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Structural flexibility of the small intestine and liver of garter snakes in response to feeding and fasting

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Summary

Garter snakes *Thamnophis sirtalis parietalis* feed frequently but also tolerate extended periods of fasting when food is unavailable. We studied the dynamics, reversibility and repeatability of size changes of the small intestine and liver using ultrasonography. We employed light and transmission electron microscopy and flow cytometry to study the tissue mechanism that drives this flexibility. We compared garter snakes that fed every other day, snakes that fed once a week and fasting snakes. In all feeding trials, the size of the small intestine and the liver increased rapidly after feeding. Constantly feeding snakes maintained an elevated level of organ size, while snakes that were fed only once a week showed a marked up- and downregulation of organ size. Histology revealed the mucosal epithelium to be a transitional epithelium that can change cell configuration considerably to accommodate organ size changes. Upregulation of small intestine and liver size was always associated with the incorporation of lipid droplets into enterocytes and hepatocytes. Cell proliferation was not involved in upregulation of organ size. In contrast, cell proliferation increased during downregulation of organ size, indicating that cells worn out during digestion were replaced. The dynamics of flexibility and the functional features of the tissue were the same as described for the Burmese python *Python molurus bivittatus*. We suggest that garter snakes employ the same energetically cheap mechanism of organ size regulation as pythons, which allows for rapid, repeated and reversible size changes with no cell proliferation involved. Comparative evidence suggests that the transitional mucosal epithelium is an ancestral character of snakes and that feeding ecology is not directly related to the cytological features of the mucosal epithelium.

Key words: phenotypic flexibility, nutrition, fasting, digestion, snake, *Thamnophis sirtalis parietalis*.

Introduction

Many snakes swallow large meals after long fasting intervals. During extended periods of fasting, there are virtually no functional demands on their intestine. Only during a relatively short period following a meal is the mucosal epithelium active and does it absorb nutrients. Burmese pythons (*Python molurus bivittatus*) and other sit-and-wait foraging snakes tolerate fasting periods of 1 year or longer, although it takes only 10–14 days to digest and absorb a large meal. Other snakes hunt actively and catch prey more frequently. Some actively hunting snakes may catch prey every other day, but they maintain the ability to tolerate long fasting periods (Greene, 1983). In snakes and other ectothermic vertebrates with intermittent feeding patterns, feeding is followed by considerable upregulation of physiological and morphological functions, i.e. elevation of metabolic rate, upregulation of enzyme activity, increased brush-border transport rates, elevated blood flow to the digestive organs and increased mass of the small intestine, liver and kidney (specific dynamic action, SDA; Brody, 1945; Secor and Diamond, 1995, 1997; Secor and Phillips, 1997; Wang et al., 1995, 2001a; Andrade et al., 1997; Overgaard et al., 1999; Busk et al., 2000a,b; Hicks et al., 2000; Secor et al., 2000; Starck and Beese, 2001). When digestion is completed, organ size and function return to fasting values. In an evolutionary perspective, Secor and Diamond (2000) and Secor (2001) suggested that up- and downregulation of the intestine in sit-and-wait foragers represent an adaptation to infrequent feeding and that selection would act against regulation of intestinal size and function in frequent feeders because of the high energetic costs of postprandial responses.

The mechanisms that drive the size changes of organs in snakes have been investigated only in Burmese pythons, a typical sit-and-wait forager. It was shown that the mucosal epithelium of the small intestine is a transitional epithelium that allows for a rapid increase in organ size after feeding. Increased blood pressure and lymphatic pressure in the connective tissue of the villi have been suggested to be the driving forces of organ size changes (Starck and Beese, 2001). The size increase of the small intestine is rapid and (supposedly) energetically cheap because it does not involve

the synthesis of new tissue. An evaluation of the energetic costs associated with gastrointestinal upregulation by Overgaard et al. (2002) supports this idea. Paradoxically, cell proliferation rates were elevated during downregulation of organ size. To explain this observation, it was suggested that new cells replace only those cells that were worn out during resorption and that cell proliferation does not contribute to organ size increase (Starck and Beese, 2001). It has remained an open question whether this mechanism of organ size change is unique to Burmese pythons to accommodate rapid upregulation of small intestine size after very long fasting intervals or whether the transitional epithelium and its functional features are more widespread among snake species.

Here, we investigate the organ size responses of a colubrid snake, the red-sided garter snake (*Thamnophis sirtalis parietalis*). The red-sided garter snake is common in the northern and central United States and occupies a variety of habitats, but with a preference for semiaquatic areas. Garter snakes feed on arthropods, snails, other invertebrates and even small vertebrates. They hunt actively and feed frequently during summer, but they fast for several months during winter dormancy. However, at any season, they tolerate long fasting intervals. We chose this species because we were interested in the dynamics, amplitude and reversibility of organ size changes in an actively feeding snake and in the tissue mechanism underlying such organ size changes. We tested the structural flexibility of the garter snakes' intestine and liver under three different feeding regimes that supposedly mimic naturally occurring differences in food availability during the active period: (i) no food=fasting; (ii) abundant food=fed every other day; and (iii) low food abundancy=fed once a week, i.e. digestion interrupted by short fasting periods. The comparison of a frequently feeding active hunter with a previously studied infrequently feeding sit-and-wait forager allowed us to determine whether the observed features of Burmese pythons are a species-specific property or whether they represent a more widespread mechanism underlying organ size changes in snakes.

Materials and methods

Animals

Sixteen half-grown red-sided garter snakes *Thamnophis sirtalis parietalis* Gray 1825 (mass range 34–82 g) were purchased from a commercial reptile farm in Germany. The animals were kept in groups of 4–6 individuals (according to feeding regime) in cages (80 cm×50 cm×50 cm) at room temperature (25 °C) and 50 % humidity and with a 12 h:12 h L:D photoperiod.

During the acclimation period and preparation for feeding trials, all snakes were fed every third day. Thus, all animals were adjusted to moderately frequent feeding when the experiment started. Animals were randomly assigned to the three experimental groups and kept under the same room conditions. Each feeding trial lasted 4 weeks. Average meal size was 12 % (range 5–25 %) of the snake's body mass. The snakes were fed earthworms or sliced fish fillet according to availability. The different groups of animals were subjected to the following feeding regimes: group 1 (*N*=4) was fasted for the entire period, group 2 (*N*=4) was fed every other day, and group 3 (*N*=4) was fed once a week. Snakes were killed at the end of the respective fasting interval, i.e. animals of group 1 were killed after 4 weeks of fasting, animals of groups 2 were killed 2 days after last feeding, and animals of group 3 were killed 1 week after last feeding.

Imaging

Ultrasonography

Ultrasonography provides reliable images of internal organs under undisturbed physiological conditions (Hildebrandt et al., 1998; Starck and Burann, 1998; Dietz et al., 1999; Starck and Beese, 2001; Starck et al., 2001). We used an ophthalmological ultrasonography system (I^3 Innovative Imaging Inc., Sacramento, CA, USA), equipped with a 10 MHz sector scanner in B-mode (scan angle 52 °; scan speed 28 frames s^{-1}; dynamic range 90 dB; image depth 45 mm; 256 gray-scale shades; cross-vector scale calibrated to 1550 m s^{-1}). The resolution of the system was 0.15 mm (axially) by 0.2 mm (laterally), and therefore size changes of approximately 0.4 mm could be detected with high reliability. Observed size changes were in the range 2–5 mm, 5–12 times greater than the lower limit of resolution.

Histology

Animals were killed with an overdose of sodium pentabarbitol. Tissue samples of the anterior part of the small intestine and liver were preserved immediately for histology.

Light microscopy

Tissue samples were preserved in 5 % paraformaldehyde in 0.1 mol l^{-1} phosphate buffer at pH 7.4 and 4 °C for at least 48 h, then washed in buffer, dehydrated through a graded series of ethanol to 96 % ethanol and embedded in hydroxyethyl methacrylate (Historesin). Embedded material was sectioned into short series of 50 sections per sample (section thickness was 2 μm), mounted on slides and stained with Methylene Blue/Thionine. Microphotographs were taken with a Zeiss Axioplan photomicroscope equipped with a digital camera.

Transmission electron microscopy

Tissue samples for transmission electron microscopy were preserved in 2.5 % glutaraldehyde in 0.1 mol l^{-1} phosphate buffer at pH 7.4 for 48 h at 4 °C and subsequently treated with 1 % osmium tetroxide in phosphate buffer for 1.5 h. Dehydration, embedding in Durcupan resin (AMC Fluka), sectioning (thickness 0.07 μm) and staining with uranyl acetate/lead citrate followed standard protocols for transmission electron microscopy. Sections were studied with a Zeiss EM 900 electron microscope.

Flow cytometry

Tissue samples were taken from the small intestine during

dissection, immediately frozen in liquid nitrogen and stored at –80 °C until processed. For flow cytometry, tissue samples were thawed and treated with 0.75 % Triton-X 100 in 0.01 mol l^{-1} Tris-HCl buffer at pH 7.4 to extract nuclei. Isolated nuclei were stained for DNA content with propidium iodide, and their fluorescence was measured in a Facs Calibur flow cytometer (Becton Dickinson & Co., San Jose, CA, USA).

The relative amount of DNA in the nuclei is indicative of the cell cycle phase of the cell. Nuclei with DNA contents between that of resting-phase cells and double this DNA content were extracted from S-phase cells. Nuclei containing twice the resting-phase DNA content came from G2-phase or mitotic cells. Because flow cytometry did not discriminate nucleus size, counts of G2-phase and mitotic cells can be inflated by G1-nuclei adhering to each other. Therefore, we refer to the proportion of S-phase nuclei as indicative of the proliferation activity of the tissue. The fraction of nuclei containing less than the resting-phase DNA content is considered to represent apoptotic cells, but it may also contain fragments of nuclei.

Morphometry and statistical analyses

Body mass measurements were taken on an electronic balance (Metzler, Germany) to 0.01 g. We used SigmaScanPro (version 4.0, Jandel Scientific, SPSS Inc., Chicago, USA) for image analysis and morphometric data acquisition. From ultrasonographs, we measured the cross section of the liver and the thickness of the mucosa of the small intestine.

Histological sections were studied using a Jenaval research microscope (Zeiss, Jena) equipped with a video camera and connected to the image-analysis and morphometry system. From each section, we measured the thickness of the muscle layer (tunica muscularis) and the height of the villi from the muscle layer to the top of the villi. We measured 10 sections per tissue sample and took 15 measurements of the thickness of the muscle layer and the height of villi per section. The length of microvilli was measured from transmission electron micrographs scanned into digital format.

Statistical analyses

Values are given as means ± S.D. (*N*=sample size). To avoid the effects of repeated measures, mean values were calculated for each individual before they were entered into an analysis of variance (ANOVA) to test for effects of feeding regime. $P<0.05$ was considered significant. The Ryan–Einot–Gabriel–Welsch multiple-range test (REGWQ) for pre-planned comparisons among means was performed at α=0.05. All statistical procedures were performed using SPSS version 10.07.

Results

Body mass and organ masses

Body mass

To account for size differences among the individual snakes, all measurements of body mass are expressed relative to the mass of each individual on the day before the experiment started. In fasting snakes, body mass declined over the 4 weeks to an average of 82 % of the initial value (Fig. 1A). In contrast, snakes that were fed every other day showed a steady increase in body mass, gaining 30 % in mass over the same 4 week period (Fig. 1B). Some individuals interrupted feeding while moulting and thus showed a short stagnation of body mass increase. In snakes that fed once a week, body mass peaked 2 days after feeding at 110–120 %, followed by a slower decline. Their body mass increased slightly over the 4 weeks (Fig. 1C).

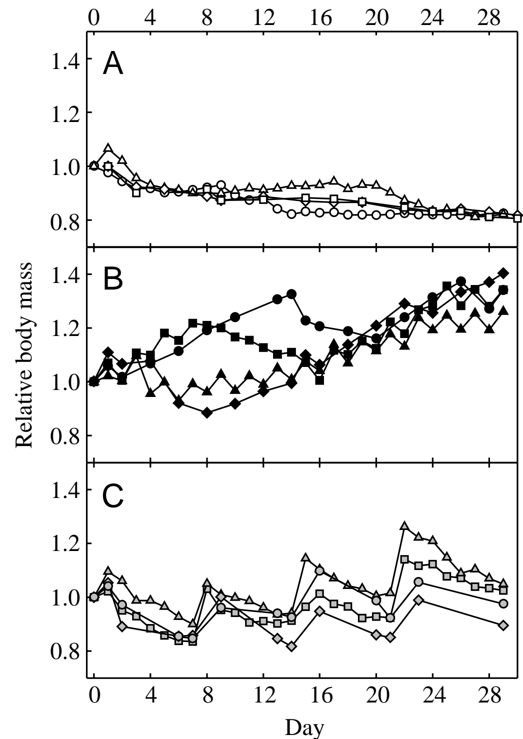

Fig. 1. Relative body mass changes of garter snakes under different feeding regimes. (A) Fasting snakes; (B) snakes fed every other day; (C) snakes fed once a week. In B and C, the body mass reported includes the mass of the ingested prey. Body mass is expressed relative to the mass of the individual on the day before the experiment started.

Small intestine

Fresh organ mass is expressed relative to body mass. We observed considerable differences in relative organ mass in relation to feeding. Small intestine mass was lowest in fasting snakes (3.11±0.22 %). Two days after feeding, the fresh mass of the small intestine (5.04±1.13 %) was significantly higher (REGWQ, α=0.05) than in fasting snakes. Seven days after feeding, the fresh mass of the small intestine (4.27±1.01 %) was intermediate between fasting and digesting values. Because of its intermediate value, it was distinguished statistically from the fasting but not from the frequently feeding snakes (REGWQ, α=0.05).

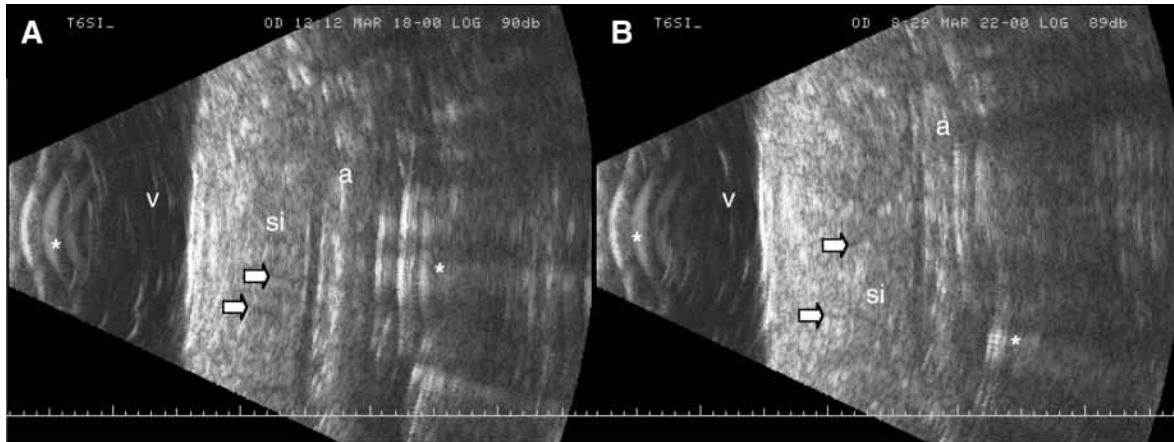

Fig. 2. Transcutaneous ultrasonographs of the small intestine of (A) fasting garter snakes and (B) snakes 2 days after feeding. Both figures are parasagittal images of the small intestine (the top of the image is cranial, the bottom is caudal, left is ventral, right is dorsal). The ventral scales and the dorsal aorta were used as morphological landmarks. The thickness of the mucosa was measured as the distance between the two dark lines of the tunica muscularis. v, ventral scales; a, dorsal aorta; si, small intestine mucosa; the arrows indicate the tunica muscularis; *imaging artifacts. Scale, major dimensions, 10 mm.

Liver

In fasting snakes, liver mass was on average 2.5±0.6 % of body mass. Two days after feeding, liver mass was increased to 4.1±0.2 % of body mass. One week after feeding, liver mass averaged at 3.3±0.4 % and, thus, was intermediate between that of fasting and digesting snakes. Differences among means were significant (REGWQ, α=0.05). The liver mass of fasting snakes was significantly lower than that of feeding snakes. Groups 2 and 3 could not be separated statistically.

Organ size changes

Small intestine

Size differences of the small intestinal mucosa between fasting and digesting snakes were clearly visible (Fig. 2A,B). The thickness of the mucosa declined when snakes were fasted (group 1). During the first 12 days of fasting, the mucosal thickness declined to approximately 80 % of that of the active mucosa (i.e. when the experiment started). The thickness of the muscosa then remained constant for the rest of the experiment (Fig. 3A). Snakes that were fed every other day (group 2) had a thicker mucosa than fasting snakes. Mucosal thickness was constant over the 4 weeks. On average, the mucosal thickness was 150 % of that when the experiment started (Fig. 3B). In snakes fed once a week, within 24–48 h after feeding the mucosal thickness increased from the fasting value of 80–90 % to approximately 120 % (Fig. 3C). This response was elicited repeatedly in exactly the same pattern. Although the range of body masses of the animals in our sample was large, the proportional response of the small intestine was consistent among animals. Also, the dynamics of up- and downregulation were consistent for all individuals. For the four feeding events in the experiment, the thickness of the mucosa did not completely return to resting values, indicating that 7 days was not long enough to downregulate the mucosal thickness completely (see below).

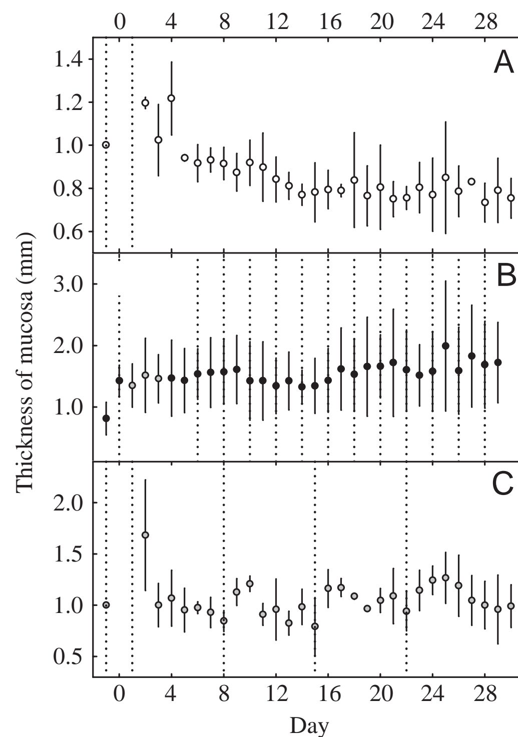

Fig. 3. Flexible response of the small intestinal mucosa to feeding measured from ultrasonographs. For comparison, multiple measurements of each individual were averaged by individual and day. Values are means ± S.D. from the four snakes in each group. Dotted lines indicate feeding events. (A) Fasting snakes, open symbols; (B) snakes fed every other day, black symbols; grey symbols are for animals that were shedding their skin and did not feed; (C) snakes fed once a week, grey symbols.

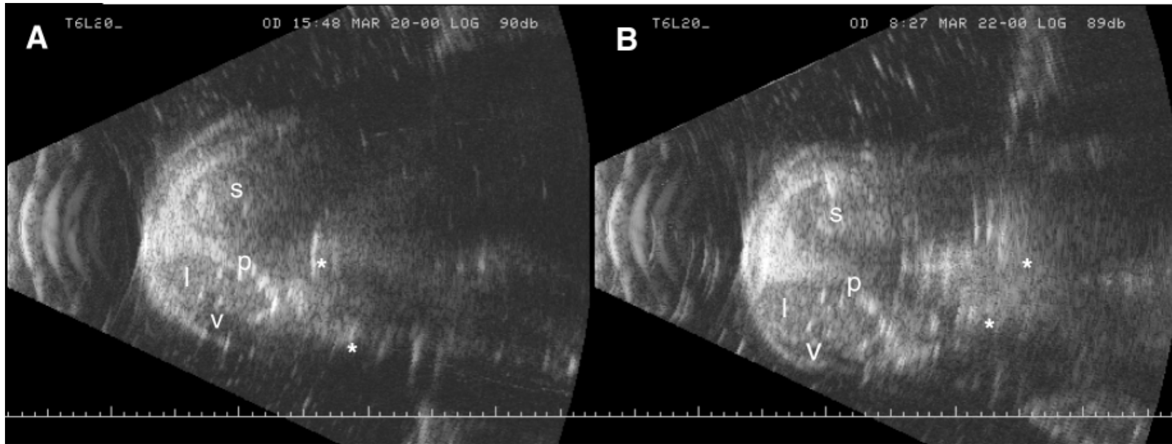

Fig. 4. Transcutaneous ultrasonographs of the liver of (A) fasting garter snakes and (B) snakes 2 days after feeding. Both figures are cross-sectional images through the snake. Left is ventral, right is dorsal. l, liver; p, hepatic portal vein, v, hepatic vein; s, stomach; *imaging artifacts. Scale, major dimensions, 10 mm.

Length of intestinal villi

For the mass range of snakes in our sample, we did not find significant effects of body size on villi length. Therefore, mean values of villi length are given. The intestinal villi of fasting snakes averaged 0.6±0.2 mm. Two days after feeding, intestinal villi measured 1.2±0.2 mm. Snakes that were fed once a week took an intermediate position, with a villous length of 0.9±0.3 mm. The differences between fasting and digesting snakes were statistically significant (REGWQ, α=0.05). Snakes of group 3 were intermediate and statistically not different from either of the other two groups.

Tunica muscularis

On average, the thickness of the muscle layer was 0.18±0.07 mm in fasting snakes, 0.19±0.1 mm in group 2 and 0.14±0.05 mm in group 3. Differences between groups were not statistically significant.

Length of microvilli

In fasting snakes, the length of microvilli was 2.4±0.6 µm. In digesting snakes, the length of microvilli was 3.4±0.6 µm. This difference was statistically significant (one-way ANOVA, d.f.=1, F=10.9; P=0.02).

Liver size

We took cross-sectional ultrasound images of the liver (Fig. 4). Images were standardized by adjusting the position of the scanner head to obtain circular cross sections of the hepatic portal vein and hepatic vein (which is their true anatomical shape).

In fasting snakes, the cross-sectional diameter of the liver increased for 4 days after the beginning of the experiment and peaked at 120 % of initial size. This increase in liver size followed a meal the day before fasting started. Thereafter, liver size declined to 90 % of the initial liver size (Fig. 5A). In snakes fed every other day, we observed an increase in liver size. The pattern of size increase showed a plateau between

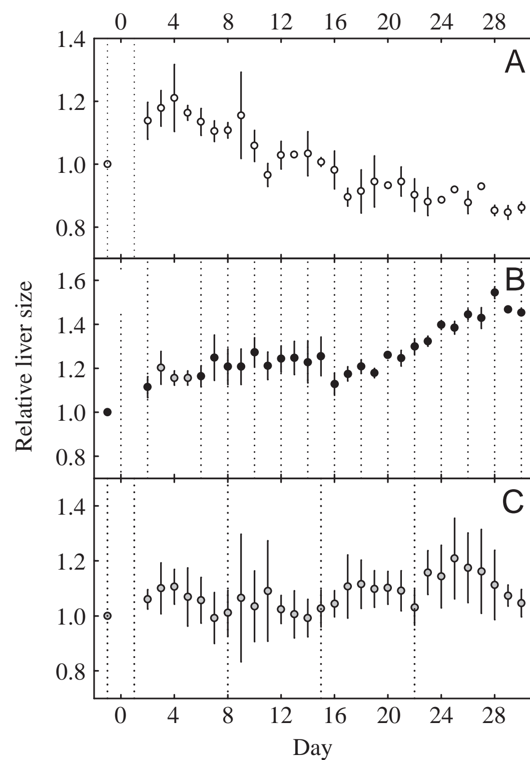

Fig. 5. Flexible response of the liver to feeding measured from ultrasonographs. Multiple measurements of each individual were averaged by individual and day; to account for size differences among individuals, measurements are expressed relative to the size (cross-sectional diameter) of the liver of each individual on the day before the experiment started. Values are means ± S.D. from the four snakes in each group. Dotted lines indicate feeding events. (A) Fasting snakes, open symbols; (B) snakes fed every other day, black symbols; grey symbols are for snakes that did not feed; (C) snakes fed once a week, grey symbols.

days 6 and 16, obviously in response to moulting, which occurred between days 3 and 5 (Fig. 5B). When snakes were fed once a week, they showed an increase in liver size in response to feeding. Liver size peaked 3–4 days after feeding. Thereafter, liver cross-sectional diameter declined (Fig. 5C). Downregulation was not complete, indicating that 1 week between feeding intervals is not enough time to return fully to original organ size. The patterns and dynamics of up- and downregulation could be elicited repeatedly.

Histology

Small intestine

The mucosal epithelium of digesting snakes (group 2; 2 days after feeding) was a single-layered epithelium with highly prismatic enterocytes (Fig. 6A,B). It showed the typical features of a fully functional vertebrate muscosal epithelium. Nuclei were located in the basal part of the enterocytes, while mitochondria and other organelles were found in the apical part. The enterocytes had a prominent brush border with a well-developed glycocalyx. The enterocytes of digesting snakes were loaded with lipid droplets (i.e. intracellular droplets that are not surrounded by a membrane and the contents of which can be extracted with ethanol; Fig. 6A–C). Within the mucosal epithelium, we also observed goblet cells and amoeboid cells with possible immune function. Enterocytes and goblet cells were rooted on a basal membrane. Digesting snakes had relatively large paracellular spaces. The connective tissue core of the villi consisted of loose connective tissue with large lymphatic spaces and blood vessels. The mucosal epithelium did not form intestinal crypts. The microvilli were long and covered with a well-developed glycocalyx (Fig. 6D).

In fasting snakes, the nuclei of the enterocytes of the mucosal epithelium were arranged in several layers. Goblet cells and amoeboid cells were present. As shown by electron microscopy, enterocytes and goblet cells were rooted on the basal membrane (Fig. 7A,B,E). This particular pseudostratified configuration of the epithelial cells is typical of a transitional epithelium. Enterocytes did not contain lipid droplets, and only

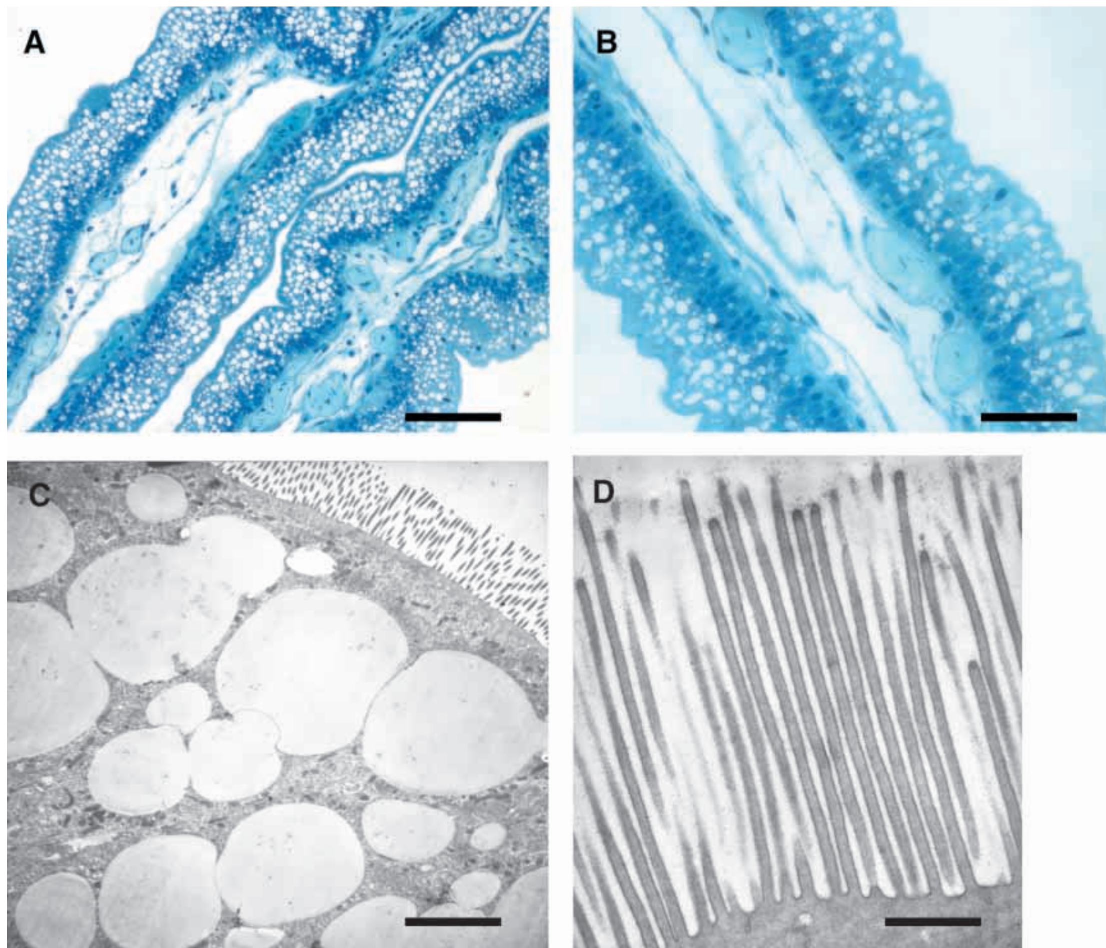

Fig. 6. Histology of the mucosal epithelium of digesting snakes 2 days after feeding. (A) Methacrylate-embedded section of the small intestine; Methylene Blue/Thionine stain. Scale bar, 50 μm. The mucosal epithelium is a single-layered columnar epithelium. (B) Methacrylate-embedded section of the small intestine; Methylene Blue/Thionine stain. Scale bar, 25 μm. Note the large lacteals, blood-filled capillaries and lipid droplets in the enterocytes. (C) Low-power electron micrograph of enterocytes filled with lipid droplets. Scale bar, 2.5 μm. (D) High-power electron micrograph of microvilli. Scale bar, 0.5 μm.

narrow paracellular spaces were visible between the enterocytes. The microvilli of the brush border were shorter and stouter than in digesting snakes (Fig. 7C; cf. Fig. 6D). The apical margin of enterocytes was tightened by desmosomes. The cell membrane of the lateral side of the enterocytes was folded or formed large coils (Fig. 7D). Also, the basal membrane of the mucosal epithelium was folded (Fig. 7E).

The histology of the mucosal epithelium of snakes in group 3 (fed once a week) was intermediate between those of fasting and digesting snakes. There were fewer lipid droplets in the enterocytes (Fig. 8A,B); in many enterocytes, there were only small residues of lipid droplets in the apical part of the cell (Fig. 8C,D). The nuclei of the enterocytes were arranged in 1–3 layers, but they were not as densely packed as in fasting snakes, indicating an intermediate stage in the transition from single-layered to pseudostratified. Small coils of membrane

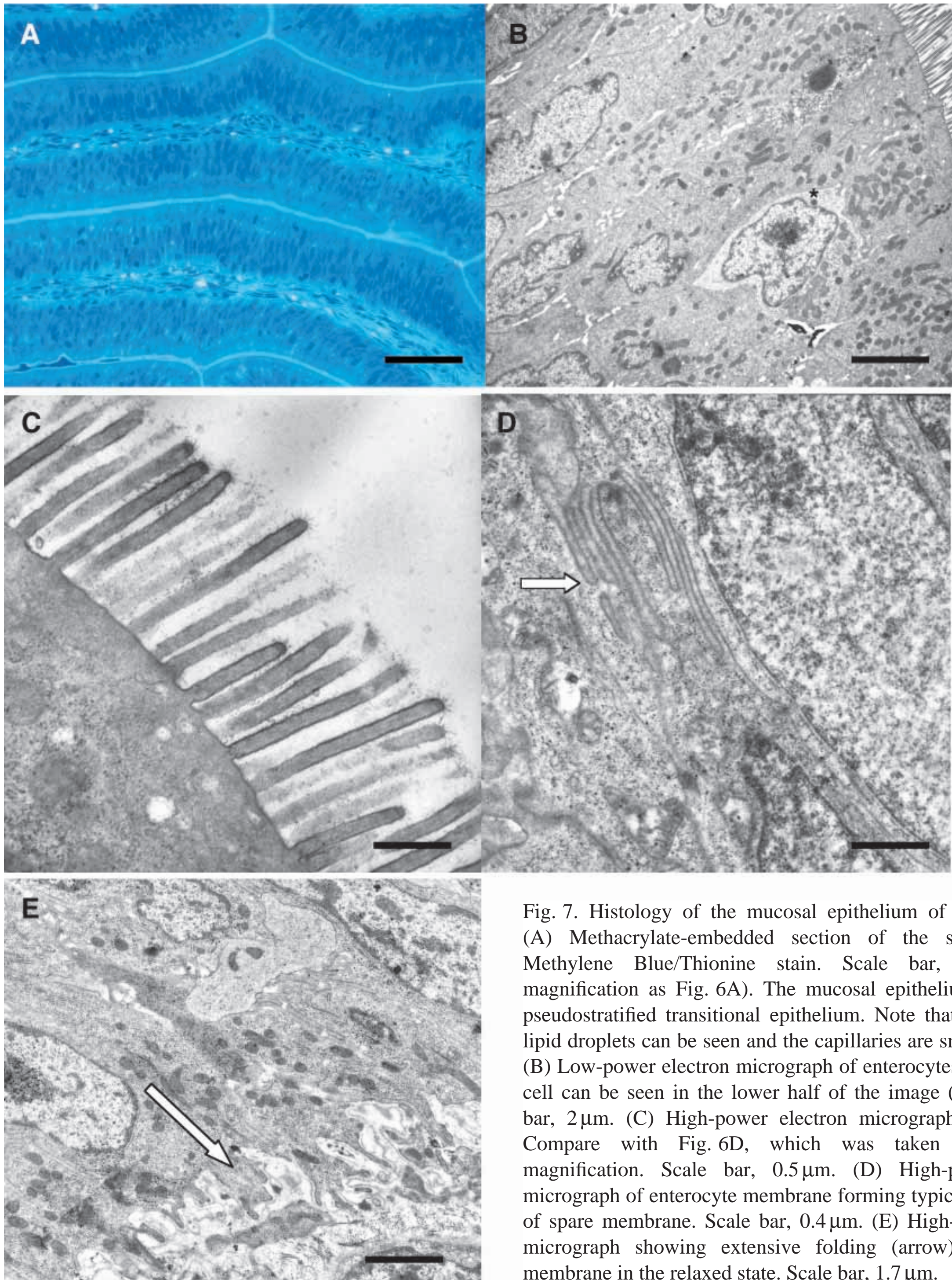

Fig. 7. Histology of the mucosal epithelium of fasting snakes. (A) Methacrylate-embedded section of the small intestine; Methylene Blue/Thionine stain. Scale bar, 50 µm (same magnification as Fig. 6A). The mucosal epithelium is a typical pseudostratified transitional epithelium. Note that no lacteals or lipid droplets can be seen and the capillaries are small and empty. (B) Low-power electron micrograph of enterocytes. An amoeboid cell can be seen in the lower half of the image (asterisk). Scale bar, 2 µm. (C) High-power electron micrograph of microvilli. Compare with Fig. 6D, which was taken at the same magnification. Scale bar, 0.5 µm. (D) High-power electron micrograph of enterocyte membrane forming typical folds (arrow) of spare membrane. Scale bar, 0.4 µm. (E) High-power electron micrograph showing extensive folding (arrow) of the basal membrane in the relaxed state. Scale bar, 1.7 µm.

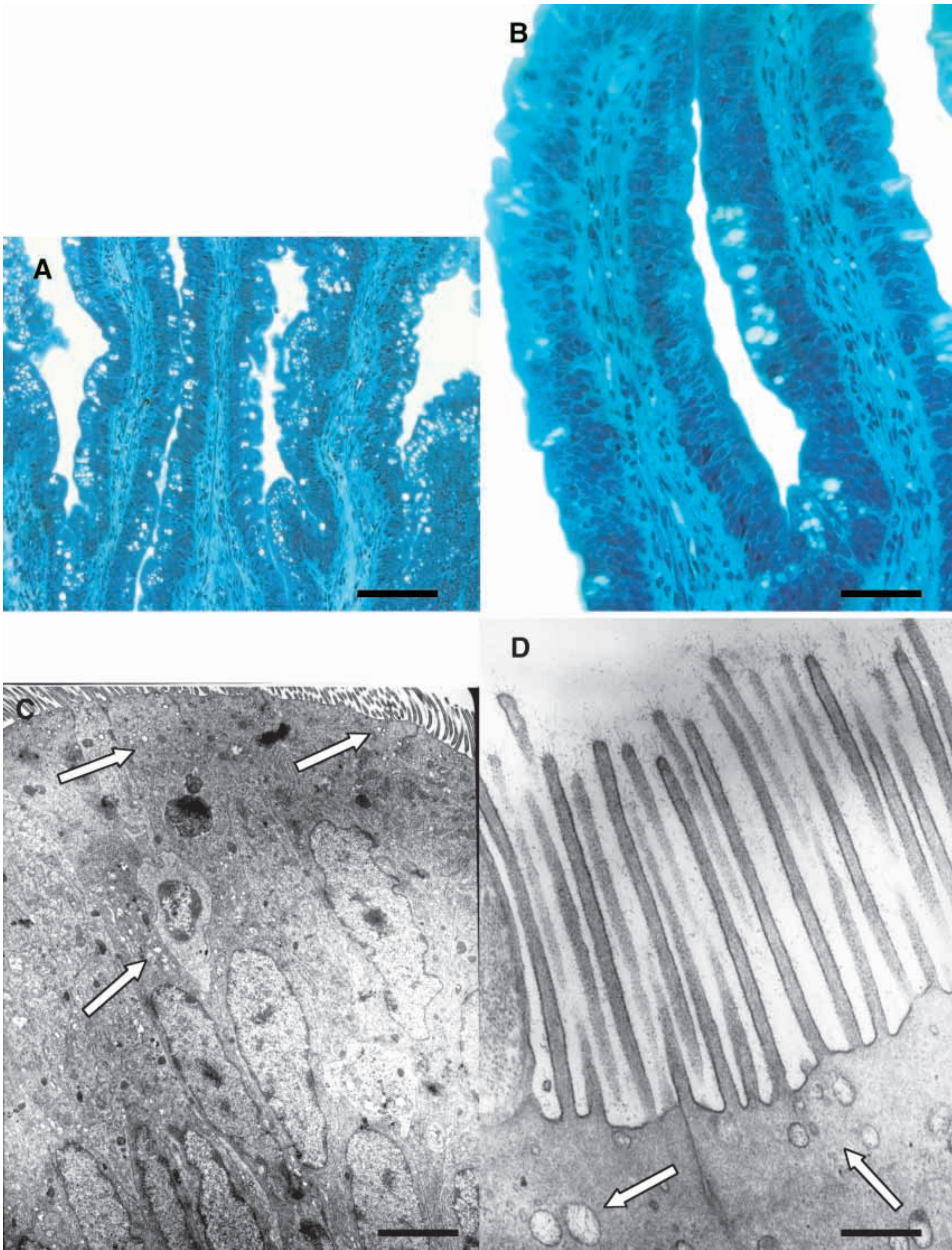

Fig. 8. Histology of the mucosal epithelium of snakes fed once a week. (A) Methacrylate-embedded section of the small intestine; Methylene Blue/Thionine stain. Scale bar, 50 µm (same magnification as Figs 6A and 7A). The mucosal epithelium has an intermediate configuration compared with Figs 6A and 7A with two layers of nuclei, few lipid droplets, reduced lacteals and reduced numbers of capillaries. (B) Histology as in A (same magnification as Fig. 6B). Scale bar, 25 µm. (C) Low-power electron micrograph of transitional epithelium. Note the small lipid droplets (arrows) in the apical part of the enterocytes. Scale bar, 5 µm. (D) High-power electron micrograph of microvilli. Compare with Fig. 6D, which was taken at same magnification. Scale bar, 0.5 µm.

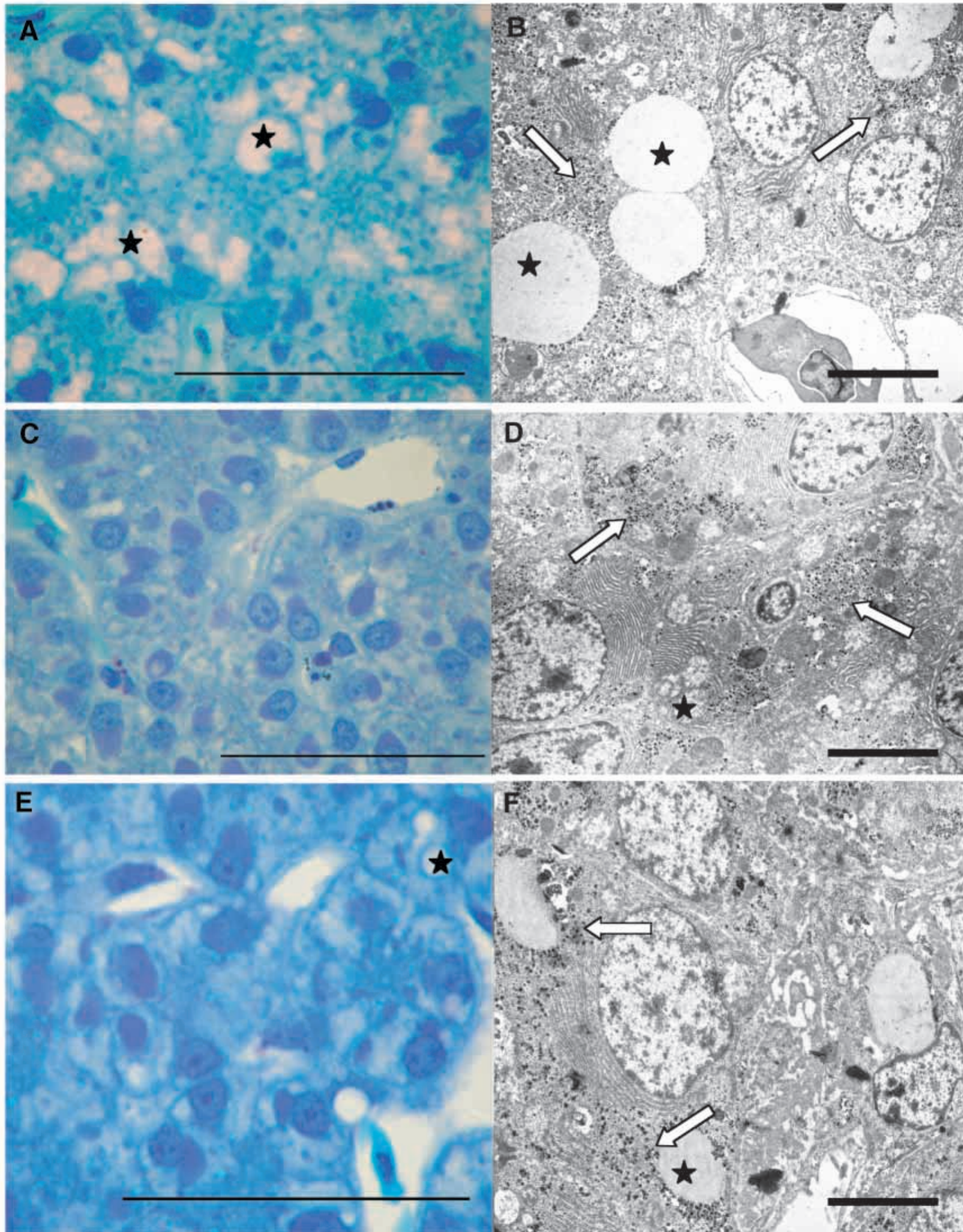

Fig. 9. Histology of the liver. (A) Digesting snake (2 days after feeding) Methacrylate-embedded section of the liver; Methylene Blue/Thionine stain. Scale bar, 50 µm. Note the many lipid droplets (stars) in the hepatocytes. (B) Digesting snake, low-power electron micrograph of hepatocytes loaded with lipid droplets and glycogen deposits. Scale bar, 4 µm. Arrows, glycogen deposits; stars k, lipid droplet. (C) Fasting snake. Methacrylate-embedded section of the liver; Methylene Blue/Thionine stain. Scale bar, 50 µm. (D) Fasting snake, low-power electron micrograph of hepatocytes. Note that no lipid droplets can be seen in the hepatocytes and that glycogen deposits are depleted. Scale bar, 4 µm. Arrows, glycogen deposits; star, lipid droplet. (E) The liver from a group 3 snake (fed once a week). Methacrylate-embedded section of the liver; Methylene Blue/Thionine stain. Scale bar, 50 µm. Star, lipid droplet. (F) Low-power electron micrograph of hepatocytes from a group 3 snake. Histology is intermediate and some lipid droplets can be seen in the hepatocytes together with glycogen deposits. Scale bar, 4 µm; arrows, glycogen deposits; star, lipid droplet.

were present in the basal parts of the enterocytes. The cytology of the enterocytes was intermediate between those of fasting and digesting snakes.

Flow cytometry

To avoid inflation of the counts of mitotic cells caused by nuclei sticking together, our analysis considered only nuclei containing between one and two times the normal DNA content (S-phase cells). In fasting snakes, we found on average 2 % of the nuclei of the mucosa epithelium in S-phase. In snakes that were fed every second day and in snakes that were fed once a week, the proportion of S-phase cells was on average 4 %.

Liver

The histological features of *Thamnophis sirtalis parietalis* liver did not differ from those described by Schaffer (1998) for sauropids in general. However, we observed differences between individual snakes in the subcellular compartment, depending on feeding status. Although hepatocyte size was not quantified, they appeared to be much smaller in the fasting than in the digesting snakes. In digesting snakes, i.e. 2 days after feeding, the hepatocytes were filled with numerous lipid droplets. Hepatocytes contained considerable glycogen deposits (Fig. 9A,B). We found no lipid droplets in hepatocytes of fasting snakes; also, glycogen deposits appeared to be smaller than in digesting snakes (Fig. 9C,D). The sinusoids of the liver appeared to be larger in fasting snakes than in digesting snakes. Snakes of group 3 showed an intermediate condition. Hepatocytes contained lipid droplets but they were fewer in number and smaller than in snakes that had been fed 2 days previously. Also, the size of the glycogen deposits was intermediate between those of digesting and fasting snakes (Fig. 9E,F).

Discussion

The frequently feeding and actively hunting garter snake regulates mucosal thickness in response to functional demands, i.e. feeding *versus* fasting. The patterns and dynamics of up- and downregulation of small intestine size were the same as described previously for Burmese pythons (Starck and Beese, 2001). Up- and downregulation of small intestine size and liver size were rapid, repeatable and reversible. In pythons, the mucosal thickness of fasting animals increased threefold within 2 days after feeding. Here, we observed only 1.5-fold size changes, but comparisons were made with an actively absorbing intestine, so the responses were relatively small. If we compare small intestinal mucosal thickness of group 1 with that of group 2, the digesting mucosa thickness is approximately 200 % of that of fasting snakes.

The mucosal epithelium is a transitional epithelium that has the same pseudostratified configuration in the fasting snake as described previously for Burmese pythons (Starck and Beese, 2001). The close similarity between garter snake and Burmese python histology extends to the ultrastructural level, where the same spiral folds, folding of the basal membrane, lipid droplets, accumulation of mitochondria and changes in micovilli length in relation to feeding can be seen. The muscle layer of the small intestine did not respond to feeding. This indicates that organ size changes are specific and restricted to those tissue components that contribute to the specific function (i.e. absorption). Cell proliferation showed paradoxical activity pattern as in the python, i.e. proliferation rate was also elevated when organ size was downregulated.

Most other studies have used invasive approaches and were, therefore, unable to trace reversibility and repeatability of organ size changes. However, it is clear from these studies that up- and downregulation of small intestine mass of a variety of snake species (Secor and Diamond, 1995, 1998, 2000; Jackson and Perry, 2000) were in the same range as those described here for the garter snake. Interestingly, the colubrid snake *Boira irregularis* shows a peak in mucosal dry mass the day after feeding that is associated with a peak in enterocyte volume on the same day (Jackson and Perry, 2000). This is consistent with our data that lipid droplets become incorporated into enterocytes. Although none of the other studies looked at cell proliferation activity, we suspect that the reported changes in mucosal epithelium mass were based on hypertrophy, i.e. increases in cell size through incorporation of lipid droplets, and not on hyperplasia (increase in cell number).

Changes in intestinal morphology in response to fluctuations in food composition or fasting have also been described in birds and mammals (for a review, see Starck, 1999). However, all the available evidence indicates that changes in the size of the mucosal epithelium of birds and mammals are based on a balance between cell proliferation in the intestinal crypts and extrusion of cells at the tip of the villi (Carey, 1990, 1992; Carey and Sills, 1992, 1996; Starck, 1996a,b; Dunel-Erb et al., 2001; Hume et al., 2002). Thus, the flexibility of the mucosal surface of the small intestine in birds and mammals seems to be based on entirely different cellular mechanisms: transitional epithelium and hypertrophy of enterocytes in snakes *versus* hyperplasy of the mucosal epithelium in birds and mammals.

For the liver, we observed a similar pattern of organ size changes. However, upregulation was somewhat slower, and the liver reached peak size only 3–4 days after feeding. Size increase was associated with an increase in liver mass and incorporation of lipid droplets into hepatocytes. Although we did not measure hepatocyte size, our data clearly showed that hepatocyte size increased with the incorporation of lipid droplets after feeding. Liver size changes in Burmese pythons show the same histological and ultrastructural pattern (J. M. Starck, unpublished data). Changes in hepatocyte size in response to feeding and fasting are known from mammals (*Rattus norvegicus*, Uhal and Roehrig, 1982; López-Navarro et al., 1996; Raul and Schleiffer, 1996; *Rangifer tarandus*, Soveri, 1993; *Rubicapra rubicapra*, Bollo et al., 1999) and are generally related to the incorporation of different amounts of lipids into hepatocytes. Therefore, we propose that the observed changes in liver size in snakes reflect the temporary storage and processing of lipids in the liver. Again, it appears

that organ size change is based on hypertrophy of cells and that no (detectable) cell proliferation is involved in these short-term changes in liver size.

The following conclusions arise from these comparisons. First, up- and downregulation of small intestine size in garter snakes are based on mechanical expansion of the intestinal villi. Stretching of the (fasting) villi will change the pseudostratified mucosal epithelium to a single-layered structure and could be associated with a switch to the functional condition. We assume that both an increase in blood flow to the tissue and and an increase in lymphatic pressure after swallowing a meal, as well as incorporation of lipid droplets into enterocytes, will cause an elongation of the villi. Increased blood pressure may be ruled out because Wang et al. (2001b) detected no increase in systemic blood pressure during digestion in *Boa constrictor*. During upregulation of organ size, a significant increase in organ mass was associated with the incorporation of lipid droplets into enterocytes, but not with cell proliferation. Two days after feeding, we found only 4 % of cells in S-phase. This low proportion of proliferating cells cannot account for the 50 % increase in the small intestinal mucosa in 2 days. Cell proliferation was also at 4 % during downregulation of small intestine size. We conclude that cell proliferation is not involved in upregulation of mucosal size but that newly formed cells replace worn-out enterocytes. The size responses are specific because other tissues of the gut, e.g. the muscle layer, do not respond to feeding. Because there is no new tissue production involved in the upregulation of organ size in garter snakes, we suggest that the increase in small intestinal mucosal thickness is energetically cheap. In our view, only a minor proportion of the elevated metabolic rate during SDA is allocated to upregulation of organ size.

Second, changes in liver size and mass in response to feeding and fasting are based on the incorporation of lipid droplets into hepatocytes. Peak liver size is reached approximately 24 h later than peak small intestine size, indicating that the transport of lipid from the small intestine to the liver takes some time. The liver acts as an intermediate site for storage and biochemical processing of lipids before they are ultimately stored in the perivisceral adipose tissue.

Third, it is unclear where the lipid droplets come from. They may originate from the meal, but they could also come from the adipose tissue of the snake to fuel the SDA. Either way, they could fuel the upregulation of enzymes and membrane-bound transport systems, including elongation of microvilli.

Fourth, garter snakes (Colubridae) and pythons (Pythonidae) are only distantly related. Because of the complexity of the transitional epithelium and the many shared features at all levels of investigation, we conclude that a transitional epithelium and its capacity for rapid up- and downregulation of the small intestine are shared traits of snakes. Additional support for this view comes from comparative histological studies of a variety of snake species and other reptiles (Luppa, 1977; Frye, 1991) in which the mucosal epithelium has been described as transitional epithelium, with no discussion of the functional properties associated with such tissue architecture. This is in contrast to the results of Secor et al. (1994), who suggested that the extreme SDA in a rattlesnake (*Crotalus cerastes*) was a 'hyperadaptation (…) but not a phylogenetic character of snakes in general'. Secor and Diamond (2000) and Secor (2001) acknowledged the increasing number of reports on SDA in snakes and other ectothermic vertebrates and assumed multiple evolutionary origins and reversals of large regulatory responses of the gut. We think, however, that the complexity of the transitional epithelium and the shared features of all their details support our interpretation that a transitional epithelium is an ancestral trait of snakes that provides the structural basis for rapid, reversible and repeated up- and downregulation of the intestine with no necessity to proliferate new cells. The ability to up- and downregulate small intestine size carries a strong phylogenetic signal and is not obviously related to the feeding ecology.

Finally, from a functional perspective, the different categories of foraging strategies (i.e. sit-and-wait *versus* active hunting) may be not very different and may impose very similar functional demands on the small intestine. In both cases, the snake must tolerate fasting intervals that are longer than the digestive periods, and they must be able to regulate small intestinal size accordingly. Evolution will select for rapid, reversible and repeatable up- and downregulation if the costs of plasticity are lower than those of maintaining a functional intestine. In such an evolutionary perspective, we would expect divergence from ancestral snake flexibility only if the costs of flexibility exceed the costs of maintaining an active intestine during periods of fasting. We have found an energetically cheap and fast mechanism that accommodates organ flexibility of frequent feeders as well as organ flexibility of infrequent feeders.

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