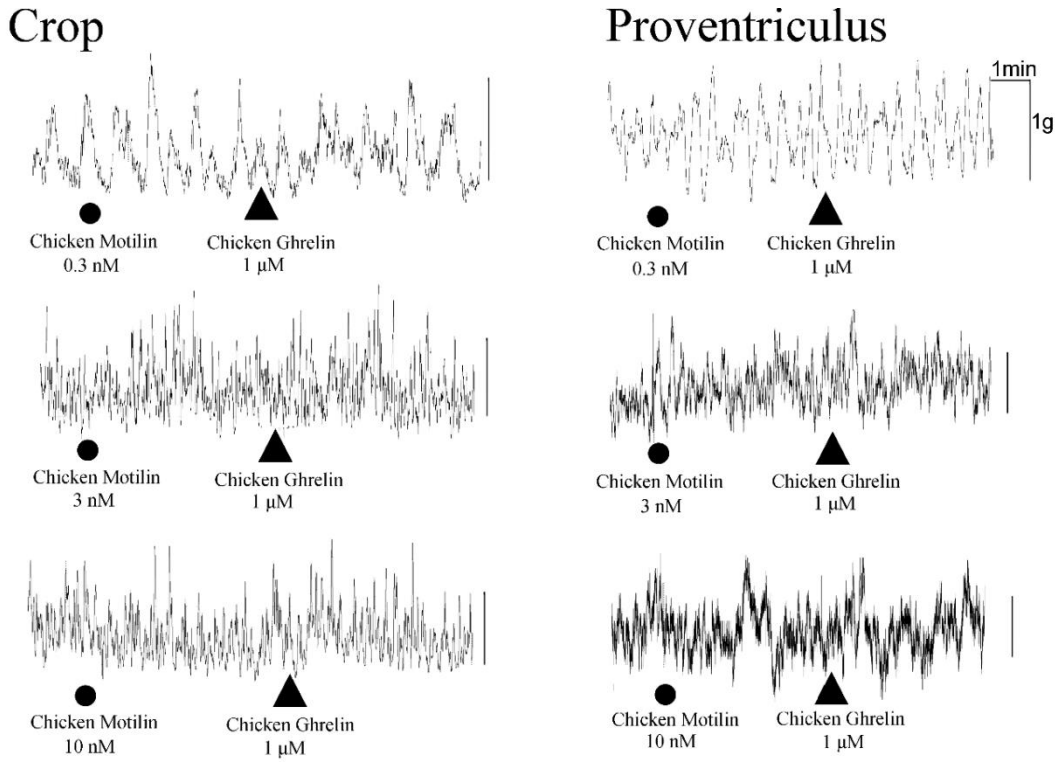

Figure 25. Representative effects of pretreatment with chicken motilin on ghrelin-induced mechanical responses in the crop and proventriculus. The crop and proventriculus were pretreated with three different concentrations of chicken motilin (0.3 nM, 3 nM and 10 nM) for 5 min and then chicken ghrelin (1 μM) was applied in the organ bath to observe contractile responses after that. Pretreatment time (5 min) was sufficient for appearance of motilin-induced response at an effective concentration.

3.4. Discussion

In the present study, ghrelin was also identified in the pheasant as in other avian species. Pheasant ghrelin was determined to be a 26-amino-acid peptide with an amino acid sequence of GSSFL SPAYK NIQQQ KDTRK PTGRLH. Compared with the structure of ghrelin in other birds, the N-terminal [1-7] sequence (GSSFLSP) is completely conserved, but the overall sequence is different from chicken ghrelin at positions 8 and 23 and quail ghrelin at positions 17, 22 and 23. Since the structure of pheasant motilin was found to be the same as that of turkey motilin, pheasant ghrelin was compared with turkey ghrelin. Only one amino

acid at position 23 was different within the 26-amino-acid sequences, though turkey ghrelin is composed of 28 amino acids in total. The structural similarity of motilin and ghrelin would be due to the close phylogenetic position between pheasants and turkeys.

In the immunohistochemical study, ghrelin-immunopositive cells were detected in the mucosal layer of the proventriculus, and their shape was a round, closed-type as observed in chickens [185,189]. Interestingly, the ghrelin-immunopositive cells were stained by a specific antibody for decanoyl ghrelin but not by a specific antibody for octanoyl ghrelin, suggesting that Ser3 of pheasant ghrelin is likely to be acylated by decanoic acid. In the case of chickens, Ser3 of ghrelin was modified by both octanoic acid and decanoic acid [78]. Ghrelin-immunopositive cells were also detected in the duodenum by an antibody for unacylated ghrelin but not by antibodies for octanoyl ghrelin and decanoyl ghrelin, suggesting that duodenal ghrelin is not acylated, although the function of unacylated ghrelin has not been clarified. In addition, the cell shape was an elongated type, which was observed in intestinal ghrelin-immunopositive cells in the chicken and rainbow trout [145,185]. The molecular cloning study revealed the amino acid structure of pheasant ghrelin, but it was not possible to identify and to determine the modification of Ser3. Purification of pheasant ghrelin from the proventriculus extract and then determination of the type of modification would be needed.

It has been demonstrated that ghrelin is located in the mucosa of the stomach in mammals [94]. In avian species, ghrelin is mainly located in the mucosa of the chicken proventriculus [78,139]. Similar to chickens, ghrelin mRNA is mainly expressed in the pheasant proventriculus but not in the gizzard, and the presence of ghrelin in the proventriculus mucosa was supported by the results of the immunohistochemical study. In the case of fish, amphibians and reptiles, high expression levels of ghrelin mRNAs were found also in the stomach [76]. Therefore, the glandular stomach (proventriculus) is the main organ in which ghrelin is produced and stored in vertebrates. The presence of ghrelin in the stomach suggests that the stomach is an endocrine organ to receive food and to secrete ghrelin for the regulation of food intake and GI functions such as contractility, secretion and absorption.

Although the amino acid sequence of pheasant ghrelin was identified, modification of Ser3 was not clarified in this study. Therefore, we used chicken ghrelin and quail ghrelin in the contraction study because the structure of pheasant ghrelin is similar to that of chicken ghrelin and quail ghrelin. Since chicken ghrelin, but not rat ghrelin, caused contraction of the

chicken GI tract [84], the effects of rat ghrelin were also examined for comparison. In the present study, three types of ghrelin (rat, chicken and quail) at 1 μM did not cause any marked contraction of the crop, proventriculus, ileum and colon of the pheasant. Since that concentration of ghrelin is sufficient to cause contraction in the chicken GI tract [84], the results suggested the ineffectiveness of ghrelin in the pheasant GI tract. The mechanical actions of ghrelin in the avian GI tract were reported to be different in chickens and quails [3,84,85]. Chicken ghrelin caused contraction of the chicken proventriculus and crop, but there was no contraction caused by quail ghrelin in the same regions of the quail GI tract even the expression levels of GHS-R1a mRNA in the quail crop and proventriculus were almost the same as those in the chicken [85]. However, the effects of a centrally injected low concentration of ghrelin on food intake were different in the chicken (inhibition) and the quail (enhancement) [142,158]. The difference in ghrelin-induced central actions suggests different functions of ghrelin in the two avian species and it is reflected in the different results in the contraction study [85]. The response to ghrelin in the pheasant GI tract was similar to that in the quail, even though the chicken, quail and pheasant belong to the same order Galliformes and the same family Phasianedae. Chicken GHS-R1a couples to the contraction apparatus, but quail GHS-R1a is not liked to contraction, suggesting that ghrelin might have other physiological roles in the GI tract of the quail and pheasant. Although other studies using different birds are needed, GI tracts of birds can be classified into ghrelin-sensitive GI tracts such as that in chickens and ghrelin-insensitive GI tracts such as those in quails and pheasants. The results suggest that control of GI motility by ghrelin varies among avian species and that ghrelin might not be a common regulator of GI motility in birds. Ghrelin might have other functions in the GI tract such as regulation of gastric acid secretion [107]. The physiological significance of this difference (ghrelin-sensitive GI tracts and ghrelin-insensitive GI tracts) is unclear at present. There is one speculation for the difference in ghrelin-induced contraction among these species (chicken, quail and pheasant). Chickens have been domestic animals for a long time, and food is generally applied consequently and they do not feel hunger. Quails and pheasants are now domestic animals, but their history as domestic animals is shorter than that of chickens. Quails and pheasants generally cannot easily get food and feel hunger sometimes. The longer history of chickens as domestic animals with constant feeding might have changed the function of the endocrine (ghrelin) system concerning regulation of GI

motility, food intake, hunger and satiety. Further study is needed to evaluate this speculation

In some mammals, such as dogs and house shrew that express both ghrelin and motilin and their receptors in the GI tract, an interaction of the two peptides in GI motility has been examined. Human ghrelin caused contraction of the house shrew stomach only in the presence of a low concentration of motilin in both *in vitro* and *in vivo* experiments, while ghrelin alone was ineffective in the absence of motilin [115]. In addition, motilin-induced contraction is also potentiated in the presence of ghrelin [115]. In conscious dogs, ghrelin applied in the phase II of the MMC inhibits phases II and III of the MMC despite the fact that ghrelin does not induce any mechanical action in phase I [122]. Measurement of both motilin and ghrelin concentrations indicated that ghrelin inhibits motilin release [121] and that the presence of ghrelin and motilin systems has been demonstrated also in birds (chicken and quail), but an interaction of ghrelin and motilin has not been observed in the quail GI tract [3]. In the present experiments, chicken motilin-induced contraction was not affected by pretreatment with quail ghrelin or chicken ghrelin in the pheasant proventriculus and ileum, and a low concentration of chicken motilin that does not cause any contraction did not affect the actions of ghrelin in the crop, proventriculus and ileum of the pheasant. These results indicate that there is no interaction between motilin and ghrelin in the pheasant GI tract as is the case in the quail at least in an *in vitro* condition. Therefore, interaction of motilin and ghrelin in the GI motility-stimulating actions reported in dogs and house shrew was not observed in the avian species. Structural similarities between motilin and ghrelin and between MLNR and GHS-R1a have been suggested [6,128]. The amino acid sequences of ghrelin and motilin are regarded as homologous, and MLNR and GHS-R1 also share 52% identity in amino acid sequences [52,162]. However, motilin and ghrelin could not activate each other's receptor [47] and pretreatment with ghrelin did not change the concentration-response curves for motilin in the rabbit duodenum (unpublished data. Kitazawa et al., Rakuno Gakuen University). Although it has been thought that motilin and ghrelin might have developed from the same ancestral gene [83], the N-terminal structures of the two peptides are quite different. This difference might help to explain why motilin and ghrelin cannot interact with each other's receptor to cause the GI contraction. On the other hand, although the structures of the receptors show some similarity, it is not possible that motilin acts on GHS-R1a and ghrelin acts on the MLNR [150]. Therefore, acting on another receptor is not a mechanism for

interaction. As previously mentioned, interaction of ghrelin and motilin has only been observed in two species, house shrew and the dog. In many mammals and avian species, interaction between ghrelin and motilin has not been observed, suggesting that interaction between ghrelin and motilin is not universal. Dogs and house shrew might be special cases and they are interesting animal species to investigate why and how the two peptides interact.

CHAPTER 4.
CONCLUSION

Motilin plays a role in the regulation of GI motility in several mammals through activation of the MLNR (GPR38) that is located on enteric neurons and smooth muscle cells [15,52,81]. Motilin is thought to be an endogenous regulator of phase III activity of the MMC in the stomach of humans, dogs and house musk shrews [70,115,143,183]. The presence of the motilin system has been reported in some birds (chickens and quails), but it has not been investigated extensively in reptiles, amphibians and fish [3,83,88,188]. In birds, motilin caused contraction of the GI tract in a region-dependent manner [3,88] and it was suggested to mediate the ROC in the small intestine observed in fasting periods [140]. Therefore, motilin is also a GI motility-regulating hormone in the avian GI tract. However, additional study using different species is needed to for an understanding of the function of motilin in the regulation of GI motility in birds.

Ghrelin, a natural ligand for 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, food intake, endocrine and exocrine pancreatic functions, cardiac function and regulation of GI motility [76,92,94,152]. Since ghrelin shows structural homology with motilin and GHS-R1a shows structural homology with motilin receptor and they are thought to be derived from the same ancestor gene [6,128], the stimulatory action of ghrelin on GI motility has been investigated in humans and some experimental animals including rodents [44,54,80,168]. Although there are some species-related difference in the actions of ghrelin, ghrelin is also thought to be a GI motility-regulatory peptide. In chickens, it was found that ghrelin causes contraction of the crop, proventriculus and colon through activation of smooth muscle receptors [84]. However, ghrelin did not cause any contraction in the GI tract of Japanese quails despite the fact that an appropriate expression level of GHS-R1a mRNA was found [3,85]. These contrastive actions of ghrelin on the contractility of the chicken and Japanese quail GI tracts prompted us to examine the effects of ghrelin on GI contractility in another avian species in order to determine what the general actions of ghrelin on contractility of the avian GI tract are.

In the present study, we selected pheasants (*Phasianus colchicus versicolor*) as another

avian species because they belong to the same order *Galliformes* that chickens and quails belong to, and it would be possible to compare the actions of motilin and ghrelin among closely related species. 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.

As described in chapter 2, motilin was identified in the pheasant by molecular cloning, and the actions of motilin on contractility of GI strips were examined *in vitro*. Molecular cloning indicated that the deduced amino acid sequence of the pheasant mature motilin was a 22-amino-acid peptide, FVPFFTQSDIQKMQEKERIKGQ, and the gene was expressed mainly in the small intestine (duodenum, jejunum and ileum). The expression levels in other regions of the GI tract and in the brain, heart, lung and liver were very low. In *in vitro* studies using pheasant GI strips, chicken motilin and pheasant motilin caused contraction of the proventriculus and small intestine, whereas the crop and colon were insensitive. The ranking order of contraction was ileum = jejunum = duodenum > proventriculus >> crop = colon. Human motilin, but not erythromycin (an MLNR agonist), caused contraction of the pheasant small intestine, but a high concentration was needed. The ranking order of motilin receptor agonists in the pheasant intestine (pheasant motilin=chicken motilin>human motilin) was different from that in the rabbit duodenum (human motilin> pheasant motilin=chicken motilin). Chicken and pheasant motilin-induced contractions in the proventriculus and ileum of the pheasant were not inhibited by mammalian MLNR antagonists, GM109 or MA2029. A similar phenomenon (ineffectiveness of erythromycin and MLNR antagonists) was also observed in chickens [88]. The results indicated that the MLNR structure in chickens and pheasants is different from that in mammals. Neither atropine (a cholinergic receptor antagonist) nor tetrodotoxin (a neuron blocker) inhibited the responses to chicken and pheasant motilins in the ileum, but both drugs decreased the responses to motilin in the proventriculus, suggesting that the contractile mechanism of motilin in the proventriculus was neurogenic, different from that in the small intestine (myogenic). The results suggested that motilin is widely present in the small intestine of avian species and causes contraction of the GI tract in a region-dependent manner (Table 5). The highest responsiveness to motilin in the small intestine suggests that motilin is a GI motility-regulating hormone and that the small intestine is a target organ of motilin in birds.

Table 5. Comparison of the presence of motilin and MLNR and the GI contractility-stimulating action of motilin in different species of birds.

Species	Motilin	MLNR	GI contractility
Chicken (*Gallus domesticus,Gallus*)	○	○	Contraction
Quail (*Coturnix coturnix,Coturnix*)	○	?	Contraction
Turkey (*Meleagris, Meleagris*)	○	?	?
Pheasant (*Phasianus colchicus, Phasianus*)	○	?	Contraction

(○) The structure has been identified. (?) No report.

As describe in chapter 3, ghrelin was identified in the pheasant proventriculus by molecular cloning, and the actions of ghrelin on contractility of GI strips were examined in the experiment *in vitro*. Molecular cloning indicated that the deduced amino acid sequence of the pheasant mature ghrelin was a 26-amino-acid peptide, GSSFLSPAYKNIQQQKDTRKPTGRLH, and ghrelin was localized mainly in the proventriculus as in other avian species. Ghrelin-immunopositive cells were detected in the mucosal layer of the proventriculus, and their shape was a round, closed type. They were stained by a specific antibody for decanoyl ghrelin but not by a specific antibody for octanoyl ghrelin. Ghrelin-immunopositive cells were also detected in the duodenum by an antibody for unacylated ghrelin but not by antibodies for octanoyl ghrelin and decanoyl ghrelin. Chicken, quail and rat ghrelin (1 μM) did not cause any contraction in any regions of the pheasant GI tract. A comparison of the effects of ghrelin in three different avian species (chicken, quail and pheasant) suggests that there is species-dependent variation in the responses to ghrelin (Table 6). Although ghrelin-sensitive (chicken) and ghrelin-insensitive (quail and pheasant) groups were identified, the physiological meanings of different actions of ghrelin in the avian GI tract was not clarified in this study.

Table 6. Comparison of the presence of ghrelin and GHS-R1a and the GI contractility-stimulating action of ghrelin in different species of birds.

Species	Ghrelin	GHS-R1a	GI contractility
Chicken (*Gallus domesticus,Gallus*)	○	○	Contraction
Quail (*Coturnix coturnix,Coturnix*)	○	○	No effect
Turkey (*Meleagris, Meleagris*)	○	○	?
Pheasant (*Phasianus colchicus, Phasianus*)	○	○	No effect

(○) The structure has been identified. (?) No report.

Since an interaction of ghrelin and motilin has been reported in the dogs and house shrews [115,121], the interaction of the two peptides was also examined in the pheasant. The chicken motilin-induced contraction was not modified by ghrelin pretreatment, and ghrelin also did not cause any contraction in the presence of motilin, suggesting that there is no contractile interaction between the two peptides in the pheasant.

In summary, the presence of both motilin and ghrelin was demonstrated in the pheasant as well as in other avian species including the chicken and quail. Motilin caused contraction of the GI tract in a region-dependent manner, but ghrelin was ineffective. 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 functional experiments have been restricted to closely related avian species, the results suggested that motilin is the common regulator of GI contractility, especially small intestinal contractility, in birds. However, further studies using avian species other than chickens, quails and pheasants are needed in the future to establish the general physiological roles of motilin and ghrelin in the avian GI tract. The birds (quails, pheasants and chickens) from the *Galliformes* used for functional studies of ghrelin and motilin are species that cannot fly for a long time, and the study using flying birds might therefore be of interest because flying birds consume a lot of energy for flying and ghrelin is a peptide regulating energy homeostasis.

LIST OF PUBLICATIONS

Related publication:

- **Zhang S**, Okuhara Y, Iijima M, Takemi S, Sakata I, Kaiya H, Teraoka H, Kitazawa T. Identification of pheasant ghrelin and motilin and their actions on contractility of the isolated gastrointestinal tract. Gen Comp Endocrinol. 2020 Jan 1;285:113294. doi: 10.1016/j.ygcen.2019.113294. Epub 2019 Oct 1. PMID: 31585115.
- **Zhang S**, Teraoka H, Kaiya H, Kitazawa T, Motilin- and ghrelin-induced contractions in isolated gastrointestinal strips from three species of frogs. Gen Comp Endocrinol. Volume 300, 2021, 113649. doi.org/10.1016/j.ygcen.2020.113649. Epub 2020 Oct 22.
- **Zhang S**, Sakata I, Kaiya H, Teraoka H, Okuhara Y, Kitazawa T. Identification of ghrelin and motilin in the pheasant, and their mechanical actions in the isolated gastrointestinal tract. NeuroGastro2019 (Lisbon), 2019 Sep 5 (Poster presentation).
- Kitazawa T, **Zhang S**, Teraoka H, Okuhara Y, Kaiya H. Does motilin regulate gastrointestinal motility of bullfrog? In vitro study using isolated GI strips. NeuroGastro 2019 (Lisbon), 2019 Sep 5 (Poster presentation)..

Unrelated publication:

- **Zhang S**, Takahashi R, Yamashita N, Teraoka H, Kitazawa T. Alpha1B-adrenoceptor-mediated positive inotropic and positive chronotropic actions in the mouse atrium. Eur J Pharmacol. 2018 Nov 15;839:82-88. doi: 10.1016/j.ejphar.2018.08.038. Epub 2018 Aug 30. PMID: 30172786.
- **Zhang S**, Kitazawa T, Teraoka H. Alpha1B-adrenoceptor-mediated positive inotropic and positive chronotropic actions in the mouse atrium. Proceedings for Annual Meeting of the Japanese Pharmacological Society 92:2-P-057, 2019 Jan. doi: 10.1254/jpssuppl.92.0_2-P-057.
- **Zhang S**, Kitazawa T, Cao J. Effects of prostaglandin E2 and F2α on expression of growth factors and proliferation of bovine endometrial cells and explants *in vitro*. 18th World Congress of Basic and Clinical Pharmacology (Kyoto), 2018 Jul. 1 – 2018 Jul. 06.
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of adult zebrafish, Danio rerio. J Toxicol Sci. 2019;44(5):347-356. doi: 10.2131/jts.44.347. PMID: 31068540.

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ABSTRACT

IDENTIFICATION OF MOTILIN AND GHRELIN AND THEIR ROLES IN REGULATION OF GASTROINTESTINAL MOTILITY IN THE PHEASANT

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Motilin, a 22-amino-acids peptide hormone, plays a physiological role in the regulation of gastrointestinal (GI) motility in several mammals through activating the motilin receptor (MLNR, GPR38) located on enteric neurons and smooth muscle cells. Motilin is thought to be an endogenous regulator of phase III activity of the interdigestive migrating motor complex in the stomach of humans, dogs and house musk shrews. The presence of the motilin system (motilin and MLNR) has been reported in some birds (chickens and quails), but it has not been investigated extensively in reptiles, amphibians and fish. In birds, motilin is able to cause contraction of the GI tract in a region-dependent manner (small intestine > proventriculus >> crop and colon) and mediates the rhythmic oscillatory complexes observed in the small intestine in fasting periods of chickens. Motilin has been also proposed to be a GI motility-regulating hormone in the avian GI tract. However, a study using different species is needed to understand the function of motilin in the regulation of GI motility in birds because previous studies have only been conducted in chickens and quails.

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 growth hormone (GH) release, glucose homeostasis, food intake, endocrine and exocrine pancreatic functions, cardiac function and regulation of GI motility. Since ghrelin shows structural homology with motilin and GHS-R1a shows structural homology with MLNR and both peptides are thought to be derived from the same ancestor gene, the GI motility stimulatory action of ghrelin has been investigated in humans and some experimental animals including rodents. Although there are

some species-related differences in the actions of ghrelin on GI motility, ghrelin is also thought to be a GI motility-regulatory peptide. In chickens, it was found that ghrelin causes contraction in the crop, proventriculus and colon through the activation of GHS-R1a. However, ghrelin did not cause any contraction in the GI tract of Japanese quails despite the fact that GHS-R1a mRNA is expressed in the GI tract. Due to the contrastive actions of ghrelin in the chicken and quail GI tracts, examination of the effects of ghrelin on GI contractility in other avian species is needed to determine the general actions of ghrelin on contractility of the avian GI tract.

In the present study, the pheasant (*Phasianus colchicus versicolor*) was selected as another avian species because pheasants belong to the same order *Galliformes* that chickens and quails belong to and it would be possible to compare the actions of motilin and ghrelin among closely related species. First, the primary structures of motilin and ghrelin in the pheasant were determined by molecular cloning. Then the effects of motilin and ghrelin and their interaction were examined in isolated GI strips of the pheasant.

In chapter 1, the background and objective of the study are introduced.

In chapter 2, identification of the motilin gene in the pheasant by molecular cloning and the actions of motilin on contractility of GI strips examined *in vitro* are described. Molecular cloning indicated that the deduced amino acid sequence of the pheasant mature motilin was a 22-amino-acid peptide, FVPFFTQSDIQKMQEKERIKGQ, and the gene was expressed mainly in the small intestine (duodenum, jejunum and ileum). The expression levels in other regions of the GI tract and in the brain, heart, lung and liver were very low. In *in vitro* studies using pheasant GI strips, chicken motilin and pheasant motilin caused contraction of the proventriculus and small intestine with almost the same affinity, whereas the crop and colon were almost insensitive. The ranking order of contraction was ileum = jejunum = duodenum > proventriculus >> crop = colon. Human motilin, but not erythromycin (an MLNR agonist), caused contraction of the pheasant small intestine, but a high concentration was needed for human motilin. The ranking order of motilin peptides in the pheasant small intestine (pheasant motilin=chicken motilin>human motilin) was different from that in the rabbit duodenum (human motilin> pheasant motilin=chicken motilin). Chicken and pheasant motilin-induced contractions in the proventriculus and ileum of the pheasant were not inhibited by the

mammalian MLNR antagonists GM109 and MA2029. These results indicated that the MLNR structure in pheasants is different from that in rabbits (mammals). Neither atropine (a cholinergic muscarinic receptor antagonist) nor tetrodotoxin (a neuron blocker) inhibited the responses to chicken and pheasant motilins in the ileum, but both drugs decreased the responses to motilin in the proventriculus, suggesting that the contractile mechanism of motilin in the proventriculus was neurogenic, different from that in the small intestine (myogenic). These results suggested that motilin is widely present in the small intestine of avian species and causes contraction of the GI tract in a region-dependent manner. The highest responsiveness to motilin in the small intestine suggests that motilin is a GI motility-regulating hormone and that the small intestine is a main target organ of motilin in birds.

In chapter 3, identification of the ghrelin gene in the pheasant proventriculus by molecular cloning and the actions of ghrelin on contractility of GI strips examined in an *in vitro* experiment are described. Molecular cloning indicated that the deduced amino acid sequence of the pheasant mature ghrelin was a 26-amino-acid peptide, GSSFLSPAYKNIQQQKDTRKPTGRLH, and ghrelin was localized mainly in the proventriculus as in other avian species. Ghrelin-immunopositive cells were detected in the mucosal layer of the proventriculus, and their shape was a round, closed type. They were stained by a specific antibody for decanoyl ghrelin but not by a specific antibody for octanoyl ghrelin. Ghrelin-immunopositive cells were also detected in the duodenum by an antibody for unacylated ghrelin but not by antibodies for octanoyl ghrelin and decanoyl ghrelin. Chicken ghrelin, quail ghrelin and rat ghrelin (1 μM) did not cause contraction in any regions of the pheasant GI tract. A comparison of the effects of ghrelin in three different avian species (chicken, quail and pheasant) suggests that there is species-dependent variation in the responses to ghrelin. Although ghrelin-sensitive (chicken) and ghrelin-insensitive (quail and pheasant) groups were identified in avian species, the physiological meaning for different actions of ghrelin in the GI tract was not clarified in this study.

Since an interaction of ghrelin and motilin has been reported in dogs and house shrews, the interaction of the two peptides was also examined in the pheasant GI tract. The chicken motilin-induced contraction was not modified by ghrelin pretreatment, and ghrelin also did

not cause any contraction in the presence of motilin, suggesting that there was no interaction between the two peptides in the pheasant GI motility.

In conclusion, motilin and ghrelin were shown to be present in the pheasant. A contraction study indicated that motilin caused contraction of the pheasant GI tract in a region-dependent manner similar with chickens and quails. However, ghrelin was ineffective, indicating that ghrelin-related modulation of GI motility that is observed in chickens might not be common in avian species. Taken together, the results suggested that motilin might be a common regulator of GI contractility, especially in the small intestine of birds. However, further studies using avian species other than chickens, quails and pheasants are needed to determine the general physiological roles of motilin and ghrelin in the avian GI tract.