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Remodeling of the intestine during metamorphosis of *Microhyla berdmorei* (Anura: Microhylidae)

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Abstract
Remodeling of the intestine of a microhylid frog, *Microhyla berdmorei* along with changes in diets between larval stages and adult stage was studied for the first time from pre-metamorphic to metamorphic climax. Elongation of the intestine from Gosner stage 25 to stage 40 significantly correlated with the elongation of the larval body. From stage 40 to stage 46, the lengths of tadpole and intestine abruptly reduced, showing perfect negative correlation between Gosner stage with that of total body length and gut length. Whereas Gosner stages 25 to 46 revealed non-significant relationship with total tadpole length and gut length. Within 10 – 17 days (during spontaneous metamorphosis), reduction of the length of *Microhyla berdmorei* tadpole intestine (from Gosner stage 40 – stage 46) by about 82% was observed. Light microscopic and transmission electron microscopic (TEM) studies showed that the primary epithelium (PE) of the larval intestine was replaced by the secondary epithelium (SE) at stage 46 where the intestinal folds (IF) appeared as several circular folds lined by mucus membrane with elaborate connective tissue and muscles. The columnar epithelial cells of the mature animal differed in their fine structural organization from their larval precursors. The present study indicated that the long small intestine with a single tubular layer of larval epithelium lined by a very little connective tissue or muscle of an omnivorous tadpole transformed into a carnivorous frog possessing the adult type of complex organ comprising of a multiple folded epithelium with elaborate connective tissue and muscle, accompanied by the programmed cell death of larval epithelium by metamorphosis.

Keywords: Intestines, remodeling, metamorphosis, *Microhyla berdmorei*

INTRODUCTION

Anuran metamorphosis is separated into three specific periods: premetamorphosis, prometamorphosis, and metamorphic climax [1,2,3]. Premetamorphosis refers to a period when embryogenesis and early tadpole growth in which some morphological changes such as limited development of the hind limbs do occur. During prometamorphosis, hind limbs undergo morphogenesis as exemplified by the differentiation of the toes and rapid and extensive growth of the hind limbs. Metamorphic climax is the period when rapid morphological changes take place and most noticeable is the complete resorption of the tadpole's tail [4].

The alimentary tract of anuran larvae has been studied by numerous workers [e.g., 5,6,7,8,9,10,11,12,13]. Fine structural changes in the intestinal epithelium during metamorphosis of the bullfrog [14], the cell specialization in the epithelium of the small intestine of feeding *Xenopus laevis* tadpoles [15], and correlated morphological features with different diets alongwith main differences in the cellular physiology were also investigated [16]. At macroscopic level, longer digestive tracts are correlated with a mainly herbivorous diet, whereas shorter ones are associated with carnivorous diets [17,18,19,20], and general morphological pattern of an almost undifferentiated tube, consisting of an esophagus, a gastric region, and a long coiled intestine [21]. Metamorphic shortening of the alimentary tract of larvae was studied on different anurans, like *Rana pipiens* [22], *Alytes obstetricians* [9], *Rana catesbeiana* [23,24]. Changes in the structure of the alimentary canal at selected stages of development of *Phrynohyas resinifictrix* [25] and *Xenopus laevis* [26] were also studied. In this paper, remodeling of the intestine of *Microhyla berdmorei* between larval stages and adult stage was studied from pre-metamorphic to metamorphic climax with changes in their diets.

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MATERIALS AND METHODS

Study areas

The breeding and development of *Microhyla berdmorei* was studied in their natural habitats, Tuitun stream (23º 58' 21.27" – 40.19" N and 92º 41' 05.51" – 10.35" E; elevations = 300 m – 325 m asl.), Kolasib district and Tlawng river (23º 48' 24.66" N - 55.04" N and 92º 38' 44.51" – 39' 08.97" E; elevations = 35 m to 50 m asl.), Aizawl district, Mizoram, India. The study areas were monitored from 2005 to 2010.

Length of intestines

Intestine of *Microhyla berdmorei* at different developmental stages were dissected out, stretched on the dissecting tray and measured with the help of threads, scale (mm) and dial vernier caliper (Mitutoyo series No. 505-671). Staging of developing larvae was done accordingly with Gosner table [27].

Histological studies
For light microscopy

Intestines were removed, washed in saline with the help of brush, fixed in bouin's fluid, dehydrated in ethanol and embedded in paraffin, serial sections at 5 µm thickness were prepared and stained with haemotoxylin eosin.

For Transmission Electron Microscopy (TEM)

Portion of the small intestine was cut and fixed in Kornovsky's fixative (Prepared in 0.1 M Sodium-Cacodylate buffer) in 0.1 M Cacodylate buffer (pH 7.4) at 4ºC for 2 hours and post-fixed with 1% Osmium tetroxide buffered (0.1 M Na-Cacodylate buffer) at 4ºC for 1 hour. The samples were then dehydrated through acetone grades (30%, 40%, 50%, 60%, 70%, 80%, 90% and 100 %) and then cleared in propylene oxide. The samples were then infiltrated in a mixture of clearing agent and embedding medium. After infiltration the tissues were embedded in the epoxy resins using beam capsules and blocks were prepared. Ultrathin sections (60 nm – 90 nm) were cut with the help of an ultra microtome (Ultratome V, LKB), stained with uranyl acetate and lead citrate and were examined with a Transmission electron microscope (JEM 100C x II Jeol) at an accelerating voltage of 80 KV.

Food items

Different developmental stages including adults of *Microhyla berdmorei* were collected from the study areas and identification on the food items of the tadpoles was made following the method of Turner [28], Edmonson [29], Needham and Needham [30] and Fritsch [31]. The feeding habit of adults was studied by removing the stomach contents and analyzed under a stereoscopic microscope (Labomed CSM2).

Statistical analysis

Statistical significance between total tadpole lengths and intestine lengths of developing *Microhyla berdmorei* were subjected to analysis of Spearman's rho (r) with the help of statistical software tools SPSS (7.5.1 version).

RESULTS
Length of intestines

The present study revealed that, in *Microhyla berdmorei,* the tadpole intestine consists of two spiral coils: the outer coil (duodenum and anterior ileum) reverses direction at the switchback point and is followed by the inner coil (posterior ileum and colon), which terminates at the rectum (Fig. 1a). During metamorphic climax, the gut is abruptly shortened into that of adult type (Fig. 1b). The length of tadpole intestine is increased from stage 25 to stage 40 (Table 1). There is significant positive correlation between the total tadpole length ($r = 0.997$) and the gut length ($r = 1.00$), where $p<0.01$. The intestine attained its maximum length (48.46 mm; SD = ±0.49 and N = 5) at stage 40 where the tadpole reached a maximum total length (25.08 mm; SD = ±0.32 and N = 5). From stage 40 to stage 46, the lengths of tadpole and intestine greatly reduced, showing perfect negative correlation between Gosner stage with that of total body length ($r = - 1.00$) and gut length ($r = - 1.00$), where $p<0.01$. Whereas Gosner stages 25 to 46 revealed non-significant relationship with total tadpole length ($r = 0.060$) ($p<0.01$) and gut length ($r = 0.038$) ($p<0.01$). Within 10 – 17 days the *Microhyla berdmorei* tadpole intestine (from stage 40 – stage 46) reduced its length by about 82%.

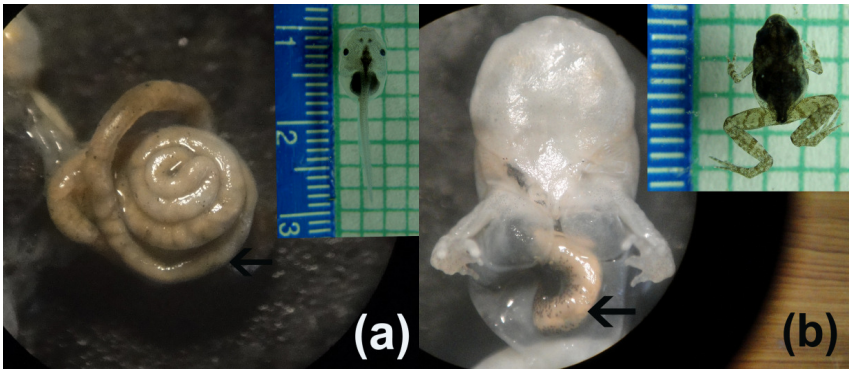


Fig 1.Guts of tadpole at premetamorphic phase, stage 27 (a) and at metamorphic climax, stage 46 (b).

Table 1. Lengths of total body and gut of *Microhyla berdmorei* at different developmental stages

Gosner Stage	Mean total length (mm) *N=5*	Standard Deviation (SD)	Mean Gut length (mm) *N=5*	Standard Deviation (SD)
25	11.13	±0.70	12.21	±0.25
26	16.74	±0.44	15.91	±1.03
27	17.30	±0.16	18.74	±0.53
28	18.31	±0.26	21.58	±0.36
29	19.05	±0.48	23.11	±0.48
30	21.41	±0.52	26.82	±1.16

31	21.50	±0.46	28.74	±0.83
32	21.79	±0.90	34.07	±1.22
33	22.52	±1.53	34.44	±0.88
34	23.43	±0.61	37.91	±0.29
35	24.48	±0.37	41.55	±0.30
36	24.44	±1.04	43.28	±0.50
37	24.64	±0.42	44.09	±0.45
38	24.91	±0.55	45.49	±0.45
39	24.92	±0.14	47.81	±0.35
40	25.08	±0.32	48.46	±0.49
41	24.39	±0.67	24.71	±0.62
42	21.94	±0.44	21.48	±0.51
43	15.16	±0.24	17.80	±0.22
44	13.98	±0.54	14.27	±0.62
45	10.76	±0.51	11.33	±0.53
46	8.11	±0.35	8.39	±0.45

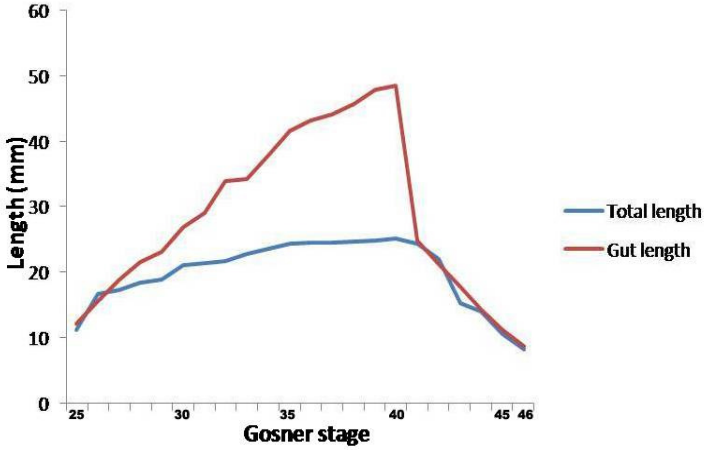


Fig 2. A graph showing changes in total length and gut length from stages 25 to 46

Histological studies
Light microscopy

Light micrographs revealed that the intestine at premetamorphic stages is a long simple tube (Fig. 3a) with a single layer of cuboidal epithelial cells and the primary epithelia are surrounded by thin layers of muscles with little intervening connective tissue, submucosa (SM) which is further enclosed by a thin serosa (S) (Fig. 3b). During prometamorphic phase (represented by stage 36), the intestinal wall is slowly thickening and the primary epithelium is still persists (Fig. 3c). At stage 46 (metamorphic climax), the primary epithelium (PE) degenerates and the secondary epithelium (SE) are formed. Intestinal folds (IF) that project into lumen (L) appear as several circular folds lined by mucus membrane (Fig. 3d).

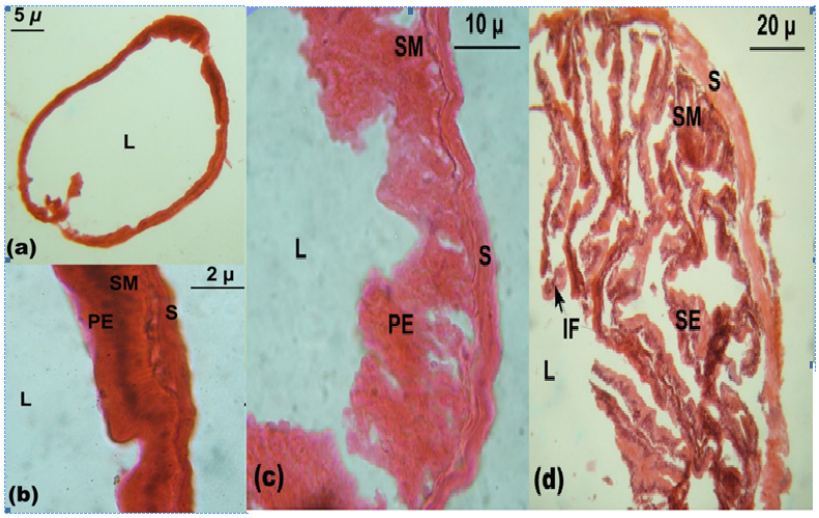
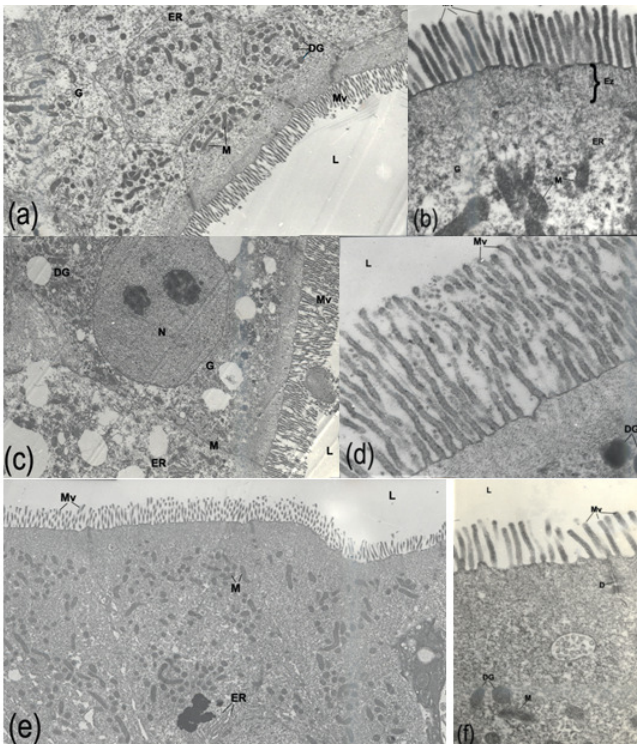


Fig 3. Light micrographs of intestinal walls (T.S)at stage 27 (a&b), stage 36 (c) and stage 46 (d). L- Lumen; PE- Primary Epithelium; S- Serosa; SM- Submucos; IF- Intestinal fold; SE- Secondary Epithelium

Transmission Electron Microscopy (TEM)

Although it is apparent from light microscopy that differentiation within the larval intestinal epithelium gives rise to absorbing cells with highly organized and complex structure, this fact is demonstrated more clearly by electron microscopy. At premetamorphic phase the striated border is resolved into a series of finger-like projections, the microvilli (Mv) that formed the brush border appears to be long and compact extend into the lumen (L) of the gut (Fig. 4a). Fine fibrillar material within the microvilli is continuous with that found in the underlying ectoplasmic zone (Ez), from which most of the commonly occurring cytoplasmic organelles are excluded (Fig. 4b). Basal to this cortical zone, mitochondria (M) are found in fairly dense array among small vesicular components of the endoplasmic reticulum (ER). Between the mitochondrial zone and the endoplasmic reticulum, the cytoplasm is typified by a surprisingly extensive Golgi region and the presence of many granules filled with dense particulate material, dense granules (DG) or lysosomes. During prometamorphic phase, the nucleus (N) is large in relation to the amount of surrounding cytoplasm. The latter contains a Golgi region (G) near the nucleus and a few mitochondria (M) and also large number of dense granules (Fig. 4c). The arrangement of microvilli (Mv) is irregular and less compact as compared to the previous phase, dense granules (DG) are found at the basal region (Fig. 4d). At metamorphic climax, microvilli (Mv) are shorter; in some instances they are fewer and are not always perpendicular to the cell surface (Fig. 4f). It does contain, however, recognizable mitochondria (M), elements of the endoplasmic reticulum (ER), and Golgi material (G). In addition, oval membrane-bounded granules (DG) are frequently observed (Fig. 4e) However, in comparison with the granules of the larval stage, the membranous element is more prominent. Furthermore, the number of granules per cell seems on the whole to be less than in the premetamorphic period. However, an examination of the fine structure of the apical cytoplasm brings to light details of their structure in which they exhibit differences from functional larval cells.



(a) At premetamorphic phase (stage 27), microvilli (Mv) that formed brush border project into the lumen (L) of the gut. Deeper in the apical cytoplasm, mitochondria (M), golgi region (G), dense granules (DG) and small vesicular components of the endoplasmic reticulum (ER) are frequently seen. X 17,000; (b) Close up view at stage 27 shows ectoplasmic zone (Ez) below the brush border. (c) At prometamorphic phase (stage 36), microvilli (Mv) that formed brush border are lesser compact. Mitochondria (M), nucleus (N), golgi region (G) dense granules (DG) and endoplasmic reticulum (ER) are seen. X 17,000; (d) Close up view at stage 36 shows the arrangement of microvilli (Mv). (e) Microvilli (Mv), mitochondria (M), dense granules (DG) and endoplasmic reticulum (ER) at metamorphic climax (stage 46). X 17,000; (f) Close up view at stage 46 shows microvilli (Mv) are lesser in number, desmosome (D), mitochondria (M) and dense granules (DG) are visible.

Fig 4. Electron micrographs of intestinal wall of *Microhyla berdmorei* at different phases.

Food items

The gut contents of premetamorphic and prometamorphic phases consists of phytoplankton, *Cymbella, Diatoma, Fragilaria, Melosira, Navicula, Pinnularia, Stauroneis, Synedra* and *Tabellaria* (Bacillariophyceae), *Cladophora, Closterium, Cosmarium, Mougeotia, Oedogonium, Sirogonium, Spirogyra, Staurastrum* and *Ulothrix* (Chlorophyceae), *Oscillatoria* (Cyanophyceae), *Anabaena, Arcella*, *Cryptomonas, Nostoc* (Cryptophyceae), *Euglena* and *Phacus* (Euglenophyceae) and zooplanktons included, *Centropyxis Euglypha, Lecane* and *Paramecium.* During metamorphic climax, as the developing larva ceased feeding, no food items were recovered from the gut. From stage 46 onwards, the animal starts to feed on other small invertebrates. Stomach contents showed that the adults feed mostly on small insects in which Hymenopterans (especially Formicidae, e.g. ants) shows the highest followed by other insects like, Isopterans (termites), small Coleopterans, Dipterans, and also

include pieces of chara, dry leaves, bamboo leaves and nematodes

DISCUSSIONS

The present findings indicated that, in *Microhyla berdmorei*, as the tadpoles continue to grow, so does the intestine. And as the tadpole regresses from stage 40 onwards, the intestine also begins to shorten. Like other anurans, *Microhyla berdmorei* tadpole intestine has a long and simple structure that remodeled during metamorphosis. It was reported that shortening of the intestine during spontaneous metamorphosis occurs uniformly along the intestine's length [24]. The abrupt shortening of the anuran gut during metamorphosis has been well documented, it was reported that in 1 week, at the climax of metamorphosis, the intestine shortens 58–90% depending on the anuran species [26], 42.3% for *Phrynohyas resinifictrix* [25], 58.15% in *Rana temporaria,* 75% in the *Xenopus laevis* [26], 82.2% in *Rana catesbeiana* [22], 84% in *Rana catesbeiana* [23] and to as high as 90% in *Alytes obstetricians* [9]. In the present study, shortening of the intestine during spontaneous metamorphosis is accompanied by a change in cross-sectional morphology from a single layer of cuboidal epithelial cells into a complicated layers consisting of secondary epithelium, intestinal folds lined with numerous microvilli which was also observed in other anurans studied so far [26]. The morphological changes that take place during intestinal remodeling are more drastic and the intestinal epithelium is a complex structure that provides an enormous luminal surface area for efficient food processing and absorption, the primary function of the organ [32,33]. Development of intestinal folds increased the absorptive surface of the small intestine [25]. As anurans transform from an omnivorous tadpole to a carnivorous frog by metamorphosing, the long small intestine, which consists of predominantly a single tubular layer of larval epithelium with very little connective tissue or muscle, reduces in its length by about 90% [15] and is replaced with the adult type of complex organ comprised of a multiply folded epithelium with elaborate connective tissue and muscle, accompanied by the programmed cell death of larval epithelium [8,14]. Both apoptotic bodies derived from larval epithelial cells and intraepithelial macrophage-like cells suddenly increase in number around the beginning of spontaneous metamorphic climax [13]. Ultra structural study of the intestines of *Microhyla berdmorei* tadpole revealed that at premetamorphic phase the microvilli composing brush border appears to be long and compact but decreases in number and height around the onset of metamorphic climax as also reported by [14,34,35]. These structural differences between larval and adult intestines presumably reflect changes in the physiological functions between herbivorous tadpoles and carnivorous frogs [4], as it was confirmed from gut contents analysis. The structural changes of musculature are formed in rearrangements of smooth muscle cells, but no proliferation of these cells with the metamorphosis [12]. It was reported that changes which take place in the alimentary canal during larval development of anurans are under control of many extrinsic (ecological features of an environment) and intrinsic (thyroxine activity) factors [25].

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