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Organisation of the musculature of the rat stomach

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30 **Abstract**

31 The strengths, directions and coupling of the movements of the stomach depend on the organisation of its
32 musculature. Although the rat has been used as a model species to study gastric function, there is no
33 detailed, quantitative study of the arrangement of the gastric muscles in rat. Here we provide a descriptive
34 and quantitative account, and compare it with human gastric anatomy. The rat stomach has three
35 components of the muscularis externa, a longitudinal coat, a circular coat and an internal oblique (sling)
36 muscle in the region of the gastro-esophageal junction. These layers are similar to human. Unlike human,
37 the rat stomach is also equipped with paired muscular esophago-pyloric ligaments that lie external to the
38 longitudinal muscle. There is a prominent muscularis mucosae throughout the stomach and strands of
39 smooth muscle occur in the mucosa, between the glands of the corpus and antrum. The striated muscle of the
40 esophageal wall reaches to the stomach, unlike the human, in which the wall of the distal esophagus is
41 smooth muscle. Thus, the continuity of gastric and esophageal smooth muscle bundles, that occurs in
42 human, does not occur in rat. Circular muscle bundles extend around the circumference of the stomach, in
43 the fundus forming a cap of parallel muscle bundles. This arrangement favours co-ordinated circumferential
44 contractions. Small bands of muscle make connections between the circular muscle bundles. This is
45 consistent with a slower conduction of excitation orthogonal to the circular muscle bundles, across the corpus
46 towards the distal antrum. The oblique muscle merged and became continuous with the circular muscle close
47 to the gastro-esophageal junction at the base of the fundus, and in the corpus, lateral to the lesser curvature.
48 Quantitation of muscle thickness revealed gradients of thickness of both the longitudinal and circular muscle.
49 This anatomical study provides essential data for interpreting gastric movements

50 **KEYWORDS**

51 Smooth muscle, gastric motility, corpus, antrum, fundus, gastric ligaments

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54 **1. INTRODUCTION**

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55 A range of disorders of stomach movements affects patients, including gastroparesis, functional dyspepsia,
56 reflux, pyloric stenosis and rapid gastric emptying (Keller et al., 2018, Tack and Pandolfino, 2018, Cheng et
57 al., 2021). These disorders commonly have downstream or associated symptoms, including early post-
58 prandial fullness and satiety, nausea, vomiting, regurgitation, bloating, upper abdominal distension,
59 abdominal pain and weight loss. Because these conditions are common and poorly treated and because co-
60 ordination of gastric movement depends on generation of electrical rhythmicity and electrical conduction,
61 there has been considerable effort made to analyze and model the electrical events on which the movements
62 of the stomach depend (Sanders and Publicover, 1989, Du et al., 2013, Cheng et al., 2021). The conduction
63 of electrical events in the stomach muscle, and the directions and strengths of forces generated when the
64 muscle is excited, depend on the organization of the musculature. However, there is no detailed quantitative
65 data available concerning the organisation of the musculature of the rat stomach, even though this species has
66 been used extensively for physiological studies and investigations of innervation and brain-gut connections
67 (Lentle et al., 2010, Lentle et al., 2016, Lu et al., 2017, Powley et al., 2019, Furness et al., 2020).

68 In humans and rodents the stomach is made up of three main regions, the fundus (proximal stomach), corpus
69 (the body of the stomach), and antrum (distal stomach tapering towards the duodenum). The proximal
70 stomach acts as a gastric reservoir, while the corpus and antrum are associated with mixing and propulsion of
71 the gastric content. The musculature of the human stomach, a single compartment stomach like rat, has three
72 layers, an external or longitudinal layer beneath which is a circular muscle layer, and an oblique muscle layer
73 that is internal to the circular muscle in the region of the gastro-esophageal junction (Hur, 2020). The
74 oblique muscle curves laterally into the corpus, where it fuses with and becomes continuous with the circular
75 muscle in human. The gastro-esophageal junction differs between species, in particular in relation to whether
76 there is esophageal striated muscle extending to the junction, and whether there is a long intra-abdominal
77 segment of esophagus (McSwiney, 1929). In human, the distal third of the esophagus is smooth muscle;
78 muscle bundles of the wall of the esophagus are continuous with the longitudinal muscle of the gastric wall
79 towards the fundus, and with the circular muscle of the stomach towards the corpus (Hur, 2020). In rat, the
80 distal esophagus is striated muscle and such continuity with gastric muscle, if it occurs, is not obvious
81 (Montedonico et al., 1999). Moreover, the esophageal muscle layers are not longitudinal and circular in the
82 rat, but are arranged as spirals at approximately 90 degrees to each other (Gruber, 1968, Neuhuber et al.,
83 1998).

84

85 **2. MATERIALS AND METHODS**

86 **2.1 Tissue sources and preparation**

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87 All procedures were approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics
88 Committee. Stomach samples were collected from female and male Sprague Dawley (SD) rats, 6-8 weeks
89 old, 185-206g for females and 220-300g for males. Rats were supplied with food and water ad libitum prior
90 to any experiments. Animals were deeply anaesthetised with either an intraperitoneal injection of
91 pentobarbital sodium (100mg/kg) or an intraperitoneal injection of a mixture of ketamine (50 mg/kg) and
92 xylazine (10 mg/kg) prior to being perfused transcardially with phosphate buffered saline (PBS: 0.15 M
93 NaCl, 0.01 M sodium phosphate buffer, pH 7.2) followed by fixative. Varying fixation methods were
94 completed to allow for comparisons, these fixation methods were: 4% paraformaldehyde (Sigma Aldrich,
95 USA), 10% neutral buffered formalin (Trajan, Melbourne, Australia) or Zamboni's fixative (2 %
96 formaldehyde plus 0.2 % picric acid in 0.1 M sodium phosphate buffer, pH 7.0). Some stomach samples
97 were collected from anaesthetised rats and placed into PBS containing nifedipine (1 μ m) before fixation.

98 **2.2 Histological staining**

99 Tissue was placed into histology cassettes and dehydrated through graded ethanol to histolene and embedded
100 in paraffin. Sections (5 μ m) were cut and stained with haematoxylin and eosin (H&E) using Leica
101 Autostainer XL and Leica CV5030 coverslipper. Slides were examined and photographed using an Axioplan
102 microscope (Zeiss, Sydney, Australia). Masson's trichrome staining was conducted manually. Sections were
103 then dehydrated, cleared in xylene and coverslipped using permanent mounting media.

104 **2.3 Histology quantification**

105 A selection of points, indicated by the pink dots in Figure 6A, were used to measure the thickness of the
106 muscle layers in rat stomach. At each point three measurements of each muscle layer (longitudinal, circular,
107 oblique (where applicable) and the muscularis mucosa) were taken and averaged. The average was taken for
108 all equivalent fiducial points. Measurements (μ m) were completed using Zeiss ZEN software.

109 **2.3 Immunohistochemistry**

110 Whollemount preparations were placed in cold fixative (2 % formaldehyde plus 0.2 % picric acid in 0.1 M
111 sodium phosphate buffer, pH 7.0) and incubated overnight at 4°C. Tissues were then washed with dimethyl
112 sulfoxide (DMSO) 3 x 10 minutes and with PBS 3 x 10 minutes. The esophago-pyloric ligament staining was
113 completed as follows: samples were then covered with normal horse serum (10% v/v with triton-X in PBS)
114 and incubated at room temperature (RT) for 1 hour, incubated with mixtures of primary antibodies including
115 sheep anti-neuronal nitric oxide synthase (nNOS; 1:1000, V205: RRID, AB_2314960) (Williamson et al.,
116 1996) and rabbit anti-tachykinin (SKSP1, raised against substance P 1-11; 1:800: RRID, AB_2814842)

117 (Morris et al., 1986) overnight at RT. The preparations were then washed three times with PBS before a 3
118 hour incubation with mixtures of secondary antibodies at RT.

119 The muscle bundle staining was completed as follows: dissected samples were covered with normal horse
120 serum (10% v/v with PBS containing 1% triton-X) and incubated at 37°C for 1 hour, followed by incubation
121 with rabbit anti- α -smooth muscle actin (α SMA; 1:200; AB5694, Abcam, Cambridge, UK: RRID,
122 AB_2223021) overnight at 37°C and for 2 days at RT. The preparations were then washed three times with
123 PBS before a 5 hour incubation with secondary antibody at 37°C.

124 Cryostat sections to determine muscle bundle widths were prepared as follows. Tissue samples were placed
125 into 30% PBS-sucrose-azide overnight at 4°C followed by overnight in a mixture of OCT compound (Tissue
126 Tek, Elkhart, IN, USA) and PBS-sucrose-azide in a 1:1 ratio before being embedded in 100% OCT and snap
127 frozen in isopentane cooled with liquid nitrogen. Sections (12 μ m) were cut and mounted onto
128 SuperFrostPlus® microscope slides (Menzel-Glaser; Thermo Fisher, Scoresby, Vic, Australia). They were
129 air dried for 1 hour then covered with normal horse serum (10% v/v with PBS containing 1% triton-X) and
130 incubated at for 30 minutes at RT, followed by incubation with rabbit anti- α SMA (1:200, overnight at 4°C).
131 The sections were then washed three times with PBS before a 1.5 hour incubation with fluorescent labeled
132 secondary antibody at room temperature.

133 Following secondary antibody incubation all preparations were then washed three times with PBS before
134 being mounted on standard microscope slides and coverslipped with Dako fluorescence mounting medium
135 (Agilent, Tullamarine, Vic, Australia). Slides were examined and imaged using an Axio Imager microscope
136 (Zeiss, Sydney, Australia) or an LSM800 confocal microscope (Zeiss).

137 **2.4 Contrast enhancement and imaging**

138 In order to visualise the circular and longitudinal muscle coats in whole stomach preparations, stomachs
139 underwent prolonged fixation in NBF, for 7 or 14 days. The stomach was then placed in 20 or 80% ethanol
140 for up to a week. Ethanol was replaced with distilled water. This procedure enhanced the outlines of the
141 muscle bundles. These were photographed in whole stomach preparations using oblique illumination to
142 enhance contrast.

144 **3. RESULTS**

145 **3.1 Anatomical features**

146 The overall anatomy of the stomach was examined in vivo, in rats that had been allowed free access to food
147 overnight and which were examined under anesthesia in the morning. The abdomen was opened in the
148 midline and the liver was retracted to reveal the stomach (Figure 1A). The stomach was located to the left in
149 the upper abdomen, and the esophago-gastric junction was to the left of the midline. The same positioning
150 was observed for the stomachs of rats that were perfused with fixative through the heart under deep
151 anesthesia, also after free access to food (Figure 1B). Perfusion with fixative euthanises the rat and the
152 internal organs are preserved in their natural positions. To investigate the regional anatomy as defined by
153 mucosal specialisation, the stomach was removed from freshly killed rats and placed in saline containing the
154 muscle relaxant, nicardipine. It was cut open along the greater curvature from the gastro-esophageal junction
155 to the pyloric sphincter and stretched flat (Figure 1C). Making a 3-dimensional surface 2-dimensional in this
156 way distorts the anatomy, but is useful to illustrate the relationships of the gastric regions.

157 The main regions of the living stomach in the anesthetised rat and of the opened stomach showed obvious
158 colour differences. The limiting ridge clearly divides the stomach into two regions, proximally the fundus
159 and esophageal groove that are lined by a stratified squamous epithelium, and distally the corpus and antrum
160 (Figure 1C). The boundary between the corpus and antrum is less clearly defined, which is consistent with
161 there being a zone of transition between the oxyntic mucosa of the corpus and the antral mucosa. There were
162 animal to animal differences in the size of the fundus and in the positioning of the limiting ridge, as also seen
163 in other studies of the rat stomach (Jaffey et al., 2021). However, we could detect no differences in anatomy
164 between stomachs of male and female rats in the weight range that we investigated (185 to 300 g). From 8
165 perfused stomachs (example in Figure 1B), we created a diagrammatic image for the purpose of mapping
166 gastric features (Figure 1D).

167 **3.2 Esophago-pyloric ligaments**

168 The rat possesses a pair of muscular ligaments that extend from a site just lateral to the esophago-gastric
169 junction to the pyloric sphincter (Figure 2).

170 The ligaments extend from an insertion at the pyloric sphincter to an attachment lateral to the base of the
171 esophagus (Figure 2). They lie parallel and lateral to the mid-line junction of the mesentery of the lesser
172 curvature (lesser omentum). The ligaments are against the gastric surface when the gastric muscle is fully
173 relaxed, but when there is a contractile wave in the antrum, a space is seen between the ligaments and the
174 antral surface (Figure 2B). The extents of the ligaments can be readily revealed by passing a sheet of black
175 plastic underneath (Figure 2C). At the esophageal end, the ligament has a triangular expansion. This is the
176 point of extensive branching of main branches of the left gastric artery (l.g. in Figure 2C) on the ventral and
177 dorsal surfaces (Jaffey et al., 2021). A prominent accumulation of fat, which is also a branching point for
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178 major blood vessels to the corpus region, lies between the ligament and the gastric surface at this point. This
179 has been partly removed in Figure 2C. The esophageal attachment is just lateral to the esophageal groove
180 and merges with the serosa close to where the corpus abuts the fundus, as defined by the limiting ridge
181 (Figure 2D). Figure 2E is an oblique section through the ligament and the surface of the stomach within the
182 region of the esophageal attachment. Between the ligament and the external muscle of the stomach is loose
183 connective tissue (Figure 2E). Immunohistochemistry revealed innervation by axons that were
184 immunoreactive for tachykinins (TK) and neuronal nitric oxide synthase (nNOS; Figure 2F). In other gastric
185 regions, TK immunoreactivity marks the axons of excitatory enteric neurons that innervate the muscle, and
186 nNOS marks axons of enteric inhibitory neurons.

187 In rat, the ligaments have been referred to as sling muscles (Powley et al., 2012), whereas in human the sling
188 muscle is internal to the gastric wall and is equivalent to the oblique muscle (Spalteholz, 1906, Stein et al.,
189 1995, Zifan et al., 2017, Hur, 2020). Powley et al (2012) found that vagal afferent endings innervate the
190 ligament.

191 **3.3 The gastro-esophageal junction**

192 When viewed from the inside of the stomach that has been fixed in situ, the orifice of the distal esophagus at
193 gastro-duodenal junction is almost hidden by closely apposed curves of the limiting ridge (Figure 3A). The
194 orifice can be seen better when the tissue lateral to it is retracted (Figure 3B). The orifice was generally
195 closed in the samples that we examined, as is seen in cross sections taken at the level of the orifice (Figures
196 3C, F).

197 In fixed tissue, the most distal part of the esophagus has the darker color typical of striated muscle (Figure
198 3D) and we confirmed by histology that striated muscle extended to the distal end of the esophagus. The
199 longitudinal muscle of the stomach forms prominent bundles in the region of the fundus approaching the
200 esophagus (Fig 3E). Thick bundles of this longitudinal muscle continue lateral to the distal esophagus at the
201 esophago-gastric junction, and can be readily seen in transverse sections of the junction (Figure 3F and H).
202 The longitudinal muscle at the lesser curvature is also thicker than at more lateral sites. The lateral extents
203 of the esophageal groove are partly obscured by a mucosal fold that includes the limiting ridge (Figure 3A,
204 F), as also described previously (Montedonico et al., 1999).

205 **3.4 The circular muscle**

206 The circular muscle is arranged in bundles that run approximately circumferential around the corpus and
207 antrum, and around the fundus (Figure 4).

208 The lesser curvature is shorter than the greater curvature and to accommodate this difference adjacent
209 bundles coming from the greater curvature coalesce as they approach the lesser curvature. For part of the
210 fundus, circular muscle bundles form rings that involve the greater curvature, but not the lesser curvature
211 (Figure 4A). At the extreme of the fundus, where the gastrophrenic ligament joins, these rings have the
212 appearance of a muscular cap. Thus, this part of the fundus has the anatomical characteristics of a gastric
213 diverticulum. The circular muscle does not follow features of the mucosa, such as the limiting ridge or the
214 boundary between corpus and antrum.

215 In histological cross sections the circular muscle bundles can be seen to be separated by connective tissue
216 (Figure 4E). Occasionally bands of muscle connect the bundles (Figure 4F), as previously described (Di
217 Natale 2021). The bundles had similar average widths in the antrum and corpus when measured in sections
218 taken at right angles to the lengths of the bundles (Figure 5). They were about half the width in the fundus.

219 **3.5 The longitudinal muscle**

220 The longitudinal muscle follows the curve of the stomach (Figure 6) and for most of the gastric surface it is
221 very thin, only about 10-20 μm . On the ventral and dorsal surfaces of the fundus, the muscle turns, creating a
222 curve that looks rather like a fingerprint (Figure 6A, E). Along the greater curvature, to the right of the
223 gastrophrenic ligament, the longitudinal muscle forms parallel thick bundles, that are seen in Fig 3E. Thus in
224 sections parallel to the greater curvature through the region of the gastrophrenic ligament the longitudinal
225 muscle is thicker to the right than to the left (Figure 6B). Some of the thick bundles coalesce and run lateral
226 to the esophago-gastric junction (Figure 6C). The longitudinal bundles follow the length of the antrum,
227 running towards the pyloric sphincter, where they end in close relation to the sphincter muscle (Figure 6D).
228 The longitudinal muscle of the ventral and dorsal surfaces are in direct continuity with the longitudinal
229 muscle of the duodenum.

230 **3.6 The oblique (sling) muscle**

231 The oblique muscle forms a crescent around and on the fundic aspect of the gastroesophageal junction
232 (Figure 4B). Its medial margin follows the line of the limiting ridge and it curves laterally to run in the same
233 direction as the circular muscle (Figure 4B), with which it eventually coalesces (Figure 6F). Where the
234 oblique muscle curves around the left side of the esophagus, which is the mid-line of the opened stomach
235 (arrow in Figure 4B), it also merges with and becomes indistinguishable from the circular muscle.

236 Some bundles of oblique muscle run laterally from their course around the gastroesophageal junction to join
237 the circular muscle after a short distance, whereas other bundles run towards the antrum before curving

238 laterally to merge with the circular muscle 6-8mm from the medial part of the lesser curvature (Figures 4B.
239 6Fi-iii).

240 **3.7 Muscle associated with the mucosa**

241 A muscularis mucosae at the inner part of the mucosa, external to the submucosal layer, was observed
242 throughout the stomach, and at the base of the esophagus (Figures 3H, 4E, 6F, 7). For most of the stomach it
243 was 30-30 μm thick. It was thicker at the base of the esophagus than in the adjacent stomach (Figure 3H)
244 and was thicker in the fundus than in other regions (Figures 4E, 7). Thin muscle bundles occurred in the
245 mucosa between the glands of the antrum and corpus, but muscle bundles were not observed within the
246 lamina propria of the fundus (Figure 7). The strands of muscle that extend between the glands in the stomach
247 may have a mixing role. In elegant experiments, the pressures in the lumens of gastric glands have been
248 measured and found to oscillate with a rhythm of 4-5 oscillations per min (Synnerstad et al., 1998). Addition
249 of VIP reduced the basal pressure and significantly reduced the amplitudes of oscillations, which is
250 consistent with an innervation of the extension of the muscularis mucosae between the glands by VIP-
251 immunoreactive inhibitory motor neurons.

252 **3.8 Relative thicknesses of layers in different regions**

253 The thicknesses of muscle layers and the mucosa throughout the stomach were measured in 2 female and 2
254 male rats (Figure 8). In some cases a greater number of rats was used. Measurements in Figure 8 are from
255 the ventral wall and the greater and lesser curvatures. The dorsal wall was also examined and appeared
256 identical in structure and dimensions to the ventral wall. For each sample, 3 measurements were taken and
257 averaged to provide the sample mean for that site for the individual rat. The circular and longitudinal
258 muscles varied considerably in thickness throughout the stomach. The circular muscle thickness ranged from
259 40-60 μm in the middle of the ventral or dorsal surface and towards the greater curvature to 150 μm at the
260 lesser curvature and distal antrum. The longitudinal muscle thickness ranged from 10-20 μm in the mid part
261 and greater curvature of the corpus to 50 μm where it passed dorsal or ventral to the esophago-gastric
262 junction (Figure 6C). The oblique muscle was thickest as it passed the esophagus (adjacent to site O of
263 Figure 8A), with an average of $201.9 \pm 29.2 \mu\text{m}$. From this site, its thickness decreased at a regular rate until
264 it merged with the circular muscle as it passed around the esophagus (arrow in Figure 4B) and in the corpus
265 (Figure 6Fi-iii). The muscularis musosae was prominent throughout the stomach. In the corpus it averaged
266 $17.9 \pm 2.9 \mu\text{m}$ and in the antrum $22.2 \pm 3.3 \mu\text{m}$. It was thicker in the fundus, $30.5 \pm 4.5 \mu\text{m}$.

267 We also measured the thicknesses of other layers. The mucosa of the fundus had similar thickness
268 throughout this region, with an average thickness of $39.5 \pm 5.6 \mu\text{m}$. The average thickness of the corpus
269 mucosa was $413.1 \pm 63.4 \mu\text{m}$ and the antral mucosa was $312.9 \pm 23.2 \mu\text{m}$.

271 4 DISCUSSION

272 4.1 Features of the rat stomach

273 The gastric muscles of the rat and their relationships are depicted in Figure 9. The rat stomach has paired
274 esophago-pyloric, smooth muscle, ligaments. We also found these in the mouse stomach, but were unable to
275 locate them in human. They are not described in authoritative textbooks of human anatomy, such as Gray's
276 Anatomy or in detailed studies of human gastric muscle (Standring, 2016, Hur, 2020). The ligaments insert
277 at one end into the pyloric sphincter and at the other end join the stomach close to the esophago-gastric
278 junction (Figure 9). The ligaments lie against the surface when the stomach is relaxed, but were observed
279 away from the surface when there was a circular muscle contraction of the antrum (Figure 2B). This gives
280 the impression that the ligaments may restrict the separation of the esophago-gastric junction and the pylorus.
281 The muscle of the ligaments was innervated by two types of nerve fibers, those immunoreactive for nNOS and
282 those immunoreactive for TK. These are established markers of inhibitory and excitatory neurons that
283 supply gastric muscle in the rat and other species (Furness, 2006), which suggests that tension in the
284 esophago-pyloric ligaments is under neural control, that could contribute to regulation of the length of the
285 lesser curvature (see Figure 9). The external muscle consists of three layers, as in human (Standring, 2016,
286 Hur, 2020), an outer longitudinal muscle, a circular muscle deep to the longitudinal, and an inner oblique
287 (sling) muscle that is closely related to the esophago-gastric junction. There is a prominent muscularis
288 mucosae. Analysis of movements of the rat stomach using spatio-temporal maps indicates that the muscularis
289 mucosae undergoes rhythmic, myogenic, contractions (Lentle et al., 2016). We found strands of muscle
290 adjacent to the gastric glands, as has also been reported for human stomach (Arai et al., 2004).

291 The gastro-esophageal junction in rat and human have distinct differences. Unlike in human (Hur 2020), the
292 circular and longitudinal muscle bundles of the rat stomach are not continuous with muscle bundles that form
293 the wall of the esophagus. This is because the external muscle of the distal esophagus of the rat is composed
294 of spirally arranged striated muscle (Gruber, 1968, Neuhuber et al., 1998), in contrast to the longitudinal and
295 circular smooth muscle coats that occur in human. A number of features may contribute to limiting the
296 passage of content across the junction. The narrowest part of the lumen at the junction is flanked by
297 thickened bundles of longitudinal muscle and circular muscle bundles continuous with those of the corpus

298 (Figure 3C, H). Contractions of these muscles would reduce the diameter of the gastro-esophageal orifice.
299 Viewed from inside the stomach, mucosal folds obscure the gastro-esophageal orifice (Figure 3A,B)
300 (Montedonico et al., 1999). This would be consistent with the mucosal folds contributing, by a valve-like
301 arrangement, to limiting gastric reflux.

302 **4.2 Muscle thickness in different gastric states**

303 The thickness of the muscle was measured from stomachs that were fixed at 10-11 am after free access to
304 food during a 6 pm to 6 am dark phase and during the light phase. The stomachs were full, but the fundus
305 was only moderately distended (Figure 1). Under different conditions, the relative thicknesses of the muscle
306 may have been different. For example, had the fundus been more distended it is anticipated that its muscle
307 layers would have been thinner. The fundus varies the most with meal size, its volume varying from about
308 400 mm³ in the empty stomach, to about 7000 mm³ in the full stomach (Jaffey et al., 2021). In contrast to
309 this almost 20 fold change in fundic volume, there was an approximately 3-fold difference in antum plus
310 corpus volume.

311 **4.3 The arrangement of the circular muscle in bundles**

312 The circular muscle coat is formed by bundles of muscle that wrap around the full circumference of the
313 stomach. Electrical conduction along the bundles is to be expected, based on the smooth muscle of the
314 bundles forming an electrical syncitium. In fact, electrical recordings indicate isochronic occurrence of
315 electrical slow waves around the circumference (Lammers et al., 2009). The slow waves initiate well co-
316 ordinated circular muscle contractile waves (gastric peristalsis), that traverse the corpus and antrum,
317 travelling perpendicular to the circular muscle bundles and partly occluding the lumen (Cannon, 1902,
318 Alvarez and Mahoney, 1922, Lu et al., 2017), although it is interesting to note that the proximal corpus
319 contracts on the side towards the greater curvature but not on the side towards the lesser curvature (Lentle et
320 al., 2016). In the small intestine and colon, conduction of peristaltic events depends on the enteric nervous
321 system (Furness, 2006). Unlike the intestine, the conduction of waves of circular muscle contraction in the
322 longitudinal direction is not impeded by cutting through the myenteric plexus (Cannon, 1912), by blocking
323 excitatory transmission with nicotine (Cannon, 1911), or by preventing nerve action potentials with
324 tetrodotoxin (Lentle et al., 2016). Thus conduction and co-ordination orthogonal to the circular muscle
325 bundles is deduced to be electrical, either through the bands of muscle that connect the circularly arranged
326 bundles (Fig 4F) or through the interstitial cells of Cajal, or through both.

327

328 **4.4 Relations between circular and longitudinal muscle**

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329 In the corpus and antrum, the circular muscle was 4-6 times the thickness of the longitudinal muscle. In vivo
330 imaging shows that the dominant movement pattern in these regions are deep circular muscle contractions
331 that partly occlude the lumen and move from the proximal part of the corpus to the distal antrum (Cannon,
332 1898, Lu et al., 2017). The longitudinal muscle may have a role to restrict the circular muscle contractions
333 being translated into elongation of the corpus and antrum. This appears to be its role in the intestine. When
334 the circular muscle contracts and shortens, it thickens. Were that thickening to be predominantly in the
335 longitudinal direction of the distal stomach, the gut would lengthen and the occlusion of the lumen would be
336 reduced. In the intestine, the longitudinal muscle contracts at the same time as the circular, restricting
337 lengthening and accentuating luminal occlusion. The simultaneous contraction of the two muscle layers was
338 reported during propulsive contractions of the canine intestine in vivo (Bayliss and Starling, 1899) and has
339 also been noted in the esophagus (Roman, 1982, Mittal et al., 2005) and in other intestinal regions and
340 species (Smith and Robertson, 1998). Simultaneous electrical records from the two muscle layers also
341 indicate that slow waves occur in synchrony (Suzuki et al., 1986). When a peristaltic reflex is induced by an
342 imposed increase in intraluminal pressure, it is observed that the contraction of the longitudinal muscle
343 begins slightly before that of the circular (Trendelenburg, 1917). This has been called the preparatory
344 contraction of the longitudinal muscle. In response to suggestions that there may be reciprocal movements of
345 the longitudinal and circular layers (that is, the longitudinal muscle relaxes and the intestine lengthens when
346 the circular muscle contracts), the relationship between their contractions has been examined, and the
347 literature reviewed (Smith and Robertson, 1998). These authors confirmed that the two layers contract
348 together during propulsive reflexes of the intestine. Imaging methods to reveal Ca^{2+} transients in the muscle
349 also show that the two layers are excited at the same time when motility reflexes are initiated (Stevens et al.,
350 2000). In the antrum and corpus of the rat stomach, the longitudinal and circular muscles contract and relax
351 at the same time during slow wave activity (Lentle et al., 2016), with the longitudinal contraction starting
352 slightly before the circular, as in the intestine. The gastric fundus relaxes in both the direction of the
353 longitudinal muscle and in the direction of the circular muscle to accommodate greater volumes of food
354 (Jaffey et al., 2021).

355 There are instances in which recordings from the intestine show that the longitudinal muscle layer elongates
356 at the same time that the circular muscle contracts (Sarna, 1993, Grider, 2003). It is possible that when the
357 contraction of the circular muscle is sufficiently strong it can overcome the longitudinal muscle contraction
358 and force the longitudinal layer to lengthen. Nevertheless, the literature does indicate that force generated by
359 longitudinal muscle restricts elongation when the circular muscle contracts.

360 **4.5 Conclusions**

361 The muscularis externa of the rat stomach has many similarities with human, consisting of an external
362 longitudinal and internal circular muscle for most of its extent. In the region of the gastro-esophageal junction
363 there is a further layer, internal to the circular muscle, the oblique or sling muscle, similar again to human.
364 Significant differences from human are the presence of the paired esophago-pyloric ligaments in rat but not
365 human, and differences in the structure of the gastro-esophageal junction. The rat and human are also similar
366 in the innervation of the gastric smooth muscle, in both cases being supplied by nitregeric inhibitory innervation
367 and excitatory neurons with the primary neurotransmitter being ACh (Furness et al., 2020). Regional
368 differences in muscle thickness suggest that the strengths of contraction differ by region. The circumferential
369 arrangement of the circular muscle bundles, and the small bands of muscle that connect adjacent bundles, are
370 consistent with rapid circumferential conduction of excitation and slower conduction orthoganol to the
371 bundles. The quantitative data that has been obtained in this study is expected to enhance modelling of
372 gastric function (Cheng et al. 2021).

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384 **Data Availability.**

385 The data that support the findings of this study are openly available in the NIH SPARC DATCORE: DOI:
386 10.26275/tlgo-dfke

388 **Author contributions**

389 JBF and MRDT conceived and designed the study; MRDT, LP, BH and JBF performed experiments; JBF,
390 XW, ZL and MRDT analyzed data and interpreted results of experiments; JBF and MRDT prepared figures,
391 JCM and MJS provided tissue specimens; JBF wrote the manuscript; JBF and MRDT edited and revised the
392 manuscript; all authors have approved the final version of the manuscript.

393

394 **Conflict of Interest**

395 The authors declare no conflict of interest

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498 **Figure Descriptions**

499 FIGURE 1: Anatomy of the rat stomach. A: The stomach in a living, anesthetised rat. The rat has been
500 opened in the ventral midline and the liver has been retracted into the abdominal cavity. A black plastic
501 membrane was placed under the fundus and corpus for image contrast. B: The stomach that has been
502 removed from a rat that was perfused through the heart. C: Fresh stomach opened along the greater
503 curvature and stretched flat. We have used the terms 'medial' and 'lateral' to refer to positions in the opened
504 stomach. D: This diagrammatic image used for mapping features is based on stomachs from perfused rats (as
505 in B).

506 FIGURE 2: The esophago-pyloric ligaments. A: Diagram to show the position of the ventral ligament. B:
507 The ligament as it appears when there is a deep peristaltic contraction of the antrum. The ligament lifts away
508 from the gastric surface. C: Both ligaments are in this image of the freshly dissected, unfixed stomach. The
509 dorsal ligament is difficult to see against the gastric surface. The ventral ligament is revealed more clearly
510 when a black plastic membrane is placed beneath it. D: Esophageal attachment of the ventral ligament
511 (fixed flattened stomach). The attachment is onto the gastric surface lateral to the esophageal groove and the
512 esophagus. E: Section through the ligament at its attachment close to the distal esophagus. The ligament is
513 composed of smooth muscle. Its join to the gastric surface is fibro-muscular (H & E staining). F: Nerve
514 fibers immunoreactive for nNOS and tachykinins (TK) innervating the ligament.

515 FIGURE 3: The gastro-esophageal junction. A: The junction seen from within the stomach in a preparation
516 that was fixed after removal and dissection in saline. The limiting ridge obscures the orifice of the
517 esophagus. The positions of the sections F (F) and G (G) are indicated. This is the most common
518 appearance: the folds of mucosa where the esophagus meets the stomach obscure the opening into the
519 esophagus. B: The same stomach as in A, with the limiting ridges retracted to reveal the esophageal orifice.
520 C: The narrow opening at the most distal end of the esophagus seen in a sagittal section view of the junction.
521 D: The base of the esophagus has the color typical of striated muscle, in comparison to the lighter color of
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522 the gastric wall. E: The bundles of longitudinal muscle of the greater curvature near the esophago-gastric
523 junction. Some bundles pass lateral to the esophagus (asterisks) and can be seen in the section of panel F
524 (enlarged in H). The dotted line marks the midline of the greater curvature of the fundus. F: Cross section
525 through the esophago-gastric junction at the level of the esophageal orifice (position of section shown in
526 panel A). At the junction, the esophageal groove is narrowed, and is bounded laterally by the mucosa of the
527 corpus and the limiting ridge (LR). G: Section adjacent to the esophago-gastric junction, at (G) in panel A.
528 The lips of the groove formed by the limiting ridge are apparent. H: Enlargement of the boxed region of
529 panel F. This shows the thickened longitudinal muscle of the stomach that skirts the esophago-gastric
530 junction. F and H stained with hematoxylin and eosin, G with trichrome.

531 FIGURE 4. Arrangement of the circular muscle. A: The organisation of circular muscle bundles when
532 visualised in the whole stomach. B: The circular muscle as seen in an opened, flattened stomach, and the
533 relation to the oblique muscle (red). Towards midline, and laterally, the oblique muscle fuses with the
534 circular muscle (red arrows). C, D: Contrast enhanced images of the circular muscle of the corpus (C) and
535 fundus (D) in preparations of whole stomach treated with prolonged fixation and ethanol to reveal the muscle
536 bundles and the connective tissue (lighter color) between bundles. E: Section showing circular muscle
537 bundles in the fundus in transverse section, separated by connective tissue (trichrome stain). F: Image of
538 circular muscle bundles in a wholemount stained with anti- α -smooth muscle actin (α SMA). Muscle bundles
539 were connected by bands of smooth muscle cells.

540 FIGURE 5. Widths of circular muscle bundles. The bundles were measured from cross sections taken from
541 the ventral stomach which were stained with anti- α -smooth muscle actin (α SMA) immunohistochemistry.
542 An example of the staining is shown in Figure 7.

543 FIGURE 6: The longitudinal muscle and the merging of the oblique muscle. A: Arrangement of the
544 longitudinal muscle and sites of images. The thicker lines indicate muscle thickening. B: Section running
545 right to left and including the base of the gastro-phrenic ligament. The longitudinal muscle to the right, that
546 runs towards the esophago-gastric junction, is thicker than the longitudinal muscle to the left of the ligament.
547 The thickened muscle bundles to the right are also seen in Fig 3E. C: The thickening of the longitudinal
548 muscle as it passes lateral to the esophago-gastric junction. D: The pyloric sphincter. The section shows the
549 longitudinal muscle of the stomach merging with the sphincter. In the first part of the duodenum large glands
550 of Brunner are prominent in the submucosa. E: Contrast enhanced image of the site where the longitudinal
551 muscle turns back on itself. Image from whole fixed stomach using oblique illumination. F: Sections taken
552 in a line along the line marked by the bar in panel A. Closest to the esophago-gastric junction, (Fi), the
553 circular and oblique layers are distinct, further away, (Fii), they come close together and towards the mid-

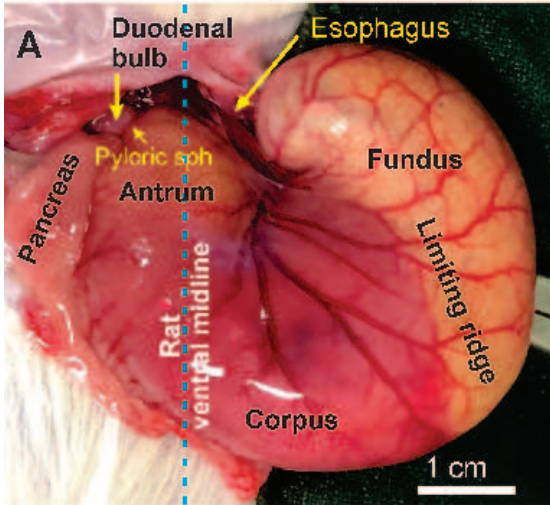
554 point of the stomach the two layers become one (Fiii). B, C, D: hematoxylin and eosin; F: Masson's
555 trichrome; E: unstained.

556 FIGURE 7. The muscularis mucosae. Stomach preparations from the ventral fundus (A), corpus (B) and
557 antrum (C) were stained with anti- α smooth muscle actin (α SMA). The muscularis mucosae, muscularis
558 externa and smooth muscle components of the muscularis mucosae that extend between the mucosal glands
559 are stained.

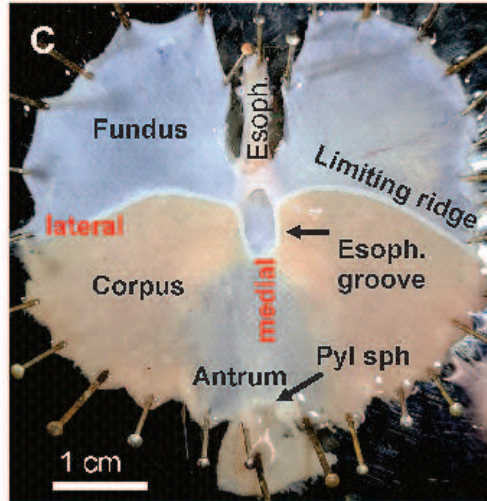
560 FIGURE 8. A: Points of measurement of thicknesses of the layers of the rat stomach. B, C: Circular
561 muscle and longitudinal muscle thicknesses at the sites from which measurements were taken. D: Mean
562 values for thicknesses of all layers \pm SEM. Measurements are from a minimum of 2 female and 2 male rats.

563 FIGURE 9. The muscles of the rat stomach, their directions and relationships. The external muscle of the rat
564 stomach has two complete layers, the longitudinal and circular layers and an incomplete layer, the oblique
565 muscle. It also has a muscularis mucosae, not illustrated, that lies adjacent to the lining mucosa throughout
566 the stomach, and paired muscular ligaments, the esophago-pyloric ligaments.

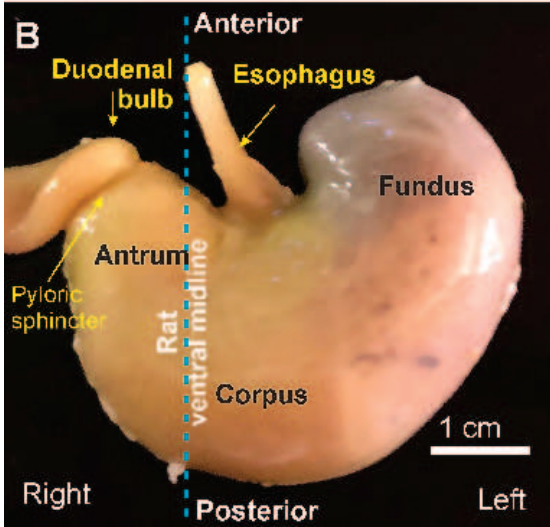
Stomach in vivo (exteriorised)



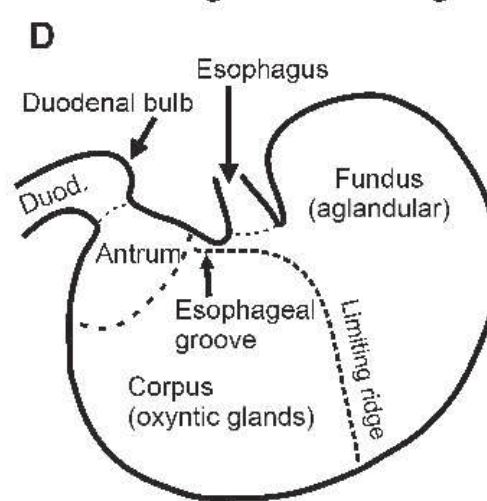
Opened at greater curvature



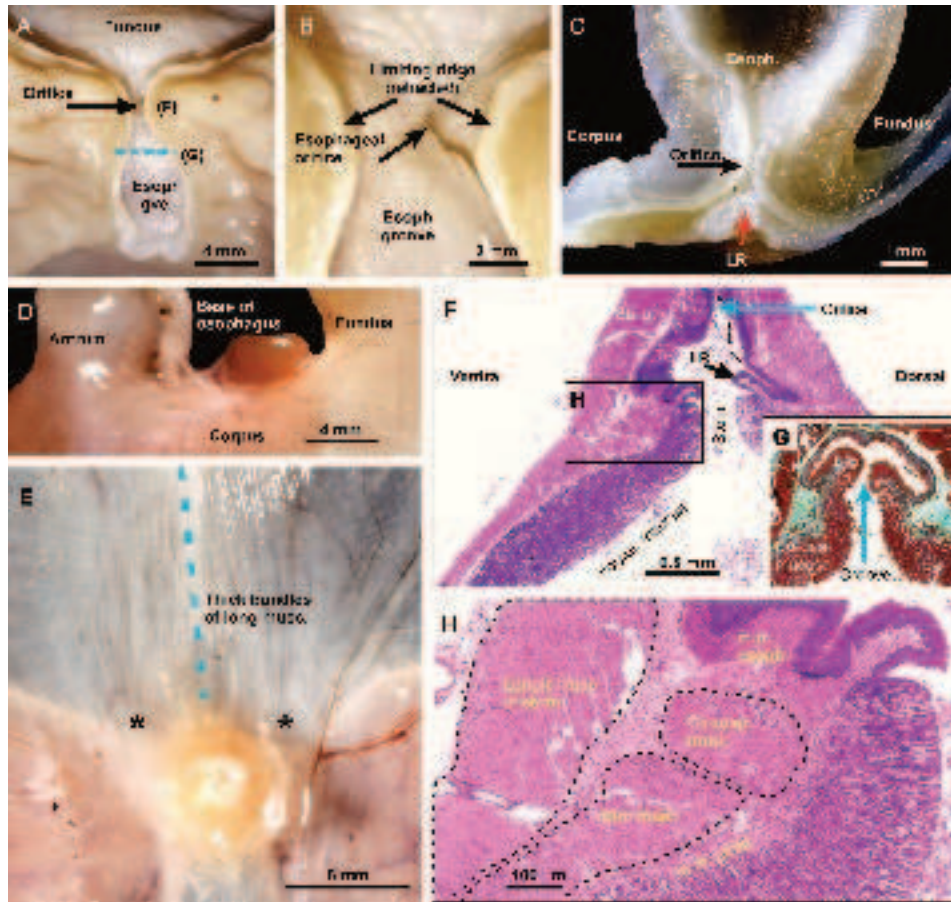
Stomach removed after fixation



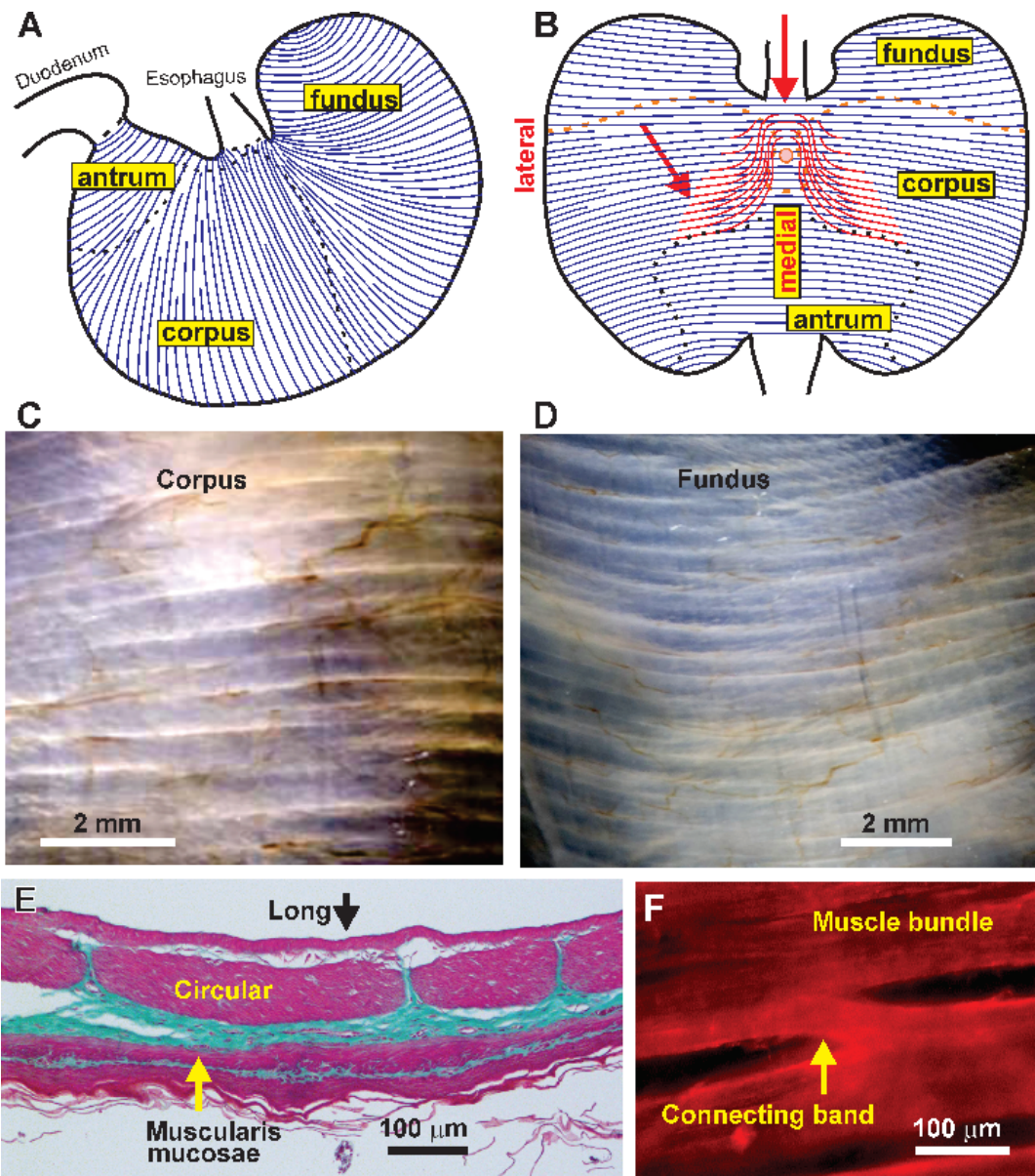
Stomach diagrammatic image



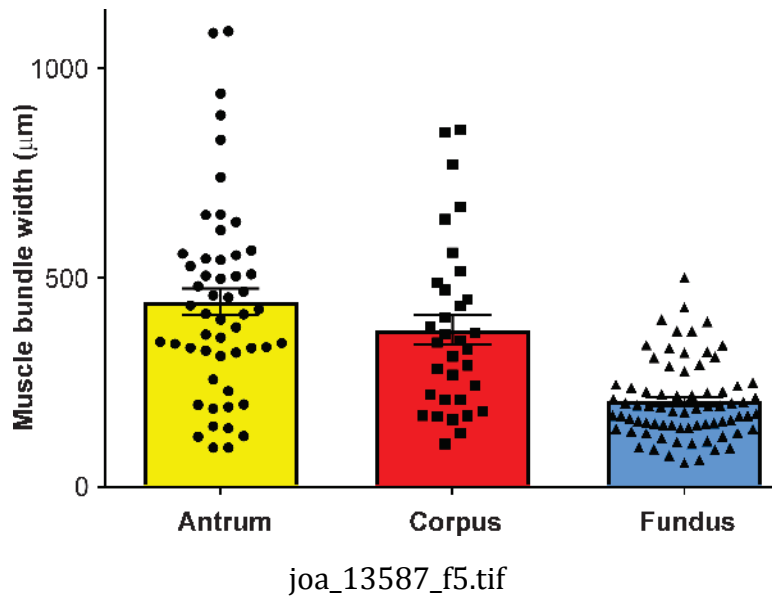
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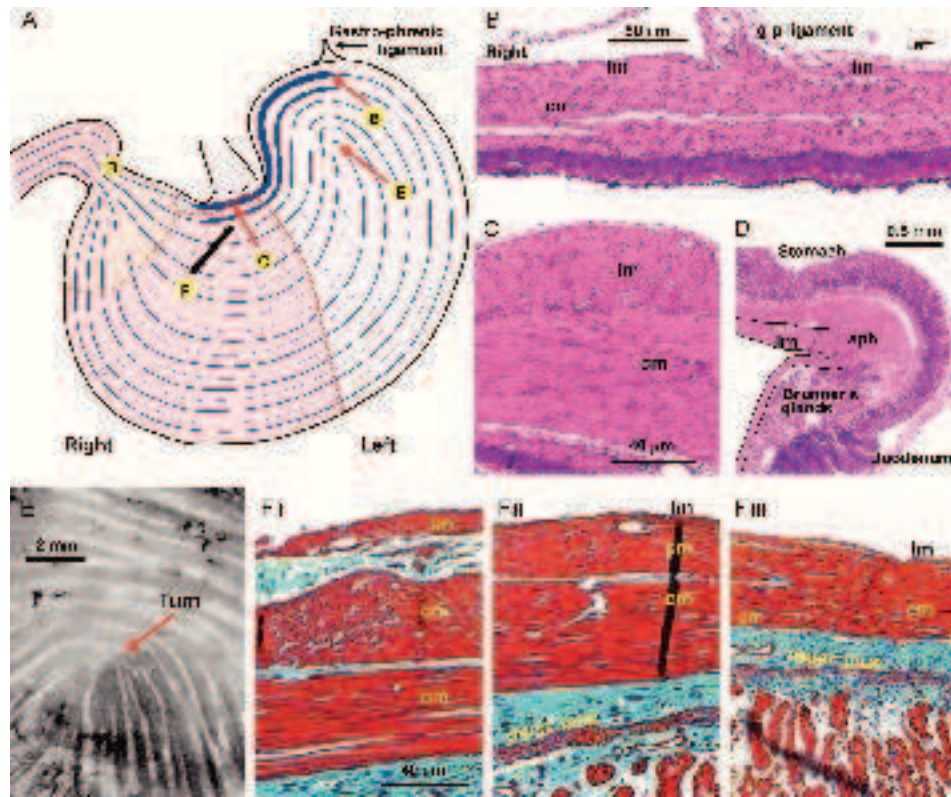


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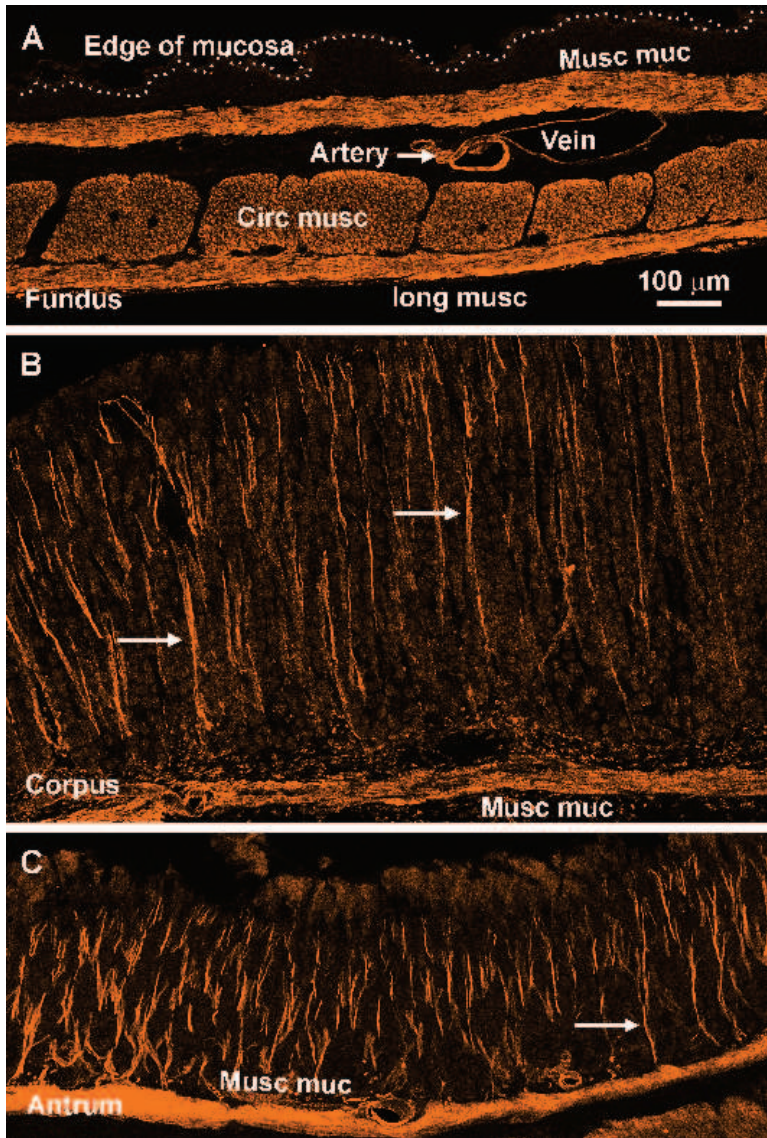


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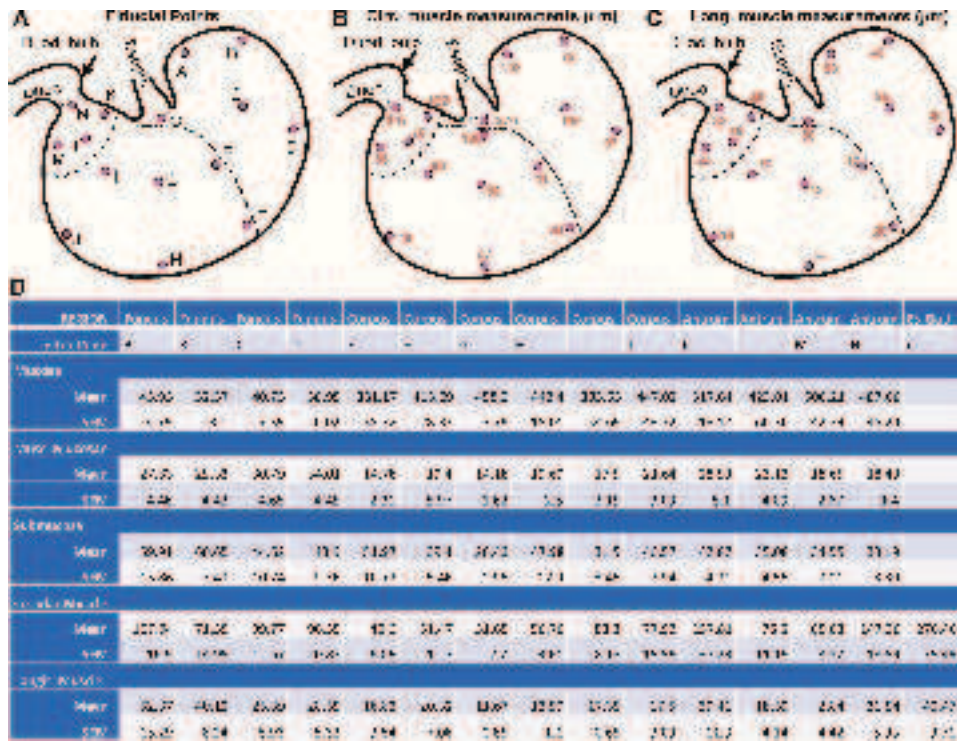




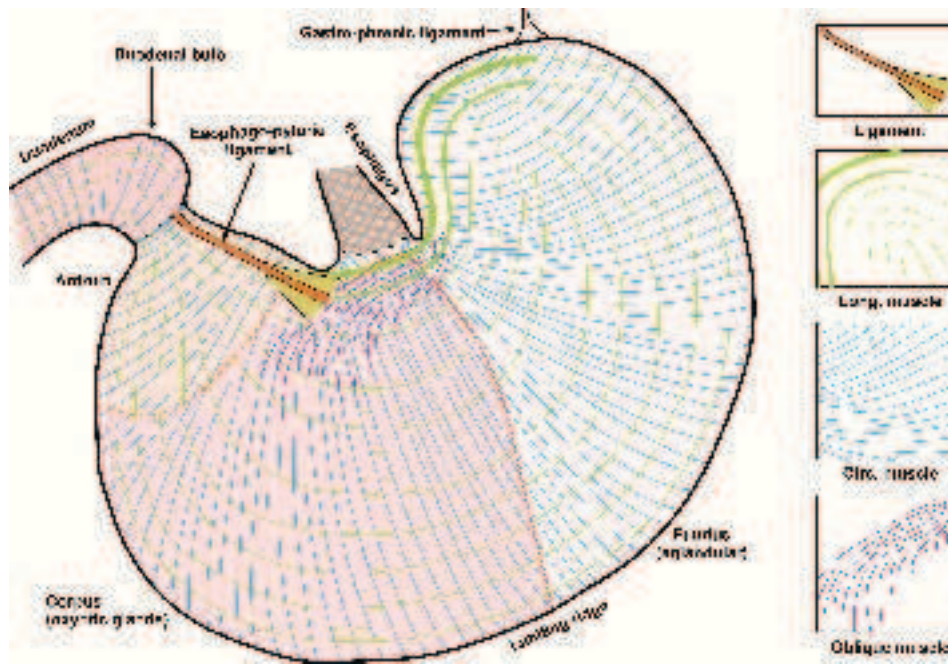
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