



## Effects of feeding on luminal pH and morphology of the gastroesophageal junction of snakes

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### ABSTRACT

At the gastroesophageal junction, most vertebrates possess a functional lower esophageal sphincter (LES) which may serve to regulate the passage of liquids and food into the stomach and prevent the reflux of gastric contents into the esophagus. Snakes seemingly lack an LES and consume meals large enough to extend anteriorly from the stomach into the esophagus thereby providing the opportunity for the reflux of gastric juices. To explore whether snakes experience or can prevent gastric reflux, we examined post-feeding changes of luminal pH of the distal esophagus and stomach, the fine scale luminal pH profile at the gastroesophageal junction, and the morphology of the gastroesophageal junction for the Burmese python (*Python molurus*), the African brown house snake (*Lamprophis fuliginosus*), and the diamondback water snake (*Nerodia rhombifer*). For each species fasted, there was no distension of the gastroesophageal junction and only modest changes in luminal pH from the distal esophagus into the stomach. Feeding resulted in marked distension and changes in tissue morphology of the gastroesophageal junction. Simultaneously, there was a significant decrease in luminal pH of the distal esophagus for pythons and house snakes, and for all three species a steep gradient in luminal pH decreasing across a 3-cm span from the distal edge of the esophagus into the proximal edge of the stomach. The moderate acidification of the distalmost portion of the esophagus for pythons and house snakes suggests that there is some anterior movement of gastric juices across the gastroesophageal junction. Given that this modest reflux of gastric fluid is localized to the most distal region of the esophagus, snakes are apparently able to prevent and protect against acid reflux in the absence of a functional LES.

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### 1. Introduction

The gastroesophageal junction joins the tubular esophagus, which functions to transport ingesta from the mouth, to the sacular stomach, where the meal undergoes chemical and mechanical breakdown (Stevens and Hume, 2004). Generally characterizing this junction is a constrictive lower esophageal sphincter (LES) that allows liquids and food to enter the stomach and prevents the reflux of gastric contents (containing HCl and pepsin) into the lower esophagus (Koelz, 1992; Mittal and Balaban, 1997; Stevens and Hume, 2004). Among mammals, the LES functions through one of three mechanisms: (i) tonic contraction of smooth muscle surrounding the distal end of the esophagus; (ii) pressure from the crural diaphragm during respiration; or, (iii) contraction of stomach sling muscles (Ingelfinger, 1958; Boyle et al., 1985; Allen and Flemström, 2004). The presence of an LES typically ensures

that there is a very steep gradient in luminal pH at the gastroesophageal junction. Luminal pH for the most distal region of the esophagus is typically 6–7 and decreases rapidly to 1.5 within the most proximal region of the stomach (Mattioli et al., 1990; Richter et al., 1992; Mekapati et al., 2008). Occasionally during feeding or while sleeping the LES will relax and gastric juices will reflux into the distal esophagus (Roush, 1990; Fletcher et al., 2001; Simonian et al., 2005; Uriona et al., 2005). To protect against the corrosive action of the acidic refluxed fluid, and to aid in meal transport, the esophageal mucosa contains mucous secreting cells embedded within a layer of stratified squamous epithelium (Orlando, 1991).

In contrast to the esophagus in mammals, the straight and elongated esophagus of snakes joins the stomach with no apparent LES or barrier mechanism between the two organs (Luppa, 1977; Helmstetter et al., 2009). Whereas for most vertebrates (with the exception of birds that possess crops) the consumed meal is passed in its entirety into the stomach, the large and elongated meal of snakes will fill not only their stomach but extend into their esophagus (Pope, 1961; Greene, 1997; Jackson et al., 2004). Another seemingly unique feature among snakes is their ability to dramatically regulate gastric acid secretion and gastric luminal pH. For

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those few species examined, gastric acid production is literally shut down with the emptying of the stomach, allowing luminal pH to increase to  $\sim 7$  (Secor, 2003; Cox and Secor, 2010). With feeding, acid production is quickly resumed and luminal pH is maintained between 1.5 and 3 for the duration of gastric breakdown (Secor, 2003; Cox and Secor, 2010). By lacking a LES, ingesting large meals that protrude into the esophagus, and possessing the capacity to widely regulate acid production, snakes are intriguing subjects to address questions regarding the pH gradient, morphology, and protective mechanisms at the gastroesophageal junction.

In the present study we used three species of snakes to examine the gradient of luminal pH and cellular morphology at the gastroesophageal junction. The three species differ in feeding habits and include the infrequently feeding Burmese python (*Python molurus*) and the frequently feeding African brown house snake (*Lamprophis fuliginosus*) and diamondback water snake (*Nerodia rhombifer*). In addition, we used Burmese pythons to explore the effects of meal size and the duration of gastric digestion on the gradient of luminal pH at the gastroesophageal junction. The objectives of the study were to (i) demonstrate the magnitude that the distal esophagus, gastroesophageal junction, and stomach are distended with feeding; (ii) identify spatial variation and postprandial changes in luminal pH of the distal esophagus and stomach and in the immediate vicinity of the gastroesophageal junction; (iii) describe changes in tissue morphology with the transition from esophagus to stomach and how such tissues respond to feeding; (iv) elucidate the mechanisms that result in a seemingly protective barrier against acidification of the distal esophagus; and (v), examine variation in structure and function of the gastroesophageal junction among snakes.

## 2. Materials and methods

### 2.1. Study animals

The Burmese python (*P. molurus*; Pythonidae), native to south-east Asia, is one of the largest snakes in the world, with lengths of up to 6 m and a body mass that can exceed 100 kg (Pope, 1961). The python employs a sit-and-wait tactic of prey capture and feeds on a variety of avian and mammalian prey (Murphy and Henderson, 1997). The African brown house snake (*L. fuliginosus*; Colubridae) is a small to medium-sized snake (up to 1 m in length and 400 g) that inhabits grasslands to woodlands of sub-Saharan Africa. An active forager, the house snake feeds on a variety of prey including mice, lizards, birds, and bats (Branch, 1998). The diamondback water snake (*N. rhombifer*; Colubridae) is a medium-size (up to 1.4 m in length and 1.4 kg) semiaquatic snake whose range includes the central region of the southern United States and northern Mexico (Gibbons and Dorcas, 2004). An active forager in streams, ponds, lakes, and rivers, this snake's diet consists largely of fish (Mushinsky and Hebard, 1977; Gibbons and Dorcas, 2004).

Pythons were purchased commercially (Bob Clark, Captive Bred Reptiles, Oklahoma City, OK, USA; Strictly Reptiles, Hollywood, FL, USA) as hatchlings and raised on a weekly diet of mice and rats. African brown house snakes were provided by Dr. Neil Ford (University of Texas at Tyler, Tyler, TX, USA) and maintained on a weekly diet of mice. Diamondback water snakes were captured by hand at a commercial catfish farm (Scotland Fisheries, Itta Bena, MS, USA) and maintained on a weekly diet of catfish. We housed pythons and house snakes individually in 20-l plastic boxes at 28–32 °C under a photoperiod of 14L:10D. Water snakes were housed communally in 3000-l circular fiberglass tanks at 24–28 °C under a photoperiod of 14L:10D. All snakes had access to water ad libitum.

Prior to the experiments of the present study, snakes were fasted for a minimum of one month to ensure they were postabsorptive. We maintained snakes at 30 °C in an environmental chamber (model DS54SD; Powers Scientific, Pipersville, PA) for 3 days (fasted snakes) and up to 8 days after feeding (fed snakes) before experimentation.

All animal care and experimentation was conducted under the approval of the University of Alabama Institutional Animal Care and Use Committee.

### 2.2. Esophageal and gastric pH

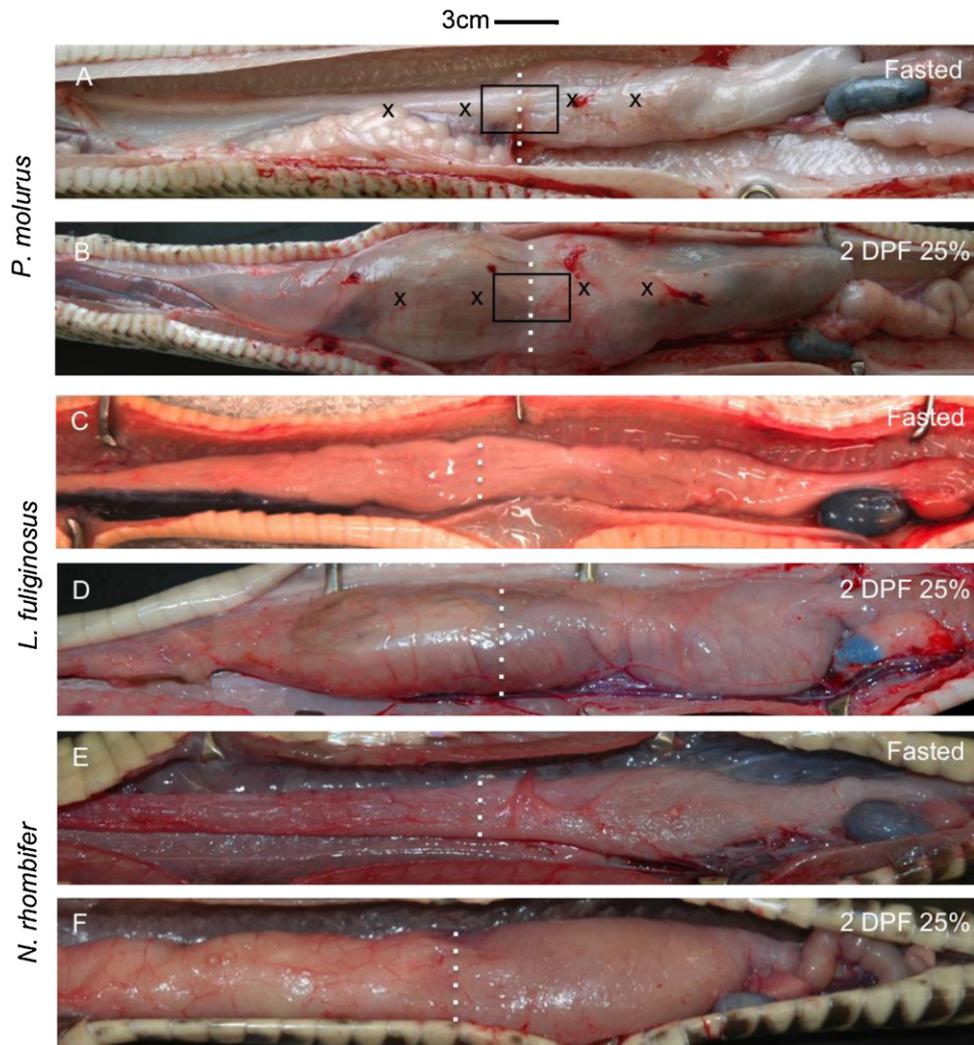
We measured distal esophageal and gastric pH in fasted and digesting Burmese pythons ( $n = 40$ , mean  $\pm$  S.E. = 801  $\pm$  9 g), African brown house snakes ( $n = 7$ , 112  $\pm$  4 g), and diamondback water snakes ( $n = 6$ , 303  $\pm$  26 g). For postprandial measurements, snakes were fed meals (a single rat or mouse for pythons and house snakes, respectively, and catfish filets for water snakes) that equaled 25% of their body mass. An additional set of Burmese pythons were fed two rats equaling (with their combined masses) 55% or 75% of the snake's body mass.

Snakes were killed by severing their spinal cord immediately behind the head at various points: when fasted (>30 days since last meal), at 0.25–15 days post-feeding for pythons, or at 2 days post-feeding for house snakes and water snakes. Following the sacrifice, a mid-ventral incision was made to expose the gastrointestinal tract. Through a small incision in the distal esophagus we measured luminal pH at two positions within the distal region of the esophagus (2 cm and  $\sim 10$  cm anterior to the gastroesophageal junction for pythons and 2 cm and  $\sim 15$  cm anterior to the gastroesophageal junction for house snakes and water snakes) and within the proximal (2 cm distal to the junction) and middle (7–9 cm distal to the junction) portions of the stomach (Fig. 1A and B). Measurements of luminal pH were made using a flexible micro tip pH electrode (Accumet 13-620-95; Fisher Scientific, Pittsburgh, PA, USA) connected to a Fisher AB15 pH meter. Prior to each experiment, we performed a three-point calibration (pH 1, 4, and 7) of the pH electrode.

To examine the fine-scale pH gradient at the gastroesophageal junction, we removed the intact distal esophagus and stomach and pinned it to a styrofoam board. The pH electrode was inserted through the esophagus to several centimeters within the stomach and then attached to a micromanipulator (Model DC3314R; WPI Instruments, Sarasota, FL, USA). The pH electrode was then advanced back through the proximal stomach and distal esophagus with luminal pH recorded at 1-mm increments over a 3-cm span including the gastroesophageal junction (Fig. 1A and B). Gastroesophageal pH measurements were recorded for fasted and fed (2 days post-feeding, meals equaling 25% of snake body mass) Burmese pythons ( $n = 15$ , 638  $\pm$  29 g), African brown house snakes ( $n = 7$ , 112  $\pm$  4 g), and diamondback water snakes ( $n = 10$ , 303  $\pm$  26 g). An additional set of gastroesophageal pH measurements was taken at 4, 6, or 8 days post-feeding, respectively, from pythons that had consumed rat meals equaling 25% ( $n = 3$ , 603  $\pm$  7 g), 55% ( $n = 3$ , 888  $\pm$  4 g), or 75% ( $n = 3$ , 781  $\pm$  19 g) of snake body mass.

### 2.3. Gastroesophageal junction

For fasted Burmese pythons, the gastroesophageal junction is easily defined externally by the meeting of the elongated tubular esophagus and the larger-diameter stomach (Fig. 1A). Internally for the python, the gastroesophageal junction is characterized by an increase in wall thickness and the appearance of longitudinal pleats or folds (i.e., rugae), originating at the proximal edge of the stomach and easily visible with distension (Fig. 1B). For fasted



**Fig. 1.** The exposed esophagus and stomach of fasting (A, C, E) and digesting (B, D, F) Burmese pythons (*Python molurus*), African brown house snakes (*Lamprophis fuliginosus*), and diamondback water snakes (*Nerodia rhombifer*). Images of digesting snakes were taken 2 days post-feeding (DPF) following the consumption of rat, mouse, and catfish meals, respectively, equal in mass to 25% of snake body mass. Sites where luminal pH was recorded are noted on the python images with “x”. The black boxes on the python images illustrate the proximal and distal limits of the fine scale measurements of luminal pH in the vicinity of the gastroesophageal junction. The gastroesophageal junction is identified by a white, vertical dotted line.

house snakes and water snakes, the gastroesophageal junction is similarly identified by the greater stomach diameter, the thicker stomach wall, and internally by the start of rugae (Fig. 1C and E).

#### 2.4. Gastroesophageal morphology

We used light microscopy to describe the postprandial changes in the morphology of the gastroesophageal junction for the three species. A 2-cm segment of the gastroesophageal region was removed from fasted and fed individuals and fixed in 10% formaldehyde in reptile Ringer's solution. These tissues were embedded in paraffin, longitudinally sectioned (6  $\mu$ m), and stained with either *hematoxylin and eosin* or Masson's trichrome. Using light microscopy, we identified for each longitudinal section the gastroesophageal junction. Using image analysis software, we calculated the thickness of five tissue layers of the gastroesophageal region (mucosa, muscularis mucosa, submucosa, and circular and longitudinal muscularis propria) by averaging 15 measurements made within 1 mm of 5 mm anterior to the junction (within the distal esophagus) and 15 measurements within 1 mm of 5 mm distal to the junction (within the proximal stomach).

#### 2.5. Statistical analysis

For each species, we used repeated measures ANOVAs to test among positional effects of pH measurements made in the esophagus and proximal and middle stomach, and fine-scale pH measurements at the gastroesophageal region ( $\pm 15$  mm proximally and distally about the gastroesophageal junction, including a measurement at the junction; cf. box in Fig. 1A and B). One-way ANOVA tests were used to assess temporal effects of fasting and digesting determined by pH measurements made in the esophagus 10 cm and 2 cm anterior to the gastroesophageal junction and in the proximal and middle stomach of all snakes. We used a one-way ANOVA to identify the temporal effects on seven sites (1.5, 1.0, 0.5 cm anterior to the junction, the gastroesophageal junction, and 0.5, 1.0, 1.5 cm distal to the junction) of the fine-scale pH measurements in fasting and 2 days post-feeding pythons, house snakes, and water snakes, and between pythons at 2 and 4 days post-feeding. Similar analyses were used to examine meal size effects on pH of the gastroesophageal junction of pythons by comparing the same seven sites among pythons digesting meals 25% (2 and 4 days post-feeding), 55% (6 days), and 75% (8 days) of body mass. For fasting and digesting snakes, we used one-way

ANCOVAs, with body mass as a covariate, to discern postprandial changes in thickness of each of the esophageal and stomach mucosa, muscularis mucosa, submucosa, and circular and longitudinal muscularis propria layers. Repeated measures ANCOVAs were used to test for differences in tissue thickness (within treatments) between esophageal and gastric sites. For both fasting and 2 days post-feeding treatments, one-way ANOVAs were used to test for interspecific differences in luminal pH measured at four sites within the esophagus and proximal and middle stomach, and luminal pH of seven sites (1.5, 1.0, 0.5 cm anterior to the junction, the gastroesophageal junction, and 0.5, 1.0, 1.5 cm distal to the junction) within the immediate gastroesophageal region of pythons, house snakes, and water snakes. We utilized one-way ANCOVA, with body mass as a covariate, to test for interspecific differences in the thickness of each of the esophageal and stomach layers among the three species for fasting and 2 days post-feeding treatments. For each ANOVA and ANCOVA resulting in a significant difference, we implemented when appropriate a pair-wise post hoc mean comparison (Tukey–Kramer procedure) to identify the level of statistical significance between time points or positions. Statistical significance was set at  $P \leq 0.05$  and we report mean values as means  $\pm 1$  standard error of the mean (SEM).

### 3. Results

#### 3.1. Gastroesophageal distension

At 6 h (not shown) and 12 h after feeding (meals 25% of body mass), the python's distalmost esophagus, gastroesophageal junction, and stomach were greatly distended due to the presence of the intact rat (Fig. 2A). The distension allowed the gastroesophageal junction to be easily identified by the presence of rugae, and the posterior half of the rat meal was visible through the partially translucent stretched esophageal wall. One and two days after feeding, and the respective passage of 17% and 42% of the rat meal into the small intestine, the distal esophagus, gastroesophageal junction, and stomach remained distended (Fig. 2B and C). After three days of digestion, 30% of the remaining rat meal was still protruding anteriorly into the esophagus (not shown). By day 4, with 88% of the meal passed into the small intestine, the esophagus had begun to return to its tubular shape (Fig. 2D). Stomachs were empty by day 6, at which time and thereafter (day 15), the gastroesophageal junction had returned to its fasting appearance (Fig. 2E and F).

For meals 55% and 75% of body mass, the python's gastroesophageal junction was still distended after 6 and 8 days, respectively (Fig. 2G and H). In both cases, the posterior portion (including hindlimbs and tail) of the second rat still extended anteriorly into the esophagus. For house snakes and water snakes two days after feeding, portions of the meal still extended anteriorly into the distal esophagus, thereby distending the gastroesophageal junction (Fig. 1D and F).

#### 3.2. Esophageal and gastric pH

For Burmese pythons, luminal pH within each of the four recorded regions adjacent to the gastroesophageal junction varied significantly ( $P's < 0.024$ ) with time post-feeding (Fig. 3A and B). Within 12 h after feeding, luminal pH within the esophagus approx. 2 cm anterior to the gastroesophageal junction had significantly ( $P = 0.046$ ) decreased and remained significantly less ( $P's < 0.003$ ) than fasting values until day 4 of digestion, at which time it returned to and remained thereafter, at values not significantly different from fasted levels (Fig. 3A). At approx. 10 cm anterior to the gastroesophageal junction, luminal pH experienced a modest postprandial change, decreasing from  $7.31 \pm 0.17$  to  $6.58 \pm 0.02$  by day

2 ( $P = 0.013$ ), returning to fasting values by day 4, and remaining unchanged thereafter (Fig. 3A). Luminal pH at both sites within the stomach varied significantly ( $P's < 0.0002$ ) with fasting and digesting (Fig. 3B). At approx. 2 cm posterior to the gastroesophageal junction, luminal pH decreased from  $6.58 \pm 0.40$  to  $1.85 \pm 0.46$  within 6 h after feeding (Fig. 3B). Thereafter, luminal pH of the proximal stomach remained significantly lower than fasting values before returning to, and not differing from, fasting levels at 10 and 15 days post-feeding ( $P's < 0.04$ ). Within 6 h after feeding, luminal pH of the middle stomach had decreased from  $6.36 \pm 0.18$  to  $2.68 \pm 0.06$ , and further declined to  $1.34 \pm 0.07$  by day 6, returning to fasting levels by 10 days post-feeding (Fig. 3B). Luminal pH varied significantly ( $P's < 0.03$ ) among the four positions from 0.25 to 6 days post-feeding. However, there was no positional variation for fasted pythons and pythons at 10 and 15 days post-feeding.

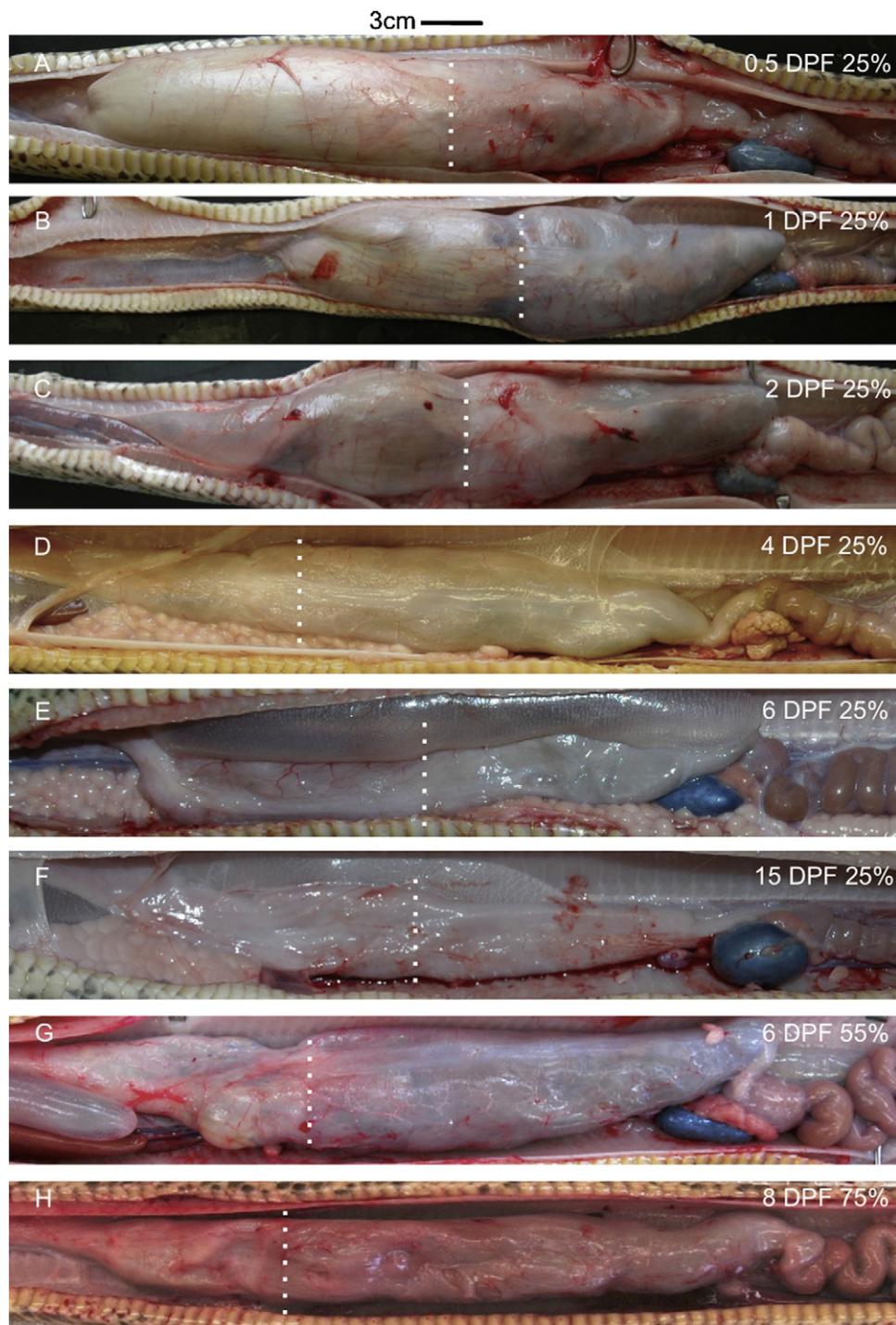
For fasting house snakes, luminal pH did not significantly vary among esophageal and gastric sites (Fig. 4A). At 2 days post-feeding, luminal pH did vary ( $P < 0.007$ ) due to the decreases ( $P's < 0.007$ ) in pH experienced by the distal end of the esophagus and proximal and middle stomach (Fig. 4A). Luminal pH of the esophageal and gastric sites for fasted water snakes varied significantly ( $P = 0.006$ ), ranging from  $6.13 \pm 0.53$  within the stomach to  $7.76 \pm 0.03$  within the esophagus (Fig. 4B). After two days of digesting, luminal pH continued to vary among sites and at each site had decreased significantly ( $P's < 0.003$ ) ranging from  $3.12 \pm 0.51$  in the middle stomach to  $6.82 \pm 0.85$  in the middle esophagus (Fig. 4B).

#### 3.3. pH gradient at the gastroesophageal junction

For fasted pythons, house snakes, and water snakes, luminal pH measured at 1-mm intervals between 1.5 cm anterior and 1.5 cm distal to the gastroesophageal junction, averaged  $7.40 \pm 0.03$ ,  $7.26 \pm 0.03$ , and  $6.69 \pm 0.07$ , respectively (Figs. 5A, 6A, and 7A). For fasted pythons and water snakes, these pH measurements varied significantly ( $P's < 0.0001$ ) spatially, decreasing by 0.73 and 0.82 pH units, respectively, from the anteriormost to the distalmost position (Figs. 5A and 7A). At two days post-feeding, luminal pH recorded at 1.5 cm anterior to the gastroesophageal junction had significantly ( $P's < 0.007$ ) decreased by an average of 1.15 and 2.12 pH units for the Burmese python and the house snake, respectively (Figs. 5B and 6B). The average 0.31 decline in pH units exhibited by water snakes at this site with feeding was not significant (Fig. 7B).

The postprandial production of gastric HCl generated steep gradients in luminal pH in the vicinity of the gastroesophageal junction. For pythons 2 days into digestion, luminal pH dropped rapidly from a point 8 mm anterior to the gastroesophageal junction ( $5.93 \pm 0.26$ ) to a point 5 mm into the stomach ( $3.38 \pm 0.44$ ) (Fig. 5B). For the next 10 mm within the stomach, luminal pH held steady (Fig. 5B). By day 4 of digestion for the python, luminal pH declined fairly consistently from the distal esophagus across the gastroesophageal junction into the stomach (Fig. 5C). From the gastroesophageal junction (pH  $4.44 \pm 0.03$ ), luminal pH had significantly decreased (to  $3.58 \pm 0.25$ ) within only 5 mm into the stomach (Fig. 5C). 6 and 8 days into digesting 55% and 75% size meals, respectively, pythons likewise exhibited steep declines in luminal pH across the gastroesophageal junction (Fig. 5D and E). Across this 3-cm span, from anterior to distal, luminal pH had dropped from  $6.10 \pm 0.73$  to  $2.83 \pm 0.42$  for the 55% meals and from  $6.05 \pm 0.10$  to  $2.13 \pm 0.12$  for the 75% meals (Fig. 5D and E).

For the house snake 2 days into digestion, luminal pH steadily decreased from  $5.31 \pm 0.28$  recorded 1.5 cm anterior to the junction, to  $4.13 \pm 0.13$  at the junction, to  $3.05 \pm 0.25$  1.5 cm distal to the junction (Fig. 6B). For digesting water snakes, luminal pH likewise declined from  $6.93 \pm 0.15$  1.5 cm anterior to the junction, to  $4.79 \pm 0.05$  at the junction, to  $3.20 \pm 0.18$  1.5 cm distal to the junction (Fig. 7B).



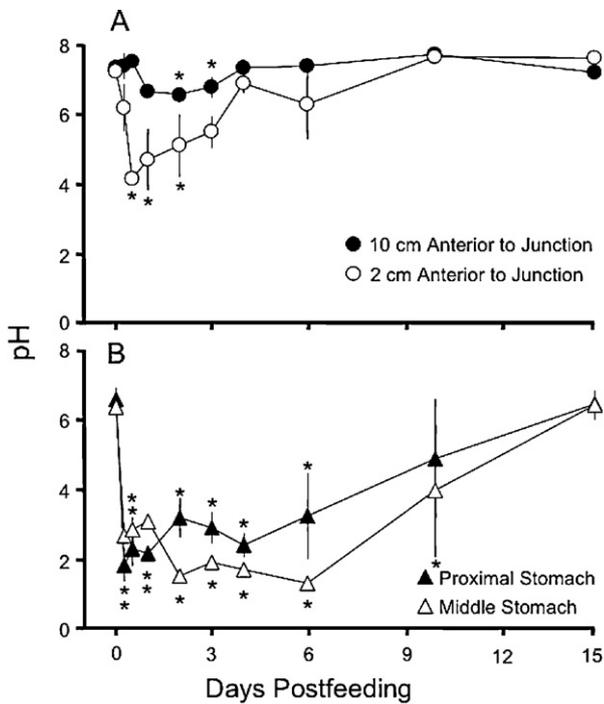
**Fig. 2.** The exposed esophagus and stomach of Burmese pythons (*Python molurus*) digesting rat meals equal in mass to 25% of snake body mass at (A) 0.5, (B) 1, (C) 2, (D) 4, (E) 6, and (F) 15 days post-feeding (DPF); and rat meals equal in mass to (G) 55% and (H) 75% of snake body mass at 6 and 8 DPF, respectively. The gastroesophageal junction is identified by a white, vertical dotted line. Note the distension of the gastroesophageal junction from 0.5 to 2 DPF of 25% meals and for snakes that consumed 55% and 75% size meals.

#### 3.4. Postprandial changes in luminal pH at the gastroesophageal junction

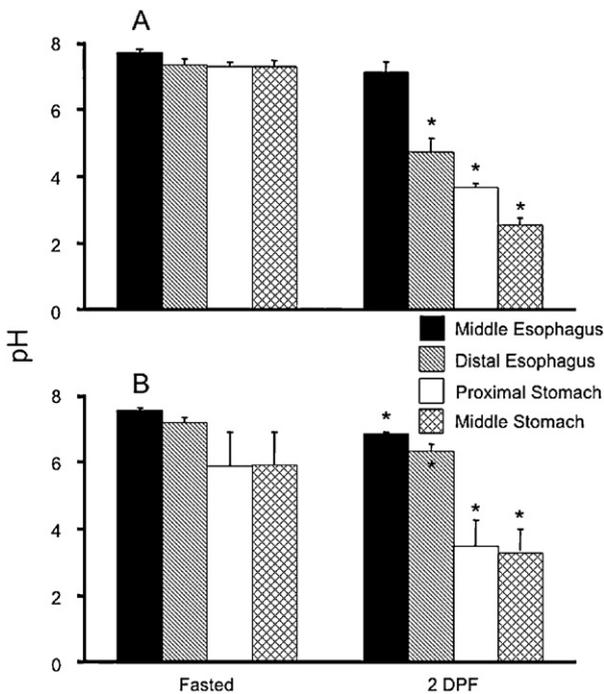
Luminal pH at seven sites of the fine-scale pH measurements decreased significantly ( $P$ 's < 0.007) with feeding for the Burmese python (Fig. 5B–E). For example, at 2 days post-feeding, luminal pH at three locations: 1.5 cm anterior to the junction, at the gastroesophageal junction, and 1.5 cm distal to the junction, had decreased by 1.24, 3.36, and 4.05 pH units, respectively. However, luminal pH

of the seven sites did not significantly differ between 2 and 4 days of digestion. For the 55% and 75% meal trials, luminal pH at the seven sites did not differ significantly from those measured for the 25% size meals at 2 and 4 days post-feeding, with the lone exception of a significantly ( $P = 0.014$ ) lower pH at 1 cm anterior to the gastroesophageal junction for the 55% meal compared to the 25% meal at 2 days post-feeding (Fig. 5B–E).

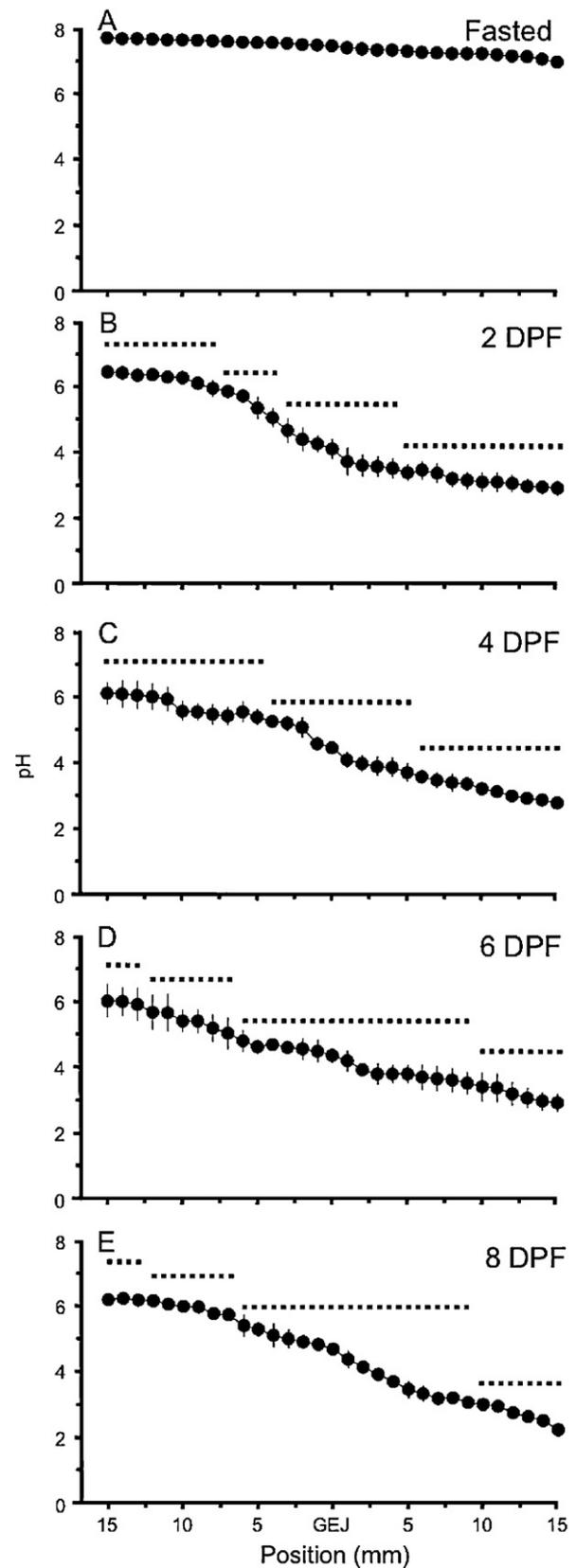
For the African brown house snake, luminal pH at all seven sites decreased significantly ( $P$ 's < 0.004) with feeding (Fig. 6A and B).



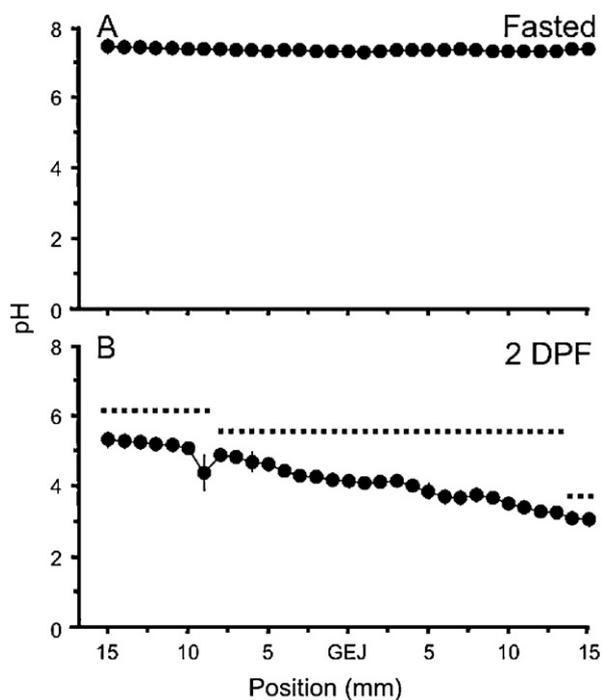
**Fig. 3.** Luminal pH (A) of the esophagus 10 cm and 2 cm anterior to the gastroesophageal junction and (B) of the proximal and middle stomach of Burmese pythons (*Python molurus*) at 0–15 days post-feeding. Note the slight decrease in pH of the distal esophagus and the marked decrease in pH in the proximal stomach upon the onset of digestion.



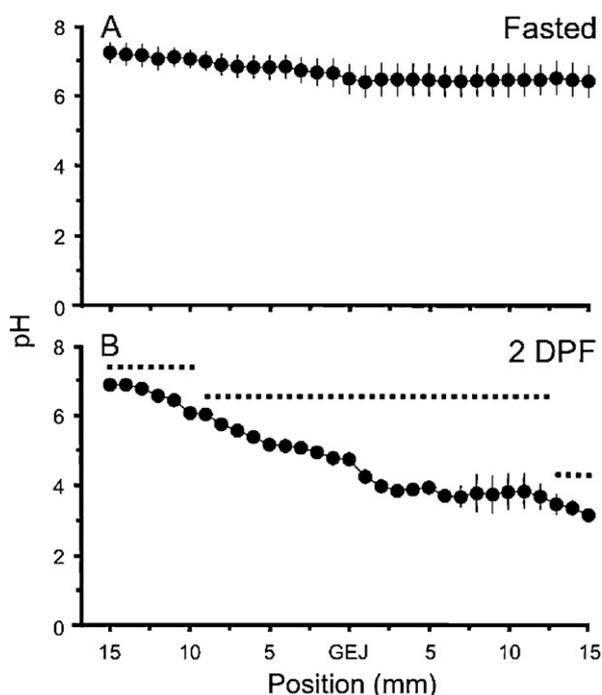
**Fig. 4.** Luminal pH of the middle and distal esophagus and proximal and middle stomach of fasting and digesting (A) *Lamprophis fuliginosus* and (B) *Nerodia rhombifer* digesting mouse and catfish filet meals equaling 25% of snake body mass, respectively. Significant postprandial decreases in luminal pH (designated by \*) occurred for the distal esophagus and the proximal and middle stomach for *L. fuliginosus*, and for all four sites for *N. rhombifer*.



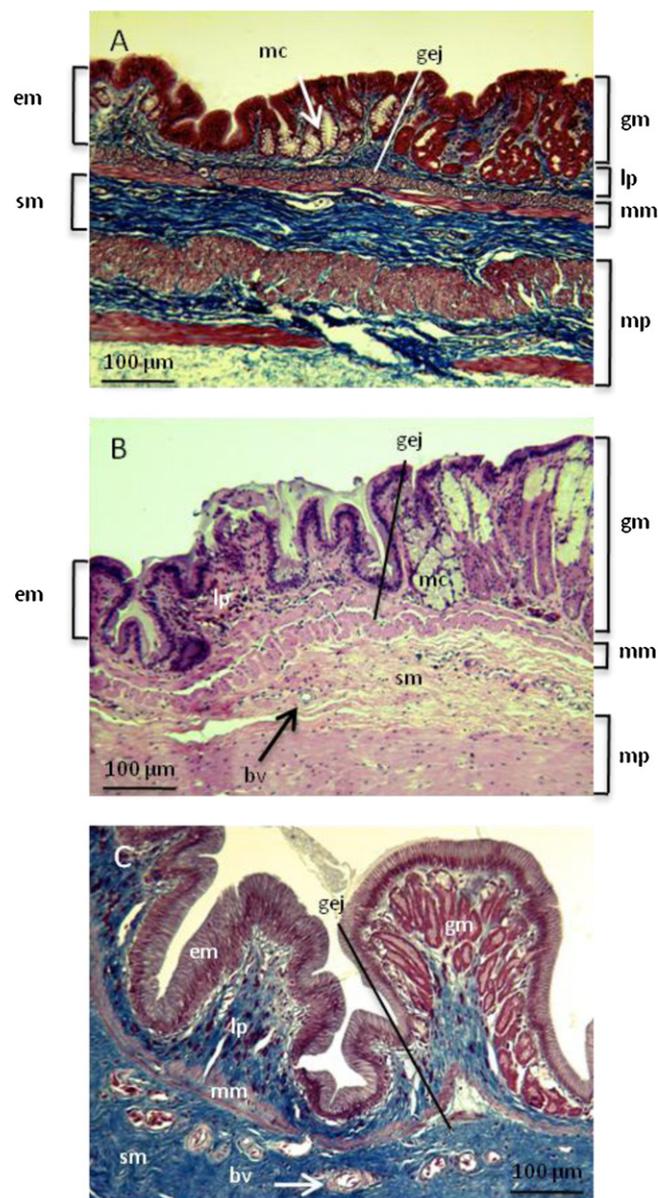
**Fig. 5.** (A–E) Fine-scale luminal pH 1.5 cm anterior to and distal to the gastroesophageal junction (GEJ) of fasting and digesting Burmese pythons (*Python molurus*) following 2, 4, 6, and 8 days post-feeding (DPF) (2 and 4 days = single rat meal equaling 25% of snake body mass, 6 and 8 days = two-rat meal equaling 55% and 75% of snake body mass, respectively). Note the strong positional gradient in pH within 1.5 cm anterior and distal to the gastroesophageal junction. The dotted horizontal lines indicate sets of luminal pH that are significantly different from each other.



**Fig. 6.** Fine-scale luminal pH measured at 1.5 cm anterior to 1.5 cm distal to the gastroesophageal junction (GEJ) of (A) fasting and (B) digesting African brown house snakes (*Lamprophis fuliginosus*) that had consumed a rodent meal equaling 25% of snake body mass two days prior. Note the linear profile of decreasing luminal pH for digesting house snakes. The dotted horizontal lines indicate sets of luminal pH that are significantly different from each other.



**Fig. 7.** Fine-scale luminal pH measured at 1.5 cm anterior to 1.5 cm distal to the gastroesophageal junction (GEJ) of (A) fasting and (B) digesting diamondback water snakes (*Nerodia rhombifer*) that had consumed a catfish filet meal equaling 25% of snake body mass two days prior. Note the linear decline in luminal pH distally for digesting water snakes. The dotted horizontal lines indicate sets of luminal pH that are significantly different from each other.



**Fig. 8.** Histological images of the gastroesophageal junction of (A) Burmese python (*Python molurus*) (Masson's trichrome, 10 $\times$ ), (B) African brown house snake (*Lamprophis fuliginosus*) (H&E, 10 $\times$ ), and (C) diamondback water snake (*Nerodia rhombifer*) (Masson's trichrome, 10 $\times$ ). Note that connective tissue is stained blue in Masson's trichrome plates. Abbreviations: bv, blood vessel; em, esophageal mucosa; gej, gastroesophageal junction; gm, gastric mucosa; lp, lamina propria; mc, mucous cell; mm, muscularis mucosa; mp, muscularis propria; sm, submucosa.

Similarly for the diamondback water snake, luminal pH at these sites, with the exclusion of the anteriormost site, had significantly ( $P < 0.004$ ) decreased after two days of digestion (Fig. 7A and B).

### 3.5. Gastroesophageal tissue morphology

The gastroesophageal junction of pythons, house snakes, and water snakes is defined histologically by the abrupt transition from thin-layered esophageal mucosa to thicker gastric mucosa (Fig. 8A–C). Although the transition is abrupt, the average distance between a clearly identifiable esophagus to gastric tissue was  $1300 \pm 77 \mu\text{m}$ ,  $884 \pm 69 \mu\text{m}$ , and  $641 \pm 75 \mu\text{m}$ , for pythons, house snakes and water snakes, respectively. For pythons and house snakes but not water snakes, spherical mucous cells in the esophagus lamina propria were present at the transition from

esophageal to gastric tissue (Fig. 8A and B). The remaining muscularis mucosa, submucosa, and muscularis propria provide little assistance in identification of the gastroesophageal junction, as their morphology was not altered in the transition. At this junction for the three snakes, we observed a continuous layer of stratified columnar epithelium that spanned the entirety of the esophageal and gastric mucosa (Fig. 8A–C). Esophageal mucosa is comprised of mucus-producing cells interspersed within the columnar epithelium, while the gastric luminal surface consists of a layer of mucous cells, beneath which are gastric pits that contain oxyntopeptic cells that produce HCl and the inactive peptidase, pepsinogen (Fig. 8A–C). Underlying the esophageal and gastric mucosa is the lamina propria, a continuous layer of connective tissue that articulates the mucosa with the continuous muscularis mucosa (Fig. 8A). Advancing outwardly is the submucosa, a layer of connective tissue containing enteric nerve plexi and blood vessels. Adjacent to the submucosa and continuous along the junction is the thicker muscularis propria, which includes an inner circular and outer longitudinal layer (Fig. 8A). Encasing these gastrointestinal tissues is the outermost serosa, a thin layer of connective tissue.

For fasted and fed (2 days post-feeding) pythons, thickness of the mucosa and the circular layer of the muscularis propria increased ( $P$ 's < 0.003) across a 1-cm transition from esophagus to stomach (Table 1). Across that same segment, the other three layers did

not significantly vary in thickness (Table 1). The mucosa of fasted and fed house snakes increased ( $P$  < 0.05) in thickness by as much as 12.6-fold in the 1-cm transition from esophagus to stomach (Table 1). Other tissues did not significantly differ in thickness from one side of the gastroesophageal junction to the other for fasted and fed house snakes (Table 1). Fasted and fed water snakes exhibited 6.9 and 9.7-fold increases ( $P$  < 0.05), respectively, in mucosa thickness from the esophagus side to the stomach side of the gastroesophageal junction (Table 1). For fasted and fed water snakes, the submucosa decreased ( $P$ 's < 0.01) in thickness (by 40%) in the transition from esophagus to stomach (Table 1).

For pythons, feeding had no significant impact on the thickness of the mucosa and submucosa layers at 5 mm anterior and 5 mm distal to the gastroesophageal junction (Table 1). Two days after feeding, the distension of the gut wall resulted in a significant reduction (by 65–86%) in thickness of the muscularis mucosa and both layers of the muscularis propria at both sites (Table 1). With feeding, house snakes experienced a significant thickening of the mucosa and submucosa at 5 mm anterior to and 5 mm distal to the gastroesophageal junction, respectively (Table 1). Other tissues at these two sites did not significantly change in thickness with feeding (Table 1). We observed no significant post-feeding change in the thickness of any of the tissues just anterior and distal to the gastroesophageal junction for water snakes (Table 1).

**Table 1**  
Thickness ( $\mu$ m) of the mucosa, muscularis mucosa, submucosa, circular and longitudinal muscularis propria 5 mm anterior (esophagus) and 5 mm distal (stomach) to the gastroesophageal junction in fasting and digesting (2 and 4 days post-feeding [DPF]) Burmese pythons (*Python molurus*), African brown house snakes (*Lamprophis fuliginosus*) and diamondback water snakes (*Nerodia rhombifer*). Sample sizes are 2–3 per fasted and fed treatments, with the exception of a single measurement of longitudinal muscularis propria of the stomach for fed *N. rhombifer*. For *P. molurus* and significant treatment effects, shared superscript letters denote non-significant ( $P$  > 0.05) differences between means as determined from pair-wise mean comparisons.

	Fasted	2 DPF	4 DPF	F	P
<b>Esophagus</b>					
<i>Python molurus</i>					
Body mass (g)	578 ± 3	698 ± 41	595 ± 12		
Mucosa	53.3 ± 7.2	44.3 ± 4.1	54.4 ± 5.1	2.93	0.129
Muscularis mucosa	62.6 ± 4.5 <sup>b</sup>	26.4 ± 2.7 <sup>a</sup>	48.3 ± 3.7 <sup>b</sup>	26.6	0.0010
Submucosa	118 ± 32	73.2 ± 13.2	106 ± 8	2.35	0.176
Muscularis propria (circular)	437 ± 20 <sup>c</sup>	84.8 ± 22.8 <sup>a</sup>	198 ± 21 <sup>b</sup>	99.0	0.0001
Muscularis propria (longitudinal)	408 ± 76 <sup>b</sup>	36.1 ± 9.4 <sup>a</sup>	86.7 ± 28.5 <sup>a</sup>	19.4	0.0024
<b>Stomach</b>					
Mucosa	421 ± 11	442 ± 13	396 ± 2	3.41	0.098
Muscularis mucosa	81.5 ± 17.4 <sup>b</sup>	34.8 ± 1.5 <sup>a</sup>	53.3 ± 9.8 <sup>ab</sup>	5.46	0.045
Submucosa	128 ± 33	73.9 ± 8.3	95.5 ± 20.0	2.05	0.210
Muscularis propria (circular)	1101 ± 98 <sup>b</sup>	312 ± 25 <sup>a</sup>	789 ± 117 <sup>b</sup>	26.2	0.0011
Muscularis propria (longitudinal)	309 ± 101 <sup>b</sup>	52.0 ± 9.5 <sup>a</sup>	95.4 ± 12.1 <sup>ab</sup>	5.80	0.040
<b>Esophagus</b>					
<i>Lamprophis fuliginosus</i>					
Body mass (g)	123 ± 4	104 ± 1			
Mucosa	30.1 ± 1.4	35.1 ± 1.6		51.4	0.019
Muscularis mucosa	35.3 ± 19.5	25.4 ± 4.7		0.08	0.809
Submucosa	89.4 ± 27.6	63.7 ± 8.1		0.21	0.689
Muscularis propria (circular)	201 ± 13	178 ± 25		0.06	0.835
Muscularis propria (longitudinal)	70.8 ± 23.8	56.1 ± 1.1		0.03	0.877
<b>Stomach</b>					
Mucosa	379 ± 84	364 ± 30		0.04	0.375
Muscularis mucosa	29.8 ± 10.8	26.8 ± 11.3		0.01	0.381
Submucosa	52.8 ± 1.6	72.3 ± 4.4		30.6	0.031
Muscularis propria (circular)	271 ± 95	285 ± 97		0.23	0.680
Muscularis propria (longitudinal)	80.6 ± 0.1	134 ± 85		0.59	0.523
<b>Esophagus</b>					
<i>Nerodia rhombifer</i>					
Body mass (g)	261 ± 22	364 ± 36			
Mucosa	49.0 ± 7.4	44.0 ± 4.4		5.77	0.095
Muscularis mucosa	28.4 ± 8.3	25.0 ± 3.5		2.05	0.248
Submucosa	186 ± 2	168 ± 35		1.42	0.320
Muscularis propria (circular)	383 ± 25	279 ± 38		5.22	0.106
Muscularis propria (longitudinal)	71.6 ± 25.1	87.0 ± 12.5		0.08	0.799
<b>Stomach</b>					
Mucosa	338 ± 65	427 ± 66		0.16	0.726
Muscularis mucosa	26.7 ± 12.5	41.9 ± 4.8		0.03	0.878
Submucosa	95 ± 9	137 ± 30		0.45	0.571
Muscularis propria (circular)	358 ± 1	421 ± 23		1.09	0.405
Muscularis propria (longitudinal)	55.4 ± 10.4	106		–	–

### 3.6. Interspecific variation in luminal pH and tissue morphology

For fasted snakes, luminal pH varied ( $P=0.02$ ) interspecifically for the middle region of the stomach as the pH recorded for house snakes ( $7.26 \pm 0.31$ ) was significantly ( $P's < 0.03$ ) greater than that for either pythons ( $6.36 \pm 0.18$ ) or water snakes ( $6.13 \pm 0.53$ ). After two days of digestion, luminal pH varied significantly ( $P=0.01$ ) once again for the middle gastric region as pythons possessed lower luminal pH ( $1.53 \pm 0.15$ ) compared to that of house snakes ( $2.55 \pm 0.20$ ) and water snakes ( $3.73 \pm 0.66$ ).

At each of the seven sites of the gastroesophageal region (0.5, 1, and 1.5 cm anterior to the junction, the gastroesophageal junction, and 0.5, 1, and 1.5 cm distal to the junction), we observed no significant variation in luminal pH among fasted pythons, house snakes, and water snakes (Figs. 5A, 6A, and 7A). Following two days of digestion, luminal pH at the sites 1 and 1.5 cm anterior to the gastroesophageal junction experienced significant variation ( $P's < 0.005$ ) among the three species, due to the lower ( $P's < 0.02$ ) luminal pH ( $5.30 \pm 0.12$  and  $5.06 \pm 0.09$ , respectively, at those sites) experienced by house snakes (Figs. 5B, 6B, and 7B).

There were notable differences in tissue thickness among the three species either fasted or fed (Table 1). For example, the thickness of the circular and longitudinal layers of the muscularis propria differed as much as 5.8-fold between species. However, when accounting for differences in body size (body mass as a covariate), analyses revealed no significant interspecific differences in the thickness of each tissue for either fasted or fed snakes

## 4. Discussion

Because snakes will consume prey that exceeds the width and length of their stomach, feeding can result in a dramatic distension of their stomach and distal portion of their esophagus. By lacking a lower esophageal sphincter, snakes digesting large meals would conceivably experience the seeping of gastric acid and enzymes into the distal esophagus. In spite of this opportunity for gastric reflux, snakes are able to maintain a steep gradient in luminal pH at the gastroesophageal junction during digestion. Due to mechanical and/or chemical processes, luminal pH on either side of the gastroesophageal junction spans 4 pH units over a distance of only several centimeters for digesting snakes. With regard to the gastroesophageal junction of snakes, we will discuss the gross and microscopic aspects of the surrounding tissues, the gradient of luminal pH between the distal esophagus and proximal stomach, and the mechanisms by which this gradient is maintained.

### 4.1. Gross morphology of the gastroesophageal junction

For fasted pythons, the gastroesophageal junction is easily distinguishable as the joining of the tubular, narrow esophagus to the wider, thicker-walled stomach. In contrast, the gastroesophageal junction for fasted house snakes and water snakes is less identifiable because their esophagus widens before joining the stomach (Fig. 1C and E). For all three species, a change in tissue thickness and color also provides a landmark for this junction when viewed externally. Snake prey items are commonly longer than the length of the stomach. For example, the body lengths (excluding the tail) of rats fed to the pythons in this study averaged  $\sim 24$  cm, whereas the length of the stomach of these snakes averaged  $\sim 10$  cm. Thus, upon consumption, the majority of the python's meal (as well as for house snakes and diamondback water snakes) resides within the esophagus, rather than within the stomach. Extreme cases of the prey extending into the esophagus occur for snakes that consume other snakes. For California kingsnakes (*Lampropeltis getula*)

that had consumed similar-sized corn snakes (*Elaphe guttata*), X-ray analysis revealed that 75–80% of the corn snake was within each kingsnake's esophagus and occupied the esophagus for at least four days (Jackson et al., 2004). We are unaware of any other group of vertebrates that routinely consume such large meals such that part of the meal also occupies the esophagus. One partial exception are the species of birds that store food (e.g., seeds, insects, and grain) within their crop (ingluvies), an expandable pouch extending from the esophagus within the thorax (Buyse et al., 1993).

Snakes often consume prey that is larger in diameter than their esophagus and stomach, thereby resulting in a gross distension of the distal esophagus, stomach, and surrounding body wall (Greene, 1997). Given that prey vary in shape and size, the extent of distension depends largely on prey diameter. The diameters of rats fed to pythons in the present study were 4.1 and 2.3 times greater than the diameters of the pythons' distal esophagus and stomach, respectively. The water snakes of the present study experienced less relative distension because they were fed long strips of catfish filet. The kingsnakes in the Jackson et al. (2004) study likewise experienced modest stretching of their esophagus and stomach with the ingestion of the narrow and elongated corn snakes.

### 4.2. Microscopic morphology of the gastroesophageal junction

The gastroesophageal junction of the three studied snake species characteristically shows an abrupt change from a single layer of columnar epithelial cells of the distal esophagus to a thicker mucosa of the stomach, composed of mucous and oxyntopeptic cells that line the upper region and lower portions of the gastric pits, respectively. The tissues of the esophagus and stomach that were most impacted by prey distension were the smooth muscle layers (muscularis mucosa and muscularis propria) that experienced a severe reduction in thickness for pythons. In contrast, the epithelial layer was generally not affected by stretching, and for the stomach this layer is folded and the distension flattens out these folds. Helmstetter et al. (2009) likewise observed a distinct flattening of the python's gastric rugae with feeding.

For snakes, a columnar epithelial layer characterizes the esophageal mucosa (Ferri and Medeiros, 1975; Luppa, 1977; Dehlawi and Zaher, 1989). We also observed for the three species a scattering of goblet cells within the distal esophageal epithelium. Similarly, goblet cells have been documented in the distal esophagus of other snake species and have been postulated to aid in the lubrication of the meal, via mucous secretion, during swallowing (Luppa, 1977). We also found the distal esophageal mucosa of pythons and house snakes to possess large concentrations of circular-shaped mucus cells that extend into the lamina propria (Fig. 8A and B). The secretions of these cells may serve to further lubricate the meal and to neutralize any gastric chyme that has refluxed into the esophagus, as noted for mammals (Orlando, 1999; Chandrasoma et al., 2000).

The transition from esophagus to stomach is abrupt in pythons, house snakes and water snakes and is readily identified histologically by the switch from a single layer of mucosa (esophagus) to the appearance of gastric pits (stomach) which become deeper distally. The gastric mucosa of snakes consists of an outer columnar epithelial layer that is dotted with openings to underlying gastric pits (Helmstetter et al., 2009). The gastric pits or crypts contain surface neck cells that produce mucus, whereas the deeper oxyntopeptic cells actively pump  $H^+$  and shuttle  $Cl^-$  across the apical membrane into the gastric lumen to produce HCl. Pythons of the present study experienced no significant increase or decrease in the thickness of the gastric mucosa from fasting to digesting. A previous study did find the gastric mucosa of the Burmese python to decrease in thickness with feeding (Helmstetter et al., 2009).

#### 4.3. Luminal pH near the gastroesophageal junction

Whereas an elevated luminal pH is expected for the esophagus, the high luminal pH ranging from 6.1 to 7.3 recorded from the stomachs of these snakes when fasting is seemingly unique (Figs. 3B, 4A and B). Snakes apparently deviate from many other terrestrial vertebrates in shutting down gastric HCl production after completing digestion (Skoczylas, 1970; Secor, 2003; Cox and Secor, 2010). We have also recorded similarly high gastric pH values from fasting individuals of other python species (e.g., *Antaresia maculosa* and *Morelia spilota*), boa species (e.g., *Boa constrictor*, *Gongylophis colubrinus*, *Eunectes murinus*), the xenopeltid *Xenopeltis unicolor*, and the colubrid *Homalopsis buccata* (S. Bessler and S. Secor, unpublished observations). In contrast, gastric pH of fasting chondrichthyes, anurans (although see Tyler et al., 1983; Taylor et al., 1985), lizards, crocodylians, birds, and mammals typically ranges from 1.5 to 3.5 (Ford, 1974; Youngberg et al., 1985; Evans et al., 1988; Gauthier-Clerc et al., 2002; Uriona et al., 2005; Papastamatiou and Lowe, 2004, 2007; Secor, unpublished observations), demonstrating that these taxa maintain gastric HCl production between meals. In addition to snakes, elevated gastric pH has been observed in fasting turtles and bony fishes (McKay, 1929; Fox and Musacchia, 1950; Montgomery and Pollak, 1988). Snakes, turtles, and bony fishes must therefore possess the capacity to depress H<sup>+</sup> release by their oxyntopeptic cells by either downregulating H<sup>+</sup>/K<sup>+</sup> pump activities or by suppressing the catalytic enzyme carbonic anhydrase, an integral component in H<sup>+</sup> production. It is hypothesized for snakes that the shutting down of HCl production between meals is an energy-saving adaptation that serves to lower their basal metabolism, allowing them to survive longer periods between meals on endogenous energy stores (Secor, 2003).

Feeding triggers the rapid production of gastric HCl in snakes. Within 6 h after feeding, the luminal pH of the python's stomach decreased from 6.6 to 1.8 (Fig. 3B). Although we did not measure gastric pH 6 h after feeding for house snakes and water snakes, we suspect that they experience a similar rapid upregulation in HCl production and subsequent decrease in gastric pH. Within 12 h after ingestion, diamondback water snakes have been noted to reduce gastric pH to 1.8 (Cox and Secor, 2010). Snakes maintain a very acidic environment within their stomach (pH 1.5–2.5), even in the face of the large buffering capacity of their meals, throughout the duration of gastric breakdown (Secor, 2003; Cox and Secor, 2010). Following the exiting of all prey remnants from the stomach, gastric HCl production is rapidly downregulated and luminal pH rises rapidly (Secor, 2003; Secor et al., 2012).

Because of the lack of gastric acid production between feeding episodes, fasting snakes exhibit modest differences in pH between the distal esophagus and stomach. This situation changes rapidly with feeding, as acidic juices surround and begin to permeate the meal. In addition to the rapid drop in gastric pH relative to esophageal levels, the distal end of the esophagus also experiences a decrease in pH as the acidic fluid diffuses around the meal and moves anteriorly. A luminal pH of 2.5–4 for the distal esophagus has been observed for fasted and digesting humans, dogs, and alligators (Fletcher et al., 2001; Kim et al., 2003; Simonian et al., 2005; Uriona et al., 2005). For snakes, luminal pH increases significantly within 1 cm anterior to the gastroesophageal junction, and continues to increase anteriorly, reaching a pH of 6.5–7.5 within 10 cm cranially to the junction. Despite lacking a functional LES, snakes effectively prevent the reflux of gastric juices and/or neutralize any of those juices that do reflux into the esophagus.

#### 4.4. Protective mechanisms of the distal esophagus

Prolonged relaxation of the lower esophageal sphincter in humans and the ensuing exposure of the distal esophagus to

refluxed gastric HCl and pepsin lead to reflux esophagitis, a condition where mucosal squamous epithelium of the distal esophagus becomes necrotic, creating painful ulcers (Spechler, 2002). With extended exposure to refluxed gastric juices, reflux esophagitis can escalate to Barrett's esophagus, a precancerous condition where squamous epithelium of the distal esophagus undergoes metaplasia (the replacement of one cell type by another mature differentiated cell type) to columnar epithelium, consisting largely of mucus-secreting goblet cells (Barrett, 1957; Cameron et al., 1985; Spechler, 2002).

The diffusion of gastric juices into the distal esophagus of snakes would likewise lead to tissue erosion and potential alteration of tissue structure. Without a distinct LES, snakes must employ other protective mechanisms to either prevent gastric reflux and/or to rapidly neutralize refluxed gastric juices. We suggest four such mechanisms that are utilized in lieu of an LES. First, the sheer size of the prey item within the esophagus and stomach, up to four days post-feeding in our study, would serve as a physical barrier and prevent gastric juices from passing cranially into the distal esophagus (Fig. 2A–C). This physical barrier hypothesis is supported by our measurements of pH at 0.25–4 days post-feeding within the gastroesophageal region, where within a 4-cm range pH drops from ~6 to ~2 from the distal esophagus to the proximal stomach, respectively (Fig. 3A and B). Second, the meal itself acts as a buffering agent, neutralizing the secreted HCl. Any gastric juices that migrate anteriorly into the esophagus would be similarly neutralized to some extent by contact with the meal. Third, the prey is slowly moved caudally as its anterior end is broken down within the stomach and passed into the small intestine. This directional motion may reduce the opportunity for gastric fluid to move anteriorly into the distal esophagus. And fourth, as described for the human esophagus, the mucus secreted from goblet and mucous cells into the esophagus acts to buffer the refluxed fluid and hence protects the epithelium (Orlando, 1991).

### 5. Conclusion

Snakes can experience dramatic distension of their gastroesophageal region immediately after feeding. This results in visible changes in tissue histology at the junction, notably the decrease in thickness of the muscularis mucosa and muscularis propria. From a relatively neutral start, feeding stimulates gastric HCl production which rapidly lowers stomach pH. Whereas some gastric juices do seep into the distal end of the snake's esophagus, a strong gradient in luminal pH is established at the gastroesophageal junction. Despite the absence of a distinct lower esophageal sphincter, snakes maintain an effective protective barrier to the reflux of gastric juices into the distal esophagus.

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