

Hierarchical loop formation in human midgut during physiological umbilical herniation

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Abstract

This study aimed to describe the morphological alterations that occur in the midgut and mesentery over time during the herniated phase of the midgut. The primary loop, a single hairpin-shaped loop, becomes recognizable at Carnegie stage (CS) 16. This loop projects toward the umbilical cord and subsequently gives rise to four secondary loops in the midgut of human embryos. As development advances, the segments corresponding to each secondary loop further develop into an increasing number of loops, referred to as tertiary loops. The mesenteric leaves and the narrowing parts, which serve as the borders of the secondary loops, remain identifiable throughout the subsequent stages of development.

A total of 47 human embryos between CS16 and CS23 and two fetuses in the physiological umbilical herniated stage were selected for high-resolution magnetic resonance imaging acquisition. Specimens were obtained from the Congenital Anomaly Research Center of Kyoto University. Serial tissue sections obtained from four embryos were subjected to histological observation. The midgut and mesentery were reconstructed in three dimensions, and the resulting morphological changes were observed and analyzed. Formation of the primary loop was observed in all specimens between CS16 and CS18. Secondary loops in the midgut were initially discerned at CS19 in segments 2 and 4 (S2 and S4). The border between S3 and S4 was identified at the apex of the midgut hernia, where traces of the vitelline artery and duct enter the mesentery. At CS21 and later stages of development, the presence of three borders at the exact location delineated by mesenteric narrowing was consistently observed, which resulted in the midgut being divided into four segments in all specimens. The formation of tertiary loops was initially identified at Carnegie stage (CS) 21, occurring in either segment S2 or S3. By CS23, tertiary loops were observed in three segments in most specimens. Notably, the initial formation of tertiary loops in S4 occurred one Carnegie stage later than in S2 or S3. Additionally, the increase in the number of folds and the length per fold in S4 was delayed compared with the number and length of folds observed in both S2 and S3. The number of loops in S1 remained constant (one secondary loop) across all specimens. Upon reaching a critical threshold length, the number of loops exhibited a marked increase, accompanied by rapid elongation in S2, S3, and S4. The number of tertiary loops increased in accordance with the crown-rump length, which exhibited a maximum of 19 tertiary loops in S2 to S4 of the midgut.

These findings support the hypothesis that tertiary loops develop biomechanically through the rapid elongation of the midgut and slow growth of the mesentery. This study describes the morphological alterations occurring in the midgut and mesentery

over time during the herniated phase of the midgut and provides a comprehensive understanding of the roles of genetic and biomechanical factors in loop formation.

Keywords: human embryo, intestinal loop formation, midgut, umbilical herniation

1 INTRODUCTION

The rate of change in the number and configuration of small intestinal loops in adults is sufficiently complex and difficult to comprehend fully. However, it is not believed to be entirely random. Therefore, examining the period during which the initial formation of the fundamental intestinal loop becomes discernible could provide valuable insights into the underlying constraints. A single hairpin-shaped loop, known as the primary loop, becomes recognizable at Carnegie stage (CS) 16, where it projects toward the umbilical cord (Kim et al., 2003; Ueda et al., 2016). Mall (1898) observed that the intestinal tracts of four embryos, measuring between 17 and 28 mm in crown-rump length (CRL), exhibited physiological umbilical herniation with intestinal loops. Six distinct loops were identified in the small intestine (the duodenum, jejunum, and ileum). Furthermore, the intestinal loop arrangements corresponding to these six loops have been observed in adult autopsy cases. Pernkopf (1925) revised the interpretation of Mall's study, particularly of the first three loops within the abdominal cavity. The initial three loops undergo elongation, forming a single extensive loop that gives rise to the duodenum and duodenojejunal flexure. Consequently, the author posited the existence of four loops in the small intestine extending from the duodenum to the cecum. A comparison of several studies (Soffers et al., 2015; Pernkopf, 1924; Pernkopf, 1925; Frazer & Robbins, 1915) revealed that the number, shape, and topography of the secondary loops remain consistent throughout embryonic development. Human anatomists and embryologists have widely accepted that four loops are formed in the small intestines of human embryos. The first loop develops and persists within the abdominal cavity, whereas the remaining three secondary loops form within the extra-embryonic coelom during the developmental period of physiological umbilical hernia.

As development progresses, each secondary loop in the extraembryonic coelom generates an increasing number of tertiary loops. In contrast, the mesenteric leaves and the narrowing parts, which delineate the borders of the secondary loops, remain discernible throughout subsequent developmental stages.

Soffers et al. (2015) argued that midgut loops arise hierarchically (Figure 1a). They proposed that the morphology of the primary and secondary loops is genetically predetermined, whereas additional tertiary loops form within the four segments corresponding to the secondary loops, exhibiting unpredictable configurations and

topographic distributions. Consequently, they suggested that biomechanical factors, particularly the differential growth between the intestine and mesentery, play a crucial role in the formation of tertiary loops. This study analyzed a relatively limited number of samples using histological sections. The precise circumstances surrounding the initial formation of secondary and tertiary loops, subsequent formation of additional loops, and currently available information on their characteristics, similarities, and differences remain insufficient. This information is crucial for a comprehensive understanding of the roles of genetic and biomechanical factors in loop formation.

Concurrent with the development of intricate midgut loops during the embryonic and early fetal stages, two significant events occur: (1) herniation into the extraembryonic coelom and (2) return to the abdominal cavity (Kim et al., 2003; Ueda et al., 2016; Nagata et al., 2019; Soffers et al., 2015; Kakeya et al., 2022). These phenomena result in a significant alteration of the midgut position, thereby rendering the precise and detailed analysis of loop formation challenging. Recent studies have visualized and observed the superior mesenteric artery (SMA) branches (Soffers, 2015; Ginzel, 2021; Kakeya, 2022) and mesentery (Soffers et al., 2015; Hikspoors et al., 2019) in conjunction with the intestinal tract, thereby providing additional anatomical landmarks and serving as a substitute for the complex course of the intestinal tract. Soffers et al. (2015) demonstrated that the mesenteric architecture accentuates the boundaries of the secondary loops. By focusing on the intestinal membrane, capturing the basic stereotypical architecture of the midgut is possible, particularly the four secondary loops that form the four segments, without being distracted by the intricate and varied course of the intestine. This study aimed to reconstruct the intestinal tract and mesentery in three dimensions (3D) to observe and quantify their morphological changes during the process of formation, from the initial formation of primary loops to secondary and complex loops. High-resolution magnetic resonance (MR) images and serial histological sections were used to reconstruct the mesenteric structure and determine the secondary loops and segments.

2 MATERIALS & METHODS

2.1 Human embryo specimens

This study was approved by the ethics committees of Kyoto University Faculty and Graduate School of Medicine (approval numbers: E986, R0316, and R2224).

A total of 47 human embryos (CRL: 7.6–32.3 mm, Carnegie stage [CS]: 16–23) and two specimens in the post-embryonic period (PE) (CRL: 32.0 mm and 36.5 mm) were selected for high-resolution MR imaging (MRI) acquisition (Table 1). The majority of the midguts of these specimens were within the extraembryonic coelom (a physiological

umbilical herniation). Other four embryo specimens at CS17, 19, 21, and 23 were selected for histological analysis, and serial tissue sections were prepared for each stage. The embryos were stored at the Congenital Anomaly Research Center of Kyoto University (Yamaguchi & Yamada, 2018). In accordance with the Maternity Protection Law of Japan, most specimens were obtained following pregnancy termination for socioeconomic reasons. The specimens were collected between 1963 and 1995 in accordance with the relevant regulations. Written informed consent from the parents was not required. The parents provided verbal informed consent for the specimens to be deposited, and the consent of each parent was documented in their medical records. All samples were anonymized and de-identified prior to analysis.

2. 2 Image acquisition

Twenty-six embryos were selected for 2.35-T MRI using previously described methods (Shiota et al., 2007) (Table 1). The MR images of 21 embryos and two fetuses were acquired using a 7-T MR system (BioSpec 70/20 USR; Bruker BioSpin MRI GmbH, Ettlingen, Germany) at the Medical Research Support Center, Graduate School of Medicine, Kyoto University.

Various MRI coils and acquisition parameters were prepared for the 7T-MRI. The appropriate settings were selected based on the specimen size (Table 2).

Serial tissue sections from the four embryos were digitized, and histological observations were subsequently performed using the Olympus virtual slide system (VS120; Olympus Corporation, Tokyo, Japan). These observations were conducted to verify how the intestinal tract, mesentery, and other tissues identified in the MR images corresponded to those observed in the histological sections.

2.3 Three-dimensional reconstruction of the midgut and mesenteries

Acquired MR images from all 49 samples were processed to create 3D reconstructions. The midgut loop (duodenum and small and large intestines) and mesentery associated with the midgut were manually segmented using Amira software (ver. 5.50, Visage Imaging GmbH, Berlin, Germany). Subsequently, 3D images of these structures were reconstructed. The AmiraSkel software module was used to determine the midlines of the intestines.

2.4 Definition of the midgut, four segments, and secondary and tertiary loops

The oral end of the duodenum was defined based on its 3D morphology, specifically the

constricted portion at the anal end of the pyloric antrum (O’Rahilly & Muller, 1987). The midgut extends from the oral end of the duodenum to the inflection point of the colon, which represents the caudal boundary of the midgut following intestinal return (Soffers et al., 2015). Soffers et al. (2015) showed that the thin sideward extensions of the mesenteric leaf on the right side of the mesenteric rod connected with loops 2 and 3, whereas the leaf on the left side connected with loop 4 and the proximal colon. The mesenteric leaves associated with the secondary loops remained recognizable during subsequent development and thus formed landmarks for these loops, which were delineated by three narrowing parts (Nps) of the mesentery. Based on the three-dimensional reconstructed mesenteric architecture, we divided the midgut loop into one segment (S1) located within the abdominal cavity and three segments (S2, S3, and S4) situated within the extraembryonic coelom and colic part in our present study (Figure 1a, Supplementary Figure 1). The secondary and tertiary loop formations were distinguished by the number of mesenteric folds per segment (Figure 1b). The secondary loop is characterized by the presence of two folds and one loop in the segment. The tertiary loop exhibited a minimum of three folds and two loops in each segment. The number of tertiary loops was calculated using the following formula: number of folds – 1. The minimum length of each segment at which more than one, two, or three tertiary loops were formed in more than 80% of individuals was defined as the threshold for the number of each tertiary loop.

2.5 Position of the midgut in the axial view

A straight line passing through two points—the tip of the herniated midgut in the umbilical cord and the center of gravity of the mesentery in the section at the umbilical ring—was defined as the axis of the midgut loop, assuming the run of the SMA trunk (Figure 1c-i). From a plane perpendicular to the axis, the areas, including S2, S3, and S4 of the midgut and their mesenteries, were determined using Amira software (Figure 1c-ii), and those areas were illustrated using fan shapes around the axis (Figure 1c-iii).

3 RESULTS

3.1 Morphology

3.1.1 Formation of the primary loop

In all nine specimens between CS16 and CS18, the midgut was elevated toward the extraembryonic coelom, forming the primary loop (Figures 2, 3a-i). The mesenteries were situated between the proximal and distal limbs of the midgut, extending directly from the base to the tip without narrowing. Except for one case at CS16, all midguts

herniated into the extraembryonic coelom.

3.1.2 Formation of the secondary loop

Secondary loops in the midgut were first detected at Carnegie stage (CS) 19. Two secondary loops with two narrowing points (Nps; Np.1 and Np.3) in the mesenteries were observed at CS19 in five of the seven cases and at CS20 in one of the eight cases (Figure 3a). Np.1 emerged around the umbilical ring and was observed to narrow progressively from the caudal to the cranial side of the ring. Np.3 was located within the herniated midgut, serving as the boundary between the proximal and distal limbs of the midgut. At CS19, the secondary loop in S1 formed a U-shaped structure, extending from the oral side of the duodenum (+) to the caudal side of the abdominal cavity. Between Np.1 and Np.3, no obvious folds were observed. Four secondary loops with three narrowing points (Nps; Np.1, Np.2, and Np.3) were observed in the remaining nine specimens at CS19 and CS20. Np.2 was positioned to divide the proximal limb of the midgut into further proximal and distal segments within the extraembryonic coelom. The three Nps were constantly observed at their exact locations. Consequently, the midgut was divided into four segments by the three Nps and ileocecum in all specimens at CS21 and in older specimens. Np.3 was noted at the tip of the midgut hernia, at the point where the traces of the vitelline artery and duct enter the mesentery (Figure 3b). S1 was formed in the abdominal cavity, S2 and S3 were formed within the extraembryonic coelom on the right side, and S4 was formed on the left side of the axis of the herniated midgut. No discernible secondary loop formation was observed in any of the specimens at the colic part.

3.1.3 Formation of the tertiary loop

Formation of the tertiary loop was initially observed at CS21 (Figure 2). The tertiary loop was observed in one segment (S2 or S3) in three specimens and in two segments (S2 and S3) in one specimen (Figure 4a). At CS21, tertiary loop formation was not observed in the remaining seven specimens. At CS22, the tertiary loop was observed in one segment (S3) in one specimen and in two segments (S2 and S3) in the remaining four specimens (Figure 4a). At CS23, the tertiary loop was observed in two segments (S2 and S3) in two specimens and in three segments (S2, S3, and S4) in the remaining five specimens (Figure 4b). The tertiary loop was observed at the PE in three segments of both specimens.

The mesentery of the midgut in S1 was not membranous, but rod-like, and a tertiary loop

was not observed in any of the specimens. In contrast, the midgut in S1 underwent caudal elongation until CS21 (Figure 5) and subsequently flexed anticlockwise in the axial view of CS22.

3.2 Length and the number of loops in the midgut

The length of the entire midgut (from the duodenal origin to the colonic inflection point) and each segment increased as the CS progressed (Figure 6a).

As the length of S2, S3, and S4 reached the thresholds indicated by "TL2" (Figure 6b, dotted line with "2"), a minimum of two folds were formed in more than 80% of the specimens, i.e., a minimum of two tertiary loops in each segment. Upon reaching the subsequent threshold lengths indicated by "TL3" and "TL4" (Figure 6b, dotted line with "3" and "4"), an increase in the number of loops was observed, forming at least three and four tertiary loops, respectively.

In each specimen at CS23 and PE, the number of tertiary loops increased in accordance with the CRL, with a maximum of 19 tertiary loops in S2, S3, and S4. S2, S3, and S4 exhibited a maximum of seven, six, and eight loops, respectively, in the CS23 and PE specimens (Figure 7a, Supplementary Table 1). The number of loops in S4 was smaller than that in S2 and S3 in specimens with a smaller CRL. This difference was not significant in specimens with a larger CRL. The number of loops in S1 remained constant (one secondary loop) across all nine specimens.

The midgut length per segment increased with the CRL (Figure 7b). The rates of increase were more rapid in S2, S3, and S4 than in S1 and the colic segment.

The mean midgut length per fold in the tertiary loop ranged between 2.5 and 3.0 mm in S2, S3, and S4 in the specimens at CS23 and PE, with the exception of S1 (Figure 7c). The mean value observed in S1 was 1.5–2.0 times higher than that observed in the other three segments.

3.3 Location of the three midgut segments along the midgut axis

The three midgut segments are located along the midgut axis.

The distributions of the three segments varied in all nine specimens (Figure 8a). S2 was situated in the segment that overlaid the third quadrant in the axial view, a configuration that was observed in the majority of the specimens. S3 exhibited a range of 180–360°, indicating that it was situated in the second and third quadrants (right-sided) of the axial view in the majority of the specimens. S4 appeared to be situated on the opposite side of S2 and S3, relative to the axis of rotation, specifically on the left or cranial side of the

axis (Figure 8b). S4 demonstrated notable inter-individual variability in the extent of segmental coverage by the central angle.

4 DISCUSSION

The present study demonstrated the stereotypical position of the Nps in the mesentery and secondary loop formation. Several anatomical structures may be associated with the development of each Np.

The 3D reconstruction revealed that the mesentery in S1 exhibited distinctive characteristics compared with those of the remaining three segments. In particular, the mesentery of S1 appeared rigid and rod-shaped and lacked a membranous leaf. In contrast, the remaining three segments exhibited membranous leaf structures. These observations are in accordance with those of a previous study (Soffers et al., 2015). Previous studies have also indicated that Np.1 is located close to the umbilical ring (Mall, 1898; Pernkopf, 1925; Soffers et al., 2015). Despite the proximity to the umbilical ring, our morphological observations do not indicate that a mechanical force can be transmitted from the umbilical ring to create Np.1. Given the potential for lethal complications resulting from mechanical forces transmitted from the umbilical ring to the midgut and its mesentery, including blood flow obstruction and intestinal injury, it is plausible that the positions of the umbilical ring and Np.1 are incidental.

Our findings indicate that discrimination of S2 and S3 by forming Np.2 occurred approximately one stage later than that of S1 and S2 and S3 and S4 by forming Nps.1 and 3, respectively. Furthermore, the midgut and mesentery characteristics in S2 and S3 exhibited more remarkable similarity than those observed in S4, even after the formation of Np.2. The anatomical structures forming Np.2 could not be identified in the 3D reconstruction or morphological observation of the mesentery. Soffers et al. (2015) posited that the distribution of the SMA sister branches flowing through S2 and S3 might contribute to the discrimination of these segments. The SMA sister branches arising in the abdominal cavity (the fourth to seventh branches) flow into the midgut in S2 of the extraembryonic coelom through the umbilical ring. In contrast, those arising in the extraembryonic cavity (the 10th to 14th branches) flow into the midgut in S3. The morphology of the SMA sister branches flowing through the small intestine differs between the jejunum and ileum in adults (Conley et al., 2009). The characteristics of the SMA branches in S2 and S3 may correspond to those of the SMA branches flowing into the jejunum and ileum, respectively. Furthermore, Soffers et al. (2015) reported that the

ileocecal artery and several short branches arise between the seventh and ninth SMA branches, which are located at the umbilical ring and do not give rise to a caudal branch flowing in either S2 or S3 of the midgut. As a result, the SMA branches for S2 and S3 were distinct in their study. However, Kakeya et al. (2022) did not detect short branches around the umbilical ring, and the boundary of the SMA branches for S2 and S3 was not discernible. Whether the distribution of the SMA sister branches determines the formation of Np.2 in the mesentery, which designates the border between S2 and S3, remains unclear.

The location of Np.3 was confirmed to coincide with the traces of the vitelline artery and duct, which led to the SMA in the MR images of the specimens at CS21 and CS23. S4 was identified on the contralateral side of S2 and S3 with respect to the midgut axis, including Np.3. This finding was consistent with the reports of Soffers et al. (2015), i.e., that the third loop was located to the right, and the fourth loop was situated to the left of the axis formed by the SMA.

Np.3, in conjunction with the vitelline artery and duct, maintained its position at the apex of the midgut loop in all specimens throughout the herniation phase. Kakeya et al. (2022) indicated that the effect of this structure was recognized until the late return phase, at which point the location of the midgut changed from the extraembryonic coelom to the abdominal cavity. The specimen demonstrated that the distribution of the midgut in the abdominal cavity was influenced by the traces of the vitelline artery and duct. The mechanism responsible for maintaining the position of Np.3 may be of great importance in the dynamic changes in the location of physiological umbilical herniation and return, which are essential for the normal midgut development.

The midgut in S1 differs from that in the other areas because it never forms tertiary loops, a phenomenon described in numerous previous studies (Mall, 1898; Bardeen, 1914; Pernkopf, 1925; Soffers et al., 2015). In the present study, the secondary loop underwent elongation as it surrounded the rod-like midgut mesentery by three-fourths, which can be described as a rotation of the duodenum by 270°. The length per fold was greater than the threshold length for tertiary loop formation in other segments. This looping mechanism is significant because an abnormality in the looping results in congenital anomalies, such as non-rotation and malrotation of the midgut (Mehall, 2002; Metzger et al., 2011; Kluth et al., 2003; Snyder and Chaffin, 1954; Vogt, 1917). The interaction between the midgut and mesentery plays a crucial role in tertiary loop formation. Nevertheless, whether the mechanism determining the loop size (degree of rotation) in S1 operates via a similar mechanism remains unclear.

Soffers et al. (2015) argued that the location where secondary loops form is genetically determined and consistent among embryos. The findings of the present study also demonstrated that Np.1 and Np.3 occur at anatomically defined locations: Np.1 near the umbilical ring, and Np.3 in association with the traces of the vitelline artery and duct. These observations support the hypothesis that the formation of Np.1 and Np.3 may be genetically regulated. Conversely, Np.2 appeared later than Np.1 and Np.3, and no anatomical landmarks were identified in this study to correlate with its location. Furthermore, the midgut and mesentery within S2 and S3, separated by Np.2, exhibited similar characteristics. Whether the formation of Np.2, marking the boundary between the second and third secondary loops, is genetically determined remains an open question.

Upon reaching a critical threshold length, the number of loops exhibited a marked increase, accompanied by rapid elongation in S2, S3, and S4. These findings indicate that midgut elongation contributes to an increase in the number of folds and support the hypothesis that intestinal loops develop biomechanically through rapid elongation of the midgut and slow growth of the mesentery (Savin et al., 2011). Segmental variations were observed between S2, S3, and S4. The initial formation of the tertiary loops was observed to occur at a later CS (one stage later) in S4 than in S2 and S3. The number of folds in S4 increased at a later CS compared with those in S2 and S3. Whether these segmental differences are significant, and what factors contribute to their emergence is currently unclear. The midgut undergoes both lengthening and an increase in diameter as a result of differentiation of its histological structures (Ueda et al., 2016; Hikspoors et al., 2019). The mesentery may undergo alterations as a consequence of the differentiation of mesenchymal structures, including structural changes in vascularity and innervation. Such growth and segmental differences may influence loop formation.

Soffers et al. (2015) described variations in the number and position of the tertiary loops in three specimens of varying sizes at CS23. The authors posited that the discrepancy in the location of the secondary region was attributable to growth-related factors, specifically the rapid transition from the right to the left. The nine specimens at CS23 and PE in our study were retested from a similar viewpoint, namely, the midgut axial view. The positions of the three segments in the extraembryonic coelom exhibited variability along the midgut axis, irrespective of the specimen size, indicating that any rapid movement of the positional changes with increasing size is unlikely at CS23. As previously demonstrated by Kakeya et al. (2022), alterations in SMA branch distribution occur before the onset of positional changes in the midgut. Considering the CRL and CS

of the specimens in the present study, we can hypothesize that alterations in SMA branch distribution may have occurred, which could influence the location of the midgut loop in the nine specimens. However, this hypothesis could not be verified because the MR image used could not detect the SMA branch distribution. To fully elucidate the SMA distribution, an analysis using 3D reconstruction from tissue sections (Soffers et al., 2015; Kakeya et al., 2022) and microcomputed tomography images (Ginzel et al., 2021) is essential.

The present study is limited in the following ways. First, the SMA was not discernible because of the intrinsic resolution of the MR images. Consequently, an examination of the relationship between the SMA distribution and intestinal tract or mesentery was not feasible. Moreover, the trajectory of the SMA could not be utilized as a reference point for the midgut axis. Second, the anal end of the pyloric antrum was defined as the oral end of the midgut. This definition does not refer to other anatomical landmarks such as the pancreatic ducts and inferior vena cava, which can provide more accurate measurements. Third, the specimens used in this study were limited to the herniated stage, wherein the midgut had herniated into the extraembryonic coelom. As the specimen develops, the midgut will undergo a change in position from the extraembryonic coelom to the abdominal cavity, and further changes occur within the abdominal cavity. Whether the number of loops, their rate of increase, and various other values are affected by the position of the midgut remain unclear.

In conclusion, this study delineated the morphological alterations that occurred in the midgut and mesentery over time during the herniated midgut phase. The mesentery displayed a fundamental, stereotypical architectural configuration that was particularly evident in the secondary loops and their delineation into the four segments of the midgut. The findings of this study are crucial for a comprehensive understanding of the role of genetic and biomechanical factors in loop formation.

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STATEMENT OF ETHICS

This study was approved by the ethics committees of Kyoto University Faculty and

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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AUTHOR CONTRIBUTIONS

Nanase Ishida: Data analysis/interpretation, Drafting of the manuscript

Yui Ueda: Data analysis

Toru Kanahashi: Data analysis

Jun Matsubayashi: Data analysis, Statistical analysis

Hirohiko Imai: Data analysis/interpretation, Data acquisition

Shigehito Yamada: Data acquisition

Tetsuya Takakuwa: Concept/design of the manuscript, Critical revision

DATA AVAILABILITY

The data used in this study are available on request from the authors.

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Table 1. MRI of the embryos

CS	ID	CRL (mm)	MRI system	7T-MRI condition
16	22322	7.6	7-T	Condition 1
	[n=2] 29261	9.2	7-T	Condition 1
17	22903	11.3	7-T	Condition 1
	[n=2] 24578	11.9	7-T	Condition 1
18	31870	11.8	7-T	Condition 1
	21314	13.1	2.35-T	
	21409	13.3	2.35-T	
	[n=5] 21142	14.1	2.35-T	
	23652	14.3	2.35-T	
19	19989	14.8	2.35-T	
	16127	14.9	2.35-T	
	21011	15.3	2.35-T	
	19605	15.7	2.35-T	
	[n=7] 26979	16.0	7-T	Condition 1
	24343	16.1	2.35-T	
	22014	16.3	2.35-T	
20	23617	16.2	2.35-T	
	22363	17.0	2.35-T	
	28070	17.0	2.35-T	
	26006	17.5	2.35-T	
	[n=8] 22606	18.0	2.35-T	
	24977	19.1	7-T	Condition 1
	25337	19.5	2.35-T	
	26354	19.8	2.35-T	
21	28066	18.8	7-T	Condition 1
	22044	19.0	2.35-T	
	29515	19.4	2.35-T	
	25858	19.7	7-T	Condition 1
	[n=11] 24787	20.0	2.35-T	
	30236	20.2	2.35-T	
	25694	20.4	2.35-T	

	32721	20.7	7-T	Condition 1
	21875	20.8	7-T	Condition 2
	24728	20.9	2.35-T	
	26896	21.4	2.35-T	
	23275	19.8	2.35-T	
22 [n=5]	35233	22.0	7-T	Condition 2
	22157	22.1	2.35-T	
	27050	22.2	7-T	Condition 1
	30809	24.5	7-T	Condition 2
23 [n=7]	20018	23.1	7-T	Condition 1
	38239	24.0	7-T	Condition 1
	52578	25.5	7-T	Condition 1
	20249	25.6	7-T	Condition 1
	25796	26.8	7-T	Condition 2
	22964	29.1	7-T	Condition 1
	15919	32.3	7-T	Condition 1
PE [n=2]	51397	32.0	7-T	Condition 2
	31423	36.5	7-T	Condition 1

CS, Carnegie stage; CRL, crown-rump length; MRI, magnetic resonance imaging; PE, post-embryonic period; 7-T, 7-T magnetic resonance system; 2.35-T, 2.35-T magnetic resonance system

Table 2. MRI coils and acquisition parameters using the animal 7T-MRI system

	Condition 1		Condition 2		
MRI coil (inner diameter)	Solenoid	coil	Quadrature	volume	coil
	(19 mm) *		(35 mm)**		
Spatial resolution (isotropic, um)	40		54		
Acquisition time	18h51mm0s		22h30m0s		
Field of view (mm×mm×mm)	20×15×15		37.8×27.0×24.3		
Matrix size	500×376×376		700×500×450		
Repetition time (ms)	30		30		
Echo time (ms)	4.7		5.7		
Flip angle (degrees)	25		25		

*Manufacturer: Takashima Seisakusyo Co., Ltd., Tokyo, Japan

**Manufacturer: Bruker BioSpin MRI GmbH, Ettlingen, Germany

FOV and spatial resolution are adjusted to cover the specimen with different sizes.

FOV, field of view; MRI, magnetic resonance imaging

FIGURE LEGENDS

Figure 1. Illustrations indicating methods and definitions used in this study

a) Schematic diagram of the hierarchical process of midgut loop formation during the herniation phase

i) Primary loop: One loop is projecting within the extraembryonic coelom.

ii) Secondary loops: The secondary loops are formed in S1 (red) in the abdominal cavity (AC) and in S2 (yellow), S3 (green), and S4 (blue) in the extraembryonic coelom (EC).

iii) The tertiary loops exhibit increased folds in S2, S3, and S4 of the extraembryonic coelom, resulting in a more complex structure.

The narrowing parts of the mesentery (Nps.1-3; purple lines) and ileocecum (purple dot) define four segments in the midgut. a, anal side; light blue line; the umbilical ring; O: oral side

+, oral end of the duodenum in the present study, corresponding to the end of the pyloric-antrum (illustrated in thick black). Note that the point is defined based on its morphological observations.

b) Definition of the tertiary loop according to the number of mesenteric folds.

i) Secondary loop: A segment with two mesenteric folds

ii) Tertiary loop: A segment with at least three mesenteric folds

The number of loops is calculated as two using the following formula: number of folds – 1. The upper and lower diagrams indicate two loops (three folds) and three loops (four folds), respectively. Note that the minimum number of the tertiary loops is two.

c) Definition of the axis of the herniated midgut for analyzing the position of the midgut segments

i) The mid-sagittal plane of the magnetic resonance imaging (MRI) indicates the axis of the herniated midgut (white dot line). The axis is lined through the tip of the herniated midgut in the extraembryonic coelom (white dot) and center of gravity of the mesentery in the section at the umbilical ring (light blue line and diamond). Pink segments show the mesentery of the midgut.

ii) Midgut and the accompanying mesentery (axial view): The white dot at the origin indicates the axis of the midgut. The midgut (duodenum, small intestine, and part of the colon) is indicated by color-coded lines according to the segments. Pink reconstruction shows the mesentery of the midgut. The black dashed lines indicate the cranial-caudal and transverse axes. L, left; R, right

iii) Diagrams showing the distribution of S2 (yellow), S3 (green), and S4 (blue) along

the axis of the midgut: The broken line indicates the middle value of each segment's distribution.

Figure 2. Summary of the primary, secondary, and tertiary loop formation by four segments

The schematic diagram illustrates the primary, secondary, and tertiary loops in each segment. The number of specimens corresponding to the loop formation is indicated according to the Carnegie stage. PE, post-embryonic period

Figure 3. Secondary loop formation with three narrowing parts of the midgut mesentery
a) Representative MR image sagittal plane, and three-dimensional (3D) reconstructions from the MR images. Histological sagittal sections stained with hematoxylin and eosin at a comparable developmental stage were analyzed to validate the findings observed in the MR images within the histological sections.

(i) One primary loop is herniated into the extraembryonic coelom. No narrowing parts of the mesentery were detected (CS17).

(ii) Two secondary loops are detected in S1 and S4. No Np.2 is detected (CS19).

(iii). Four secondary loops are formed with three Nps in the mesentery's midgut (CS21).

The color-coded asterisks and tubes indicate the midgut (the duodenum, small intestine, and colon), and the color corresponds to the segments indicated in the legend. The scale bar indicates 1 mm.

Light blue line, umbilical ring; arrowheads, mesentery of the midgut; purple lines, mesenteric narrowing parts (Nps); +, anal end of the pyloric antrum

A, anal side; AC, abdominal cavity; EC, extraembryonic coelom; Li, liver; O, oral side

Note that the mesentery is clearly discernible between the midgut on MR image, exhibiting a signal intensity that is either equal to or slightly lower than that of the midgut.

b) The magnetic resonance images indicating the relationship between the narrowing part of the midgut mesentery (Np.3) and the traces of the vitelline artery and duct

Specimens at CS21 (left) and CS23 (right) are provided. The traces of the vitelline artery and duct (Vad), which are similar in intensity to the intestine, run into the mesentery on Np.3.

CS, Carnegie stage; MRI, magnetic resonance

Figure 4. Formation of the tertiary loops

(a) Representative three-dimensional reconstruction indicating the tertiary loop formation in S2 and S3 (CS22: left lateral view). S1 and S4 have secondary loops.

(b) Representative histological sagittal sections with hematoxylin and eosin staining, MR image sagittal plane, and three-dimensional reconstructions from the MR images indicating the tertiary loop formation in S2, S3, and S4 (CS23)

The color-coded asterisks and tubes indicate the midgut (the duodenum, small intestine, and colon). Arrowheads, mesentery of the midgut; light blue line, umbilical ring; purple dot, ileocecum; purple line, mesenteric narrowing parts (Np); +, end of the pyloric antrum A, anal side; AC, abdominal cavity; CS, Carnegie stage; EC, extraembryonic coelom; Li, liver; MR, magnetic resonance; O, oral side; P, pancreas; S, stomach

Figure 5. Representative three-dimensional reconstructions indicating the change in the secondary loop in the S1 of the midgut at CS20 and CS23

The axial (left) and caudal views in the abdominal cavity (AC) (right) are provided.

The color-coded tubes or lines indicate the run of the midgut. The pink-colored reconstruction indicates the mesentery of the midgut. Light blue line, umbilical ring; +, oral end of the duodenum, EC, extraembryonic coelom; L, left; R, right

Red arrows indicate the bending direction in S1 (red tubes). Gray arrows indicate the direction of the colon (proximal to distal).

Marked left caudal flexion is observed. The mesentery of the abdominal cavity is rod-like rather than membranous.

Figure 6. Midgut length

(a) Mid-gut length of each specimen

The data are presented in order by the Carnegie stage and by the crown-rump length of the specimens within each Carnegie stage. Colored bars in the graph correspond to the segments indicated in the legends. CS, Carnegie stage; PE, post-embryonic period;

(b) Midgut length in S1 (i), S2 (ii), S3 (iii), and S4 (iv) The data are presented in order first by the Carnegie stage and then by the crown-rump length (CRL) of the specimen within each stage. The colored bars in the graph correspond to the number of folds in each segment of the specimen.

The red dashed horizontal lines (with "TL2") in the graph indicate the threshold length for the tertiary loop formation. A minimum of two loops are formed when the midgut length in the segment is greater than the threshold. The dotted lines (with "TL3" and "TL4")

indicate the threshold length for forming a minimum of three and four tertiary loops in the segment, respectively. The number of tertiary loops is calculated using the following formula: number of folds – 1.

Figure 7. Number of folds and the midgut segment length at CS23 and in the post-embryonic period

i) Number of folds

ii) Midgut length per segment

iii) Midgut length per fold by each segment

The data are presented in order by the Carnegie stage and by the crown-rump length of the specimens within each Carnegie stage. The colors in the graph correspond to the segment indicated in the legend.

Figure 8. Location along the axis of the midgut loop at CS23 and the post-embryonic period

(a) Diagrams showing the distribution of the S2 (yellow), S3 (green), and S4 (blue) along the axis of the midgut loop

A diagram of all the nine specimens at CS23 and PE is shown. The broken line indicates the middle value of each segment's distribution. CRL and the number of loops in the midgut for each specimen are provided.

(b) Graph showing the distribution of the midgut by S2, S3, and S4 at CS23 and PE

The data are presented in order first by the Carnegie stage and then by the specimen's crown-rump length (CRL) within each stage,. The bars indicate the range of each segment. The broken lines represent the middle value of each segment's distribution. In specimens in which the segments extend across 0°, 360° is added or subtracted from the cranial or caudal angle of the segment, depending on the size of the central angle.

CS, Carnegie stage; PE, post-embryonic period

Supplementary Figure 1 Three-dimensional reconstructions of the mesentery and three narrowing parts

(i) No narrowing parts (Nps) of the mesentery were detected.

(ii) Two Nps are detected (Np.1 and 3). No Np.2 is detected (CS19).

(iii). Three Nps are detected (CS21).

(iv) Three Nps can still be identified after forming the tertiary loop (CS23).

Pink reconstructions represent the mesentery, and lines indicate the centerlines of the

660 midguts (the duodenum, small intestine, and colon), and the color corresponds to the
661 segments indicated in the legend. The scale bar indicates 1 mm.
662 Light blue line, umbilical ring; purple lines, mesenteric narrowing parts (Nps); +, anal end
663 of the pyloric antrum

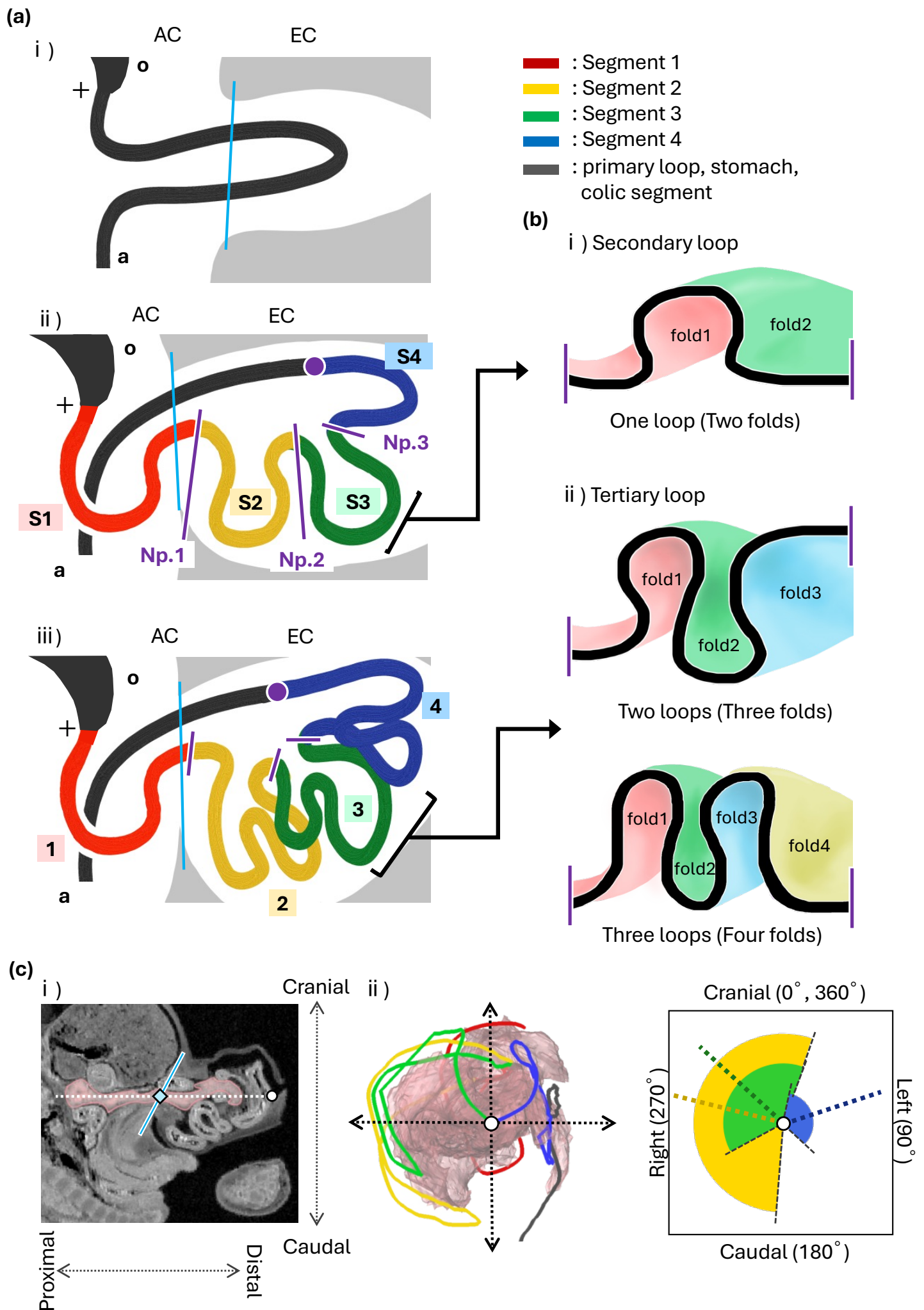


Figure 1.

(a)

Segments				Carnegie stages								
1	2	3	4	16	17	18	19	20	21	22	23	PE
				2	2	5	0	0	0	0	0	0
				0	0	0	5	1	0	0	0	0
				0	0	0	2	7	7	0	0	0
				0	0	0	0	0	1	0	0	0
				0	0	0	0	0	2	1	0	0
				0	0	0	0	0	1	4	2	0
				0	0	0	0	0	0	0	5	2
Number of specimens				2	2	5	7	8	11	5	7	2

■ : No secondary loop
■ : primary loop
■ : Segment 1
■ : Segment 2 and 3
■ : Segment 2
■ : Segment 3
■ : Segment 4

Figure 2.

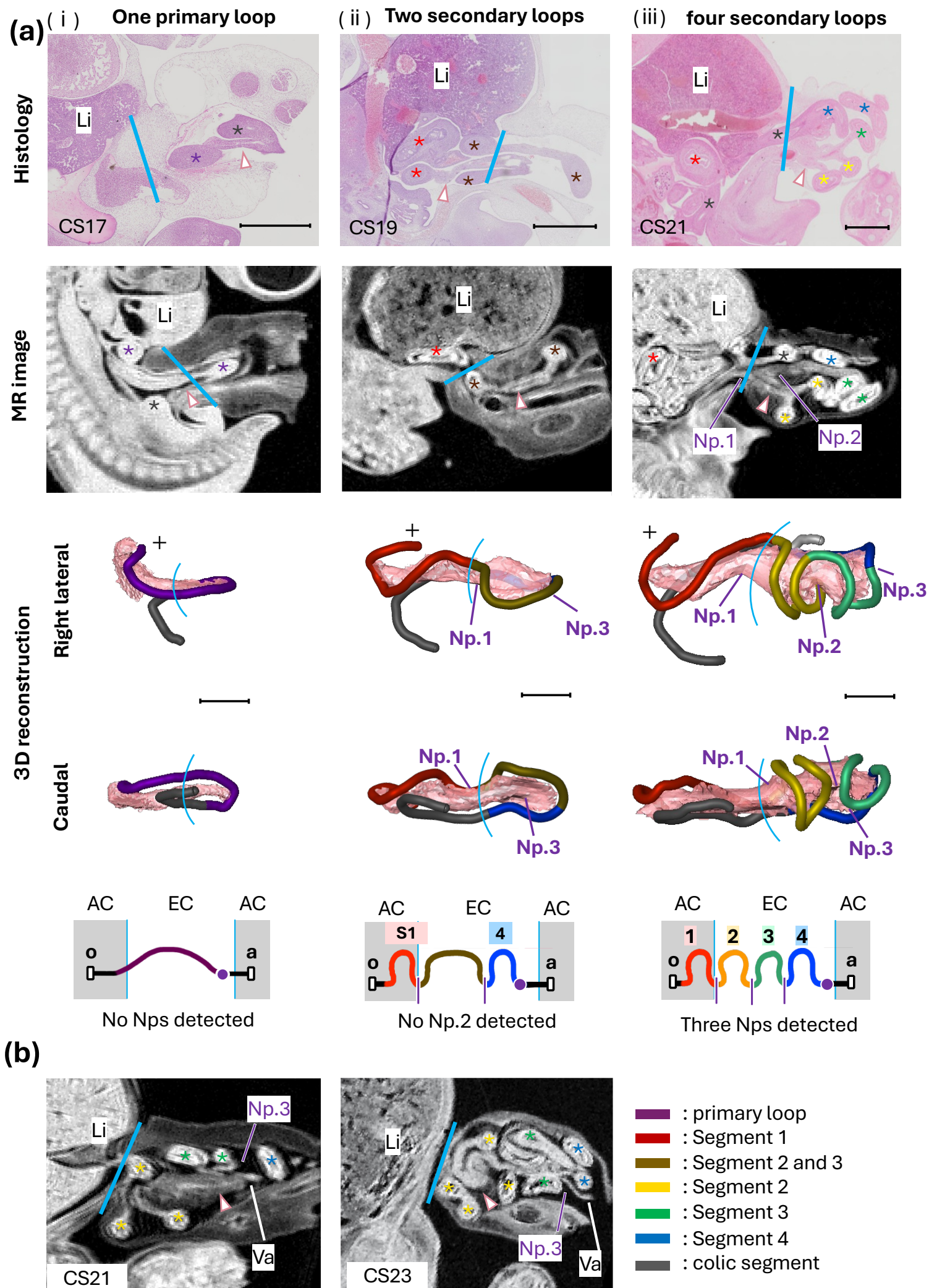
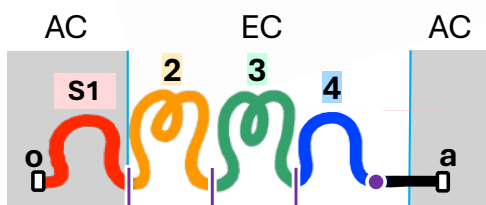
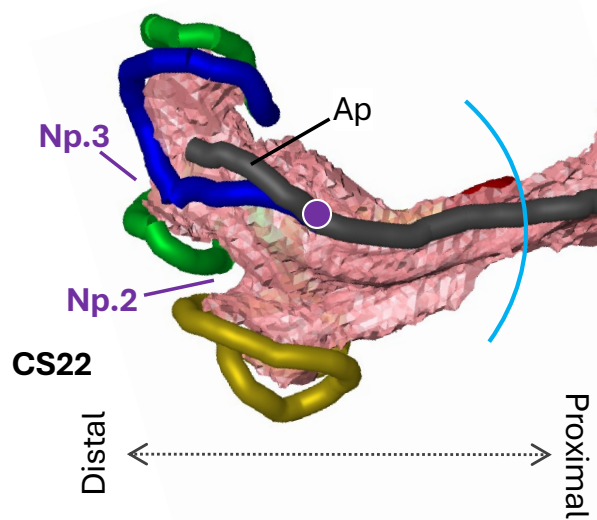


Figure 3.

(a) Tertiary loops in two regions



- : Segment 1
- : Segment 2
- : Segment 3
- : Segment 4
- : colic segment

(b) Tertiary loops in three regions

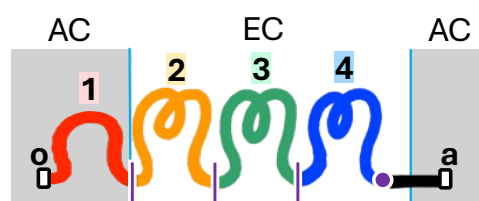
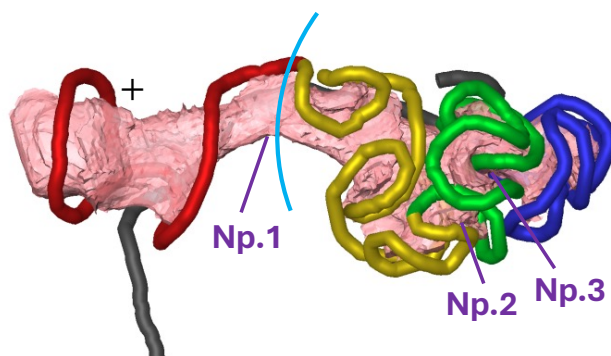
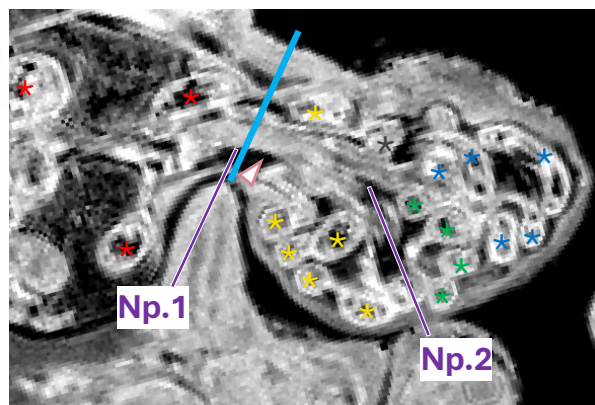
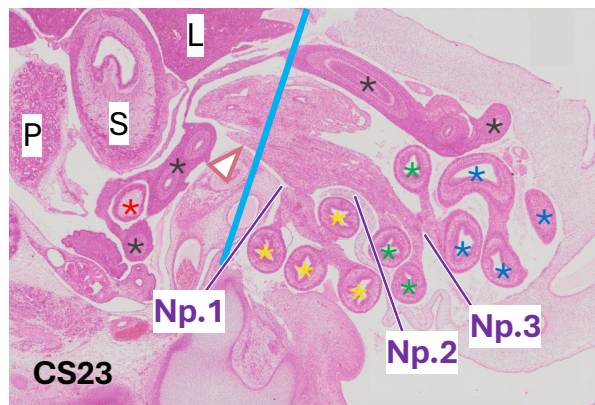


Figure 4.

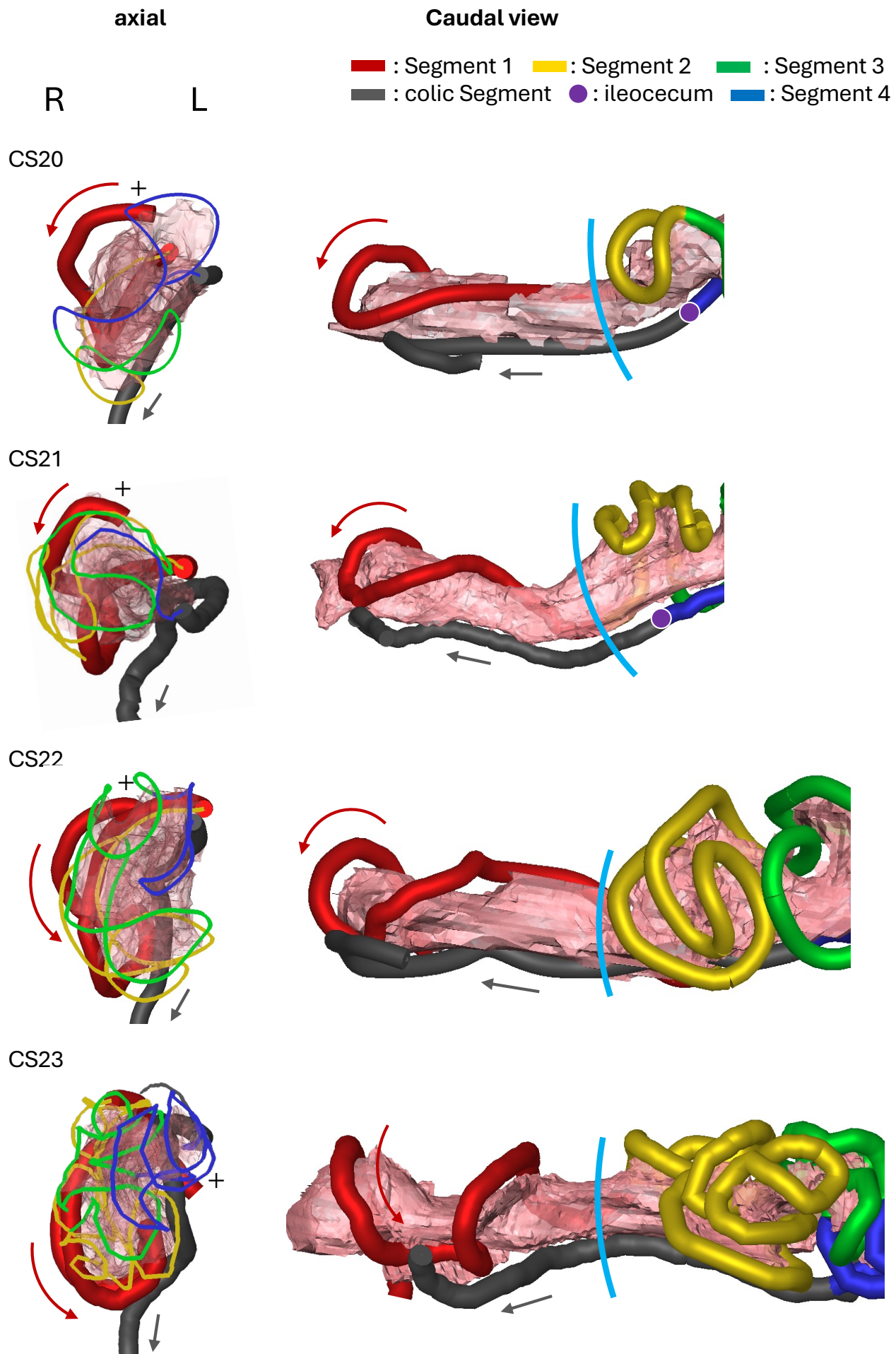


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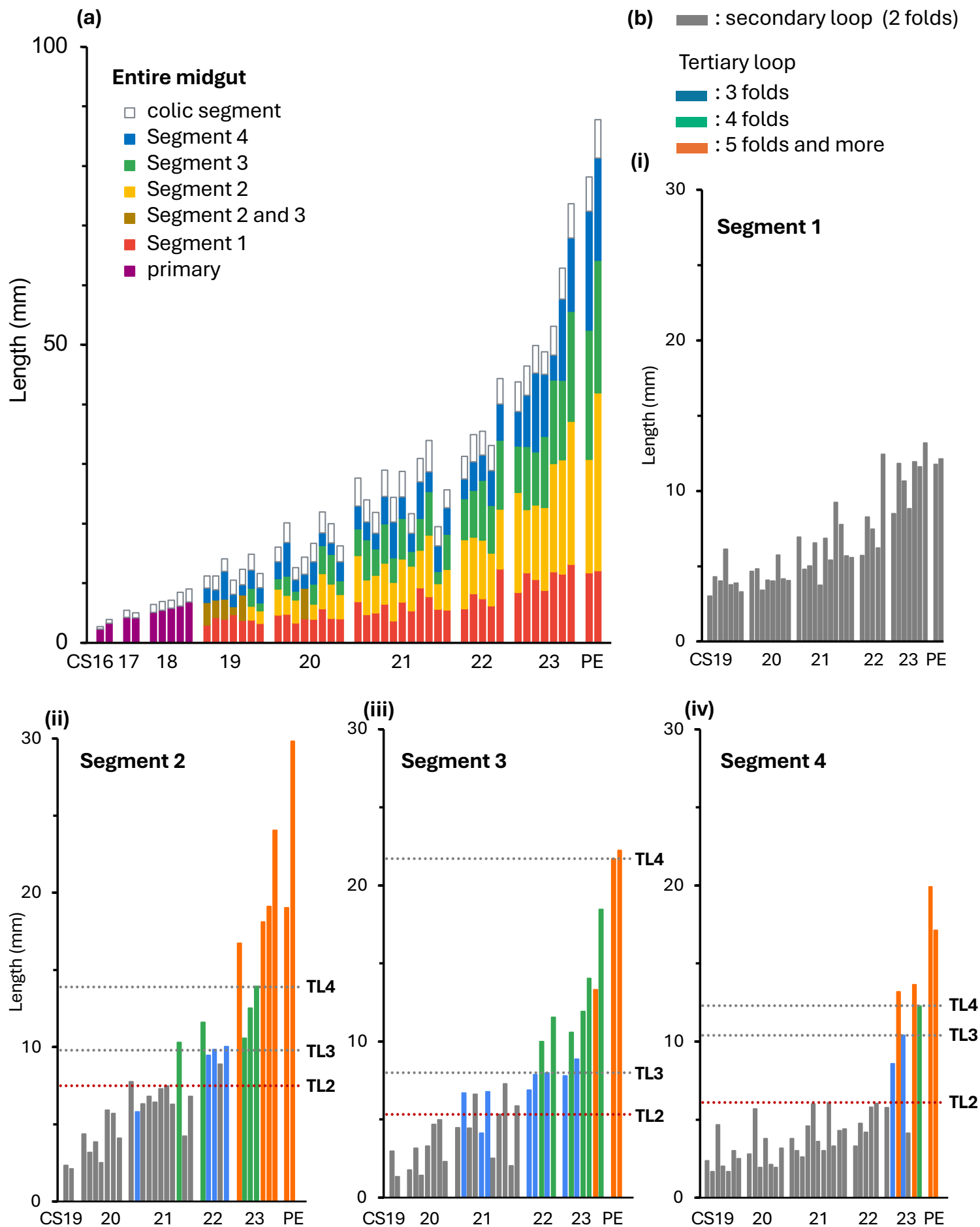


Figure 6.

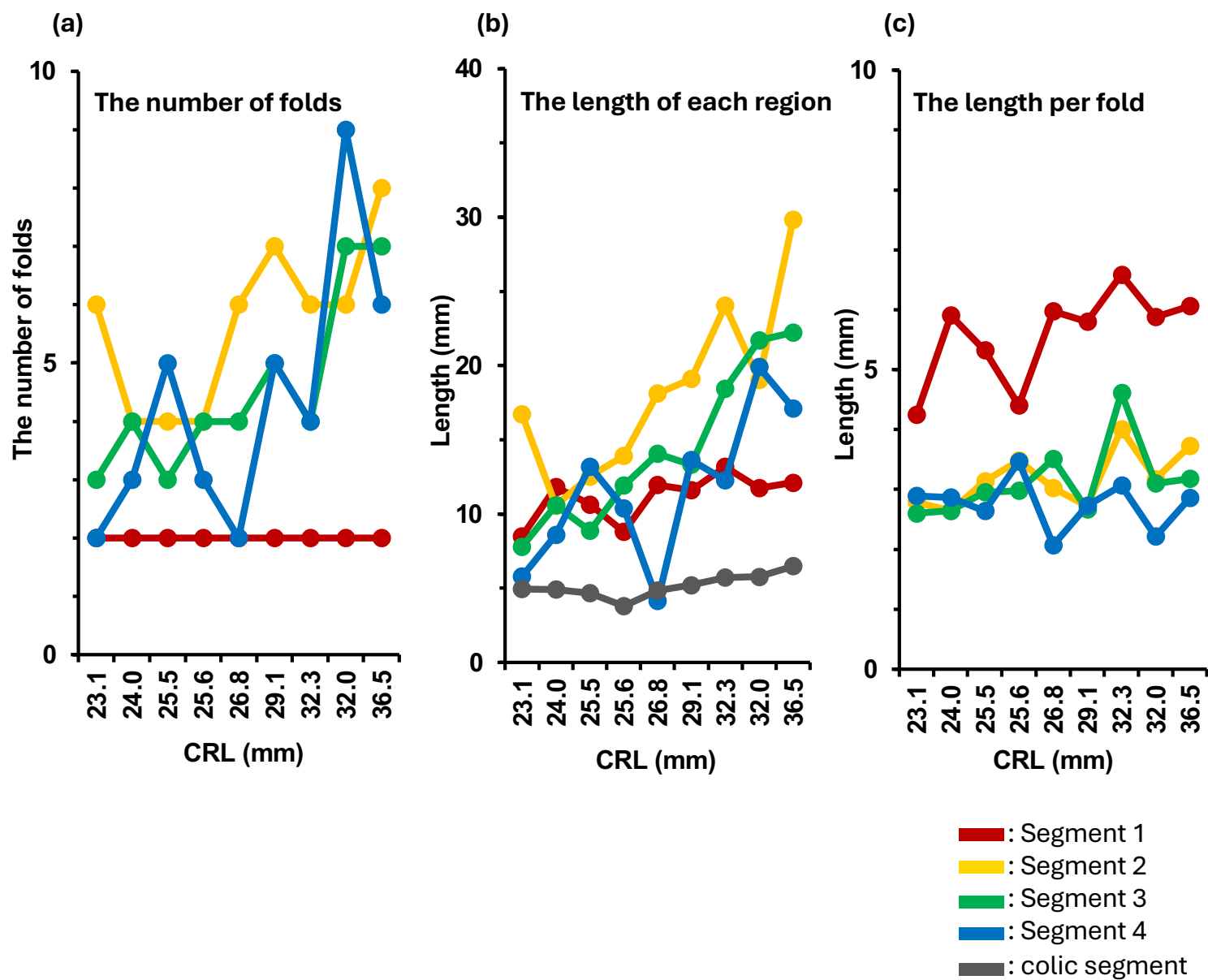
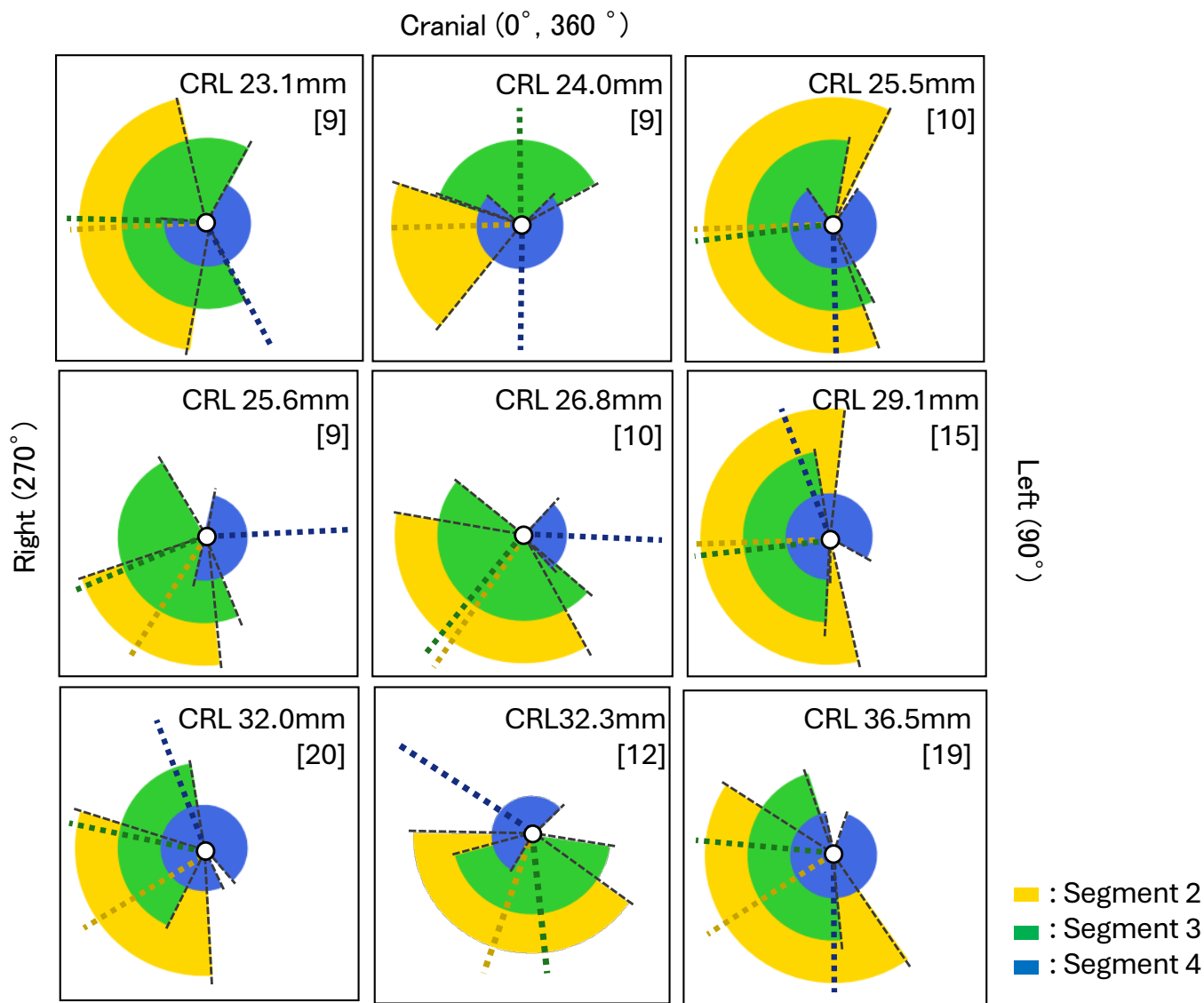


Figure 7.

(a)



(b)

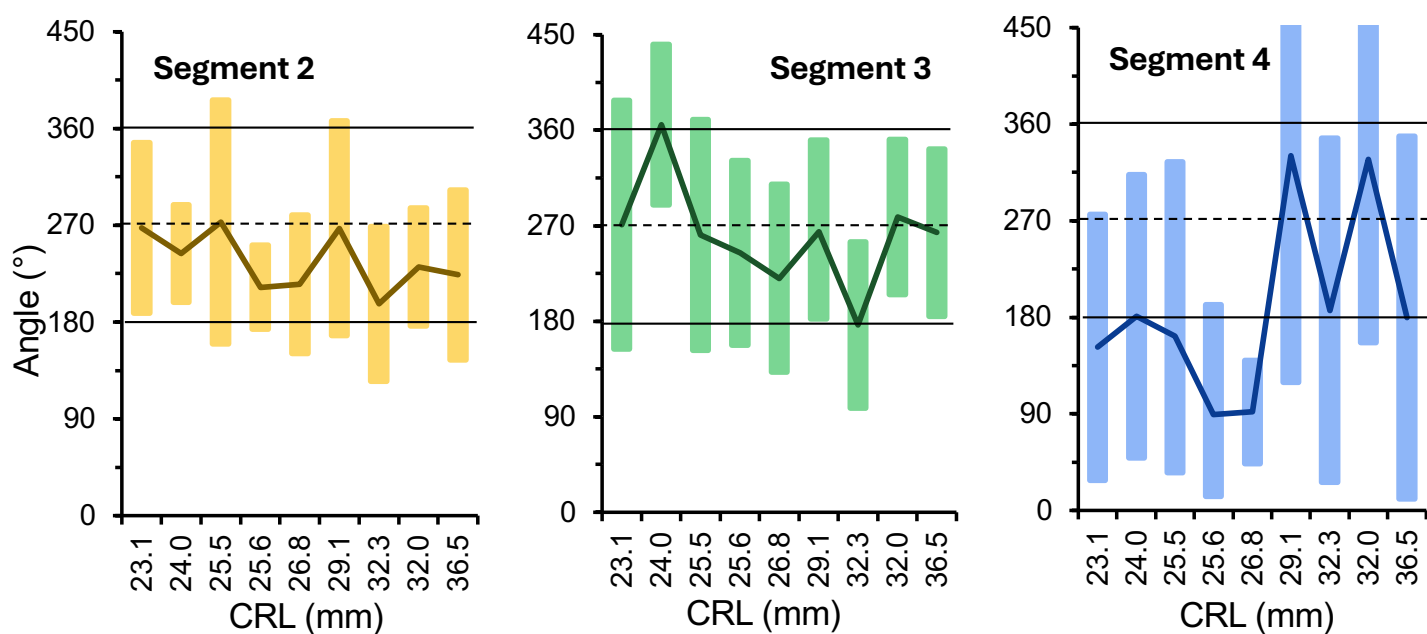


Figure 8.