THE UNIVERSITY OF CALGARY

GIARDIASIS IN GROWING MICE

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Giardiasis in Growing Mice" submitted by Andre G. Buret in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Giardia is recognized as an important cause of diarrhea and malabsorption in man and other animals. Despite the high prevalence of this disease, the pathogenesis of such disturbances in gut function and the influence of the infection on food intake and growth remain unclear. A study was conducted to determine the effect of giardiasis on food intake, growth, small intestinal mucosal architecture, jejunal ultrastructure and brush border enzyme activity. Weanling mice were orogastrically inoculated with 1000 *Giardia muris* cysts suspended in saline. Course of infection was characterized by cyst output pattern and distribution of trophozoite population for a period of 65 days. Age and weight matched control and pair fed animals were given saline only. Growth and food intake were recorded for 50 days post inoculation. On day 0, 8, 30 and 50, segments of duodenum, jejunum and ileum were obtained and prepared for histopathological evaluation under. On day 0, 2, 8, 24 and 65, mucosal scrapings were obtained from duodenum, jejunum and distal small gut and assayed for lactase, sucrase and maltase activity. On day 8 of the infection, segments of jejunum were exteriorized, prepared for transmission electron microscopy and microvillous brush border height was assessed. The greatest numbers of trophozoites were found in the proximal small intestine although during the peak of infection (days 4 to 10) substantial numbers were present in

the distal small gut. Food intake of infected mice was intermittently reduced throughout the 50 days of study. Growth in infected mice was significantly retarded when compared to controls up to the 40th day of infection. Infected animals also exhibited a more severe growth retardation than those pair fed during the first 8 days of infection. At the peak of infection, giardiasis significantly reduced villus to crypt ratio in the duodenal and jejunal mucosae. In infected mice, sucrase, maltase and lactase deficiencies were observed in all areas of the small intestine, but most significantly in the duodenum and jejunum, 8 and 24 days post inoculation. Microvillous height was significantly decreased throughout the infected jejunum when compared to control and pair fed animals. Pair feeding also resulted in enzyme deficiencies and microvilli shortening, but to a lesser degree than infection. Such findings demonstrate that giardiasis impairs food intake and growth. Diffuse injury is inflicted to the small intestinal architecture and the jejunal brush border, and its extent corresponds to the where and when trophozoites are most numerous. The latter may explain our observation of generalized small intestinal enzyme deficiencies and these abnormalities may significantly contribute to production of malabsorption in giardiasis.

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1. INTRODUCTION

Giardia, a flagellated protozoan enteroparasite, has been described as early as 1681 by Van Leevenhoek who found it in his own stools (1,2). In 1859, Lambl defined the organism in detail and called it Cercomonas intestinalis (1,3). Early reports presented Giardia as a normal, non pathogenic microorganism, living in the human small intestine (3,4). An observation often cited in favour of this view was the frequent occurrence of this parasite in the absence of symptoms. In 1954 however, experiments on volunteer prisoners demonstrated that Giardia was an infectious parasite (5). It is only within the past two decades that this protozoan has come into prominence following the occurrence of localized outbreaks of epidemic proportions, the importation of the infection by travellers and the recognition of Giardia as a common parasite not only in the tropics but also in temperate zones (6,7,8). It became clear that giardiasis was a worldwide endemic infection and Giardia lamblia is now recognized as the most commonly confirmed etiologic agent of waterborne disease in the United States (6).It is estimated to occur in the gastrointestinal tract of up to 7.4 % of otherwise healthy Americans (9). Also, a recent study showed that in some areas of Peru, 100 % of the tested population are or have been infected (10).

Giardia organisms exist in two forms: the trophozoite and the cystic form. *In vitro* experiments have shown that excystation is triggered by brief (10 to 30 minutes) exposure of the cyst to acid followed by transfer to a solution similar in pH, temperature, and nutrient content to that of the duodenum (11). Trophozoites are the motile, feeding stage. They inhabit the small gut and attach themselves to the epithelium by means of their ventral adhesive disk or swim in the intestinal fluid (12). Cysts are thought to develop between the lower ileum and the upper colon and are eliminated with feces. Some trophozoites may be passed out in particularly fluid stools. The infection is transmitted by cysts but trophozoites also have the capacity to infect laboratory animals (13,14,15), thus raising the possibility that this mode of transmission may occur in nature. Fecal-oral and waterborne transmissions are the most common ways of transmitting *Giardia* infections (6,16,17). Food-borne (18,19) and sexual transmission (20,21) have also been documented.

The debate over *Giardia* speciation and interspecies transmission, especially between humans and other animals, has been intermittently active for more than 60 years. However, many studies based on cross-transmission experiments have now shown that *Giardia* of several host species is similar (22,23,24,25). This accumulating data refutes the hypothesis of rigid species specificity. Thus,

the high prevalence of human type giardiasis in domestic animals, and the close proximity in which man and these animals live, provides ample opportunity for zoonotic transmission of the infection.

1.1 HUMAN GIARDIASIS

In human giardiasis, the parasite is either eradicated naturally or cyst excretion may persist for periods of time which may exceed one year (26). The infection can be symptomatic or asymptomatic (5,16,27). Epidemics of giardiasis resulting in malabsorption and diarrhea can occur (16,27,28,29). Where the infection is common, the incidence appears to be highest in children (30,31). Reports on giardiasis in children with protein-calorie malnutrition (32,33) and in adults after gastrectomy (34) suggest that susceptibility to infection might be influenced by nutritional factors and changes in gastrointestinal physiology. These observations indicate that some of the differences between infection and disease are inherent not in the parasite but in the host. However, the reasons for the extreme individual variation in the response to the parasite remain unelucidated. Diarrhea and malabsorption in giardiasis may be caused by blanketing of the intestinal epithelium by the trophozoite population (35,36), secondary pancreatic insufficiency (37), competition for nutrients

between Giardia and host (35), production of toxins by the parasite (38), bile salts abnormalities due to the parasite itself (39) or to bacterial overgrowth associated with the infection (40,41), abnormalities in intestinal motility (42), and/or enzymatic and immunological deficiencies due to morphological and ultrastructural changes in the gut (43,44,45). Furthermore, two recent studies demonstrated binding of Cholera Toxin to Giardia muris (46) and Giardia lamblia (47), thus raising the possibility that Giardia may interact with toxigenic organisms. However, contradictory reports are numerous and the mechanisms whereby giardiasis causes malabsorption remain very poorly understood.

1.1.1. Course of infection

Because of the difficulties involved in repeated investigation on human giardiasis, information on the distribution of the parasite and the course of infection in man are rare. However, it is now accepted that, following a prepatent period of about 9 days, cysts will be excreted intermittently for a variable duration by infected subjects (5,48).

1.1.2. Histopathology

To this day, despite the fact that several authors

found trophozoites in various extraintestinal sites (49,50,51), mucosal invasion beyond the epithelial basement membrane has not been clearly demonstrated. The parasite adheres to the epithelial brush border and leaves typical circular marks on the mucosal surface after departure (52). A few studies report localized shortening and bending of the microvilli caused by penetration of the adhesive disk into the brush border (35,53,54,55). Villus atrophy and proliferation of acute (polymorphonuclear leukocytes and eosinophils) and chronic (intraepithelial lymphocytes) inflammatory cells have been demonstrated (56,57,58,59,60). While several authors have reported a correlation between the degree of malabsorption and the severity of villus abnormality, such pathology alone cannot explain malabsorptive giardiasis since patients with normal villus architecture can also show clinical signs of the infection (56,57).

1.1.3. Disaccharidase activity

Several studies reported jejunal sucrase, maltase and lactase deficiencies associated with human giardiasis (45,54,62). In most cases, these abnormalities occurred independently of any changes in villus architecture and reverted to normal after successful treatment. There is no information available on changes of duodenal and ileal

disaccharidases levels in human giardiasis.

1.1.4. Host response

High incidence of giardiasis has been reported in patients with immunodeficiency syndromes (63,64,65,66). Also, it was observed that infected subjects became resistant to subsequent infection (5,67). Such reports demonstrate the importance of host immune response in the defense against giardiasis. The synthesis of such studies and others suggest that the early immune response to the infection is associated with an increase in IgM and IgE production in the small intestinal mucosa, followed by IgA and IgG synthesis (66,68,69). Serological studies also demonstrated a production of specific anti-*Giardia* IgG antibodies in symptomatic subjects (70,71) although serum immunoglobulin levels in individuals with active infections seem to be within normal limits (72). Intraepithelial lymphocyte counts were found to be high during the infection and low after treatment. It has also been demonstrated that human peripheral blood monocytes-macrophages are spontaneously cytotoxic for *Giardia lamblia* (73). From all these findings it is tempting to conclude that chronic infection may result from immunodeficiencies in the host and that part of the host-immune response may be cell-mediated.

Giardia most commonly infects children in endemic

areas. Interestingly however, the prevalence seems to be lower during the first six months of life (74). It has been recently suggested that this may result from the protective effect of breast milk (75), an effect mediated by lipolysis of milk lipids (76,77). Finally, recent findings report activation of a parasite lectin by a host protease (78). This novel mechanism may contribute to the affinity of Giardia lamblia to the infection site and therefore help us in our understanding of the host-parasite interaction.

1.2 MURINE GIARDIASIS

Giardia organisms are not specific to man. They are found worldwide in all classes of vertebrates, in both tropical and temperate climates (30). Two well documented animal models can now reliably be experimented on: the murine model using laboratory mice infected with Giardia muris (79) and the Mongolian gerbil model infected with Giardia lamblia (13). The use of such models allows pathophysiological study of the disease under controlled conditions not possible in humans, and therefore greatly facilitates research on giardiasis.

1.2.1 Course of infection

Oral inoculation of Giardia muris cysts into Swiss

albino mice results in proliferation of trophozoites in the small gut and excretion of cysts in stools (79). When infected with 1000 cysts, adult mice demonstrate a pattern of cyst excretion which peaks during the second week of infection and cysts become undetectable in most animals 4 weeks post inoculation (48,79). Total numbers of trophozoites show a parallel pattern (48,79). However, the small gut still harbors a substantial population of trophozoites 6 weeks post-inoculation, a time when cysts are no longer detected in feces of most mice (48). Studies using electron microscopy (12) and various counting techniques (43,48) demonstrated that the trophozoite population was largest in the proximal jejunum throughout the course of infection.

1.2.2. Histopathology

At the peak of an infection with Giardia muris, mice demonstrate a significantly reduced villus to crypt ratio in the upper jejunum (79). This decrease results from the combined effects of villus atrophy and crypt hyperplasia observed at that time (48). At a later stage of a primary infection (8 and 10 weeks post-inoculation), jejunal villi were longer in infected than in control animals, whereas both villi and crypts were shorter in infected ileum (48). An increase of intraepithelial lymphocytes was demonstrated

in infected mice six weeks post inoculation and their number remained significantly high thereafter (43,48,80). In chronic giardiasis, jejunal crypt cell production rate rose in infected animals and reached a peak three weeks post-inoculation (80). Interestingly, this rate decreased in infected ileum (48). However, studies of epithelial cell kinetics during a primary infection were not able to confirm any increase in crypt cell production associated with the infection (48). Since Sha Sha mice, used in the study of chronic giardiasis, are also chronically infected with Hexamita muris (101), the effect of giardiasis alone on crypt cell proliferation deserves to be clarified. As in research on human giardiasis, most studies using the murine model focus on the jejunum and no information is available on duodenal mucosal injury associated with the infection.

Another interesting finding is the increase in mucosal damage in infected mice fed an elementary diet (protein only) when compared to mice on a carbohydrate-rich diet (43,81). This demonstrates the importance of nutritional factors in giardiasis. Trophozoite adhesion marks on the mucosal surface were also demonstrated in the murine intestine (12,52), and the observation of overlapping adhesion marks indicated that Giardia can release and re-attach, an event which may be triggered by epithelial cell migration. Giardia can penetrate the epithelium at point of damage or cell desquamation (12,82), but trophozoites have

only been observed between cells in the epithelium, never within columnar cells. When Giardia-infected mice were supra-infected with Plasmodium berghei, trophozoites were also found in organs other than the intestine (liver, heart, brain) (83). However, the infrequency of such entry implies that the flagellate, so well adapted for intraluminal existence, took advantage of a weakened epithelial barrier, and was lost rather than invading.

1.2.3. Disaccharidases activity

Decrease in jejunal lactase, sucrase and maltase activity has been observed in adult mice two weeks after being infected with Giardia muris (48,80). Jejunal enzyme contents reached supra-normal levels by the sixth week of infection, a time when lactase and sucrase activity was significantly reduced in the ileum (43,48,80). It has been suggested that high numbers of trophozoites may damage the mucosal surface and thus provoke a reduction in disaccharidase activity (80). But the observation of localized shortening and bending of microvilli alone (12,52) does not adequately explain such deficiency, and the exact nature of brush border injury remains to be determined.

1.2.4. Host response

Prior infection with Giardia muris results in resistance to subsequent challenge with the same organism (12,84). During clearance of the parasite in murine giardiasis, lymphocytes can cross the epithelium and attach to Giardia in the lumen (12). This direct interaction of lymphocytes and trophozoites may contribute to elimination of the protozoans. While damage to the parasite by these encounters has not yet been reported, such observation could suggest that recognition of microvillous or similar attachment receptors may be prevented by this cellular interaction, hence facilitating clearance of the infection. As mentioned above, cellular immune response in murine giardiasis was further supported by the demonstration of increased intraepithelial lymphocyte counts (both in jejunum and ileum) six weeks post-inoculation, a time when parasite numbers are falling (48). However, it has been suggested that clearance of Giardia muris from the mouse intestine was not mediated by natural killer cells in an experiment where both immunocompetent mice and beige mice (which are deficient in natural killer cells) cleared infection at similar rates (85). The thymus dependent nature of the immune response to Giardia in mice has been demonstrated by infection studies in nude mice (86). These T-cell deficient animals maintained a persistent infection and failed to

acquire resistance to further challenge demonstrating the importance of cell-mediated immunity in giardiasis. Also, in athymic mice, macrophages have been shown to extend pseudopods to phagocytose invading trophozoites (82), thus counterbalancing the lymphocyte deficiency of the host. It should be noted however that activated macrophages in this model did not imply increased resistance to giardiasis. Studies of the antibody response to Giardia muris in the mouse seem to indicate that the host response in murine giardiasis involves mainly secretory and serum IgA, some serum IgG but no IgM activity (87,88). Another aspect of research in murine giardiasis was the discovery that mice ingesting milk from immune mothers were protected against infection with Giardia muris (89).

1.3. GENERAL CONSIDERATIONS

From our review, we conclude that most research dealing with pathogenesis and malabsorption of giardiasis in man and in the murine model has been limited to the study of the jejunum in adult animals. Also, while it has been demonstrated in a few studies that giardiasis can cause weight loss, the effect of this disease on food intake and growth remains to be assessed. The variability in pathological signs associated with giardiasis suggest that the pathogenesis of this disease is multifactorial. The

extent of such insults to all areas of the small intestine and their evolution during the course of the infection need to be described for a better understanding of how and when giardiasis can cause malabsorption.

1.4. OBJECTIVES

In order to examine in detail the relationships between host food intake and growth, time course of infection, number of parasites in the small gut and various intestinal pathological features, the effect of a primary Giardia muris infection in growing mice was studied. The objectives of this research were:

1. To describe the course of a Giardia muris infection over a period of 65 days in a model of weanling Swiss Webster mice by assessing the patterns of the following criteria:

   a) Cyst release
   b) Total trophozoite population in the small intestine
   c) Trophozoite population in the duodenum
   d) Trophozoite population in the jejunum
   e) Trophozoite population in the distal small gut

2. To assess whether giardiasis can result in growth retardation and changes in food intake. Experimentation will include three groups of animals: Control, infected and,

since protein-calorie malnutrition alone can impair intestinal absorption (90,91), a group of pair fed animals.

3. To describe chronologically factors in the pathogenesis of malabsorption during the course of infection by investigating:

a) Histopathological changes of the small intestine under light microscopy. Alterations in villus height, crypt depth and crypt-villus ratio will be assessed for the duodenum, jejunum and ileum, 0, 8, 30 and 50 days post-inoculation.

b) Ultrastructural changes in the jejunal epithelium at the peak of the infection.

c) Changes in the activity of three disaccharidases (lactase, maltase and sucrase) in the duodenum, jejunum and distal small intestine, 0, 2, 8, 24 and 65 days after inoculation.

2. METHODS AND MATERIAL

2.1. ANIMAL MODEL

The animal model described by Roberts-Thomson *et al* (79) was slightly modified for the present study. Weanling male Swiss Webster mice (CLR:(SW)BR), weighing between 20 and 24 grams (X=22.1) were used in all experiments. The animals were protozoan free and free of any previous protozoan infection. Examination of feces and duodenal scrapings of five randomly selected mice were undertaken before any experimentation. Animals were kept individually in suspended metabolic cages, given water *ad libitum* and fed commercial rodent chow (Rodent Blox, Wayne Pet Food Div., Chicago, ILL.). Room environment was maintained at 22° C and 40% relative humidity, with 12-hour photoperiods. Maintenance of animals and experimental procedures were carried out in accordance with the guidelines of the Canadian Council of Animal Care.

2.2. GIARDIA MURIS INFECTION

Experimental animals were orogastrically infected with *Giardia muris* (originally obtained from Roberts-Thomson). Inocula contained 1000 cysts suspended in 0.1 ml. of normal saline and were administered with an 18 gauge blunt needle.

Cysts were inoculated into experimental animals within one hour of isolation. The Giardia strain was maintained in protozoan free mice reinoculated weekly and kept in cages equipped with filter tops.

2.3. EXTRACTION AND QUANTITATION OF CYSTS

Cysts were isolated from a two hour stool collection by sucrose flotation as described by Roberts-Thomson et al (79) with a few modifications: Fecal suspensions were layered over 5 ml. of 1M sucrose with a specific gravity of 1.13 g/mm^3. In order to locate the sucrose-supernatant interface more easily, 0.5 ml. of 0.5% trypan blue in 0.85% saline was added to 500 ml. of the sucrose solution. Samples were centrifuged at 800 g for 5 minutes. Isolated cysts were resuspended in 1 ml. of normal saline, and counted with a hemocytometer. Cyst output was calculated every second day for 50 days and expressed in log 10 No. per gram of feces. Eleven animals were studied for this part of the study.

2.4. EXTRACTION AND QUANTITATION OF TROPHOZOITES

Infected mice (n=35) were killed by cervical dislocation at intervals until 60 days post inoculation. The entire small intestine was carefully removed and divided in three segments: 1) duodenum, between the pylorus and the

ligament of Treitz, 2) jejunum, the proximal half of the remaining small gut and 3) distal, the distal half of the remaining small intestine. Each segment was slit longitudinally and placed into 4 ml. of PBS pH 7.2. After incubation for 2 hours at 37° C in a shaking incubator (120 RPM), trophozoites were counted with a hemocytometer. This technique allowed us to assess the total number of trophozoites in the small intestine as well as the population of parasites in each segment studied. Total numbers of trophozoites were expressed in log 10 and separate populations from each segment in log 10 No. per centimetre of gut.

2.5. FOOD INTAKE AND WEIGHT GAIN

Weight gain and food intake were assessed for all animals once every second day for 50 days. Weight gain was calculated as a percentage of the starting weight (100%). In an initial part of our study, a group of control (n=10), pair-fed (n=11) and infected (n=11) animals were observed for 30 days. Thereafter, food intake and weight gain were measured for 50 days in another 10 control, 10 pair fed and 10 infected animals. Additional weight gain data were obtained for each group from animals used later in research.

2.6. HISTOLOGY

At 0, 8, 30 and 50 days post inoculation, animals were killed by cervical dislocation. Immediately thereafter, gut specimens were taken from the duodenum (3 cm. distal to the pylorus), jejunum (15 cm. distal to the pylorus) and ileum (3 cm. proximal to the ileo-cecal junction). Specimens were fixed in Carnoy's fixative (92), dehydrated in ethanol, infiltrated and embedded in plastic JB-4 medium (Polysciences Inc., Warrington, PA. 18976). 1.5 um sections were stained with Lee's methylene blue and basic fuchsin (93) and histopathology was evaluated under a Wild (Heerbrugg, Switzerland) light microscope. Crypts and villi were measured from 10 crypt-villus units in each specimen. Crypt-villus ratio was calculated from each observation. The number of animals studied was distributed as follows: 3 control animals on day 0, 5C, 5PF and 5I on day 8, 6C, 6PF and 6 I on day 30 and 10C, 5PF and 5I on day 50.

2.7. TRANSMISSION ELECTRON MICROSCOPY

Eight days post inoculation, mice (3C, 3PF, 3I) were killed by cervical dislocation and a segment from the jejunum was immediately removed and immersed into 5% gluteraldehyde in 0.1 M cacodylic buffer, pH 7.2, osmolality 300 mOsmol at 20° C. Specimens for electron microscopy were

cut into 1 mm cubes while immersed in the original fixative at 4° C and stored in the fixative overnight. Specimens were then washed in cacodylic buffer, postfixed in 1% OsO_4 , dehydrated in distilled acetone, cleared with propylene oxide, and finally infiltrated and embedded in Spurr's low viscosity medium (J.B. EM Services Inc., Dorval, Quebec, CDN). Sections (0.25 um) were obtained and stained with basic toluidine blue for light microscopy to select areas for thin sectioning for electron microscopy. Thin sections were stained with saturated uranyl acetate in 50% ethanol followed by 0.4% lead citrate (94) and examined at 50 KV on a Hitachi H600 transmission electron microscope. Three to five tissue blocks from control, pair fed and infected jejunum were prepared and 10 to 15 sections from each block were examined, representing ultrastructural assessment of 90 to 200 villus-crypt units. Micrographs of the microvillous brush border (12000 X) were obtained from three villous areas (upper, mid and low).

2.8. MUCOSAL ENZYME ACTIVITIES

At 0, 2, 8, 24 and 65 days post inoculation, infected (n=5), pair-fed (n=5) and control mice (n=5) were killed by cervical dislocation. Mucosa from duodenum, jejunum and distal small intestine was scraped with a microscope slide, homogenized in 2.5 mM EDTA (10% weight/volume), frozen in

liquid nitrogen and stored in a -70° C freezer until assayed for lactase, sucrase and maltase activity. A glucose-oxidase reagent was used for assay of the glucose liberated from the substrate according to the method of Dahlqvist (95). Protein contents from the substrate were measured as described by Lowry *et al.* (96) and disaccharidase activity was expressed as units per gram of protein.

2.9. STATISTICAL ANALYSIS

All data were compared using Student's *t* test and one-way analysis of variance (ANOVA) followed by a multiple comparison test (Student-Newman-Keuls-test). Probability level of $P < 0.05$ was considered significant.

3. RESULTS

3.1. COURSE OF INFECTION

Throughout our study, all (100%) experimental mice inoculated with 1000 cysts of Giardia muris (n=100) produced infection, as demonstrated by high numbers of trophozoites in the small intestine (fig.1) and the release of cysts in the feces (fig.2). None of the infected animals died as a result of infection and none exhibited abnormal stool texture. While high numbers of trophozoites could already be found in the small gut 2 days post inoculation, cysts were first detected in feces on day 4 (fig.3). Cysts were then released for 30 to 46 days and their number became undetectable thereafter, despite the presence of trophozoites in the small intestine throughout the 65 days of observation (fig.3). Peak cyst output was observed between day 6 and day 16 and was highest at day 10 with a mean number of cysts per gram of feces of 1.6×10^6. Highest trophozoite population was observed between day 4 and 16, with a peak at day 6 when the small intestine contained an average of 1.8×10^8 trophozoites. Starting at day 20, intermittent cyst release was observed in some animals. The greatest number of trophozoites was found in the proximal small intestine although substantial numbers were present in the distal small gut between day 4 and 10 (fig.4). A peak in

the trophozoite population was found between day 4 and 10 of the infection in all areas of the small intestine (fig.4). Trophozoite levels in specific areas of the small gut were also documented: in the duodenum of some animals, trophozoites were undetectable very late (day 50 and 60) and very early (day 2) in the infection. In the ileum however, trophozoites were undetectable on day 2 in some animals, but were always present thereafter. In the jejunum, trophozoites were found throughout the time of study.

3.2. FOOD INTAKE

Infected animals intermittently showed decreased food intake throughout the time of study (fig.5). The most significant decrease was seen between day 2 and 14 of the infection. After day 26, infection resulted in decreased food intake on day 36 and 44 only. The number of animals studied in each group varied between 10 and 30 at any time during the study.

3.3 WEIGHT GAIN

Weight gain in infected animals when compared to controls was significantly reduced up to the 40th day of infection (fig.6). Infected mice resumed normal growth thereafter. Differences in growth between control and

infected appeared greatest between day 2 and 10 of the infection (fig.6). Observation of weight gain in a group of pair fed animals allowed us to assess the effect of malnutrition associated with giardiasis. Infected mice showed a more severe growth retardation than malnourished pair fed mice during the 8 first days of infection (fig.7). On day 12 and thereafter, infected mice gained significantly more weight than pair-fed animals (fig.7). By day 50, animals from all groups exhibited similar weight gain (fig.7). The number of animals studied in each group varied between 10 and 46 at any time of the study.

3.4. HISTOPATHOLOGY

Gross inspection of the entire intestine of infected mice revealed no pathology. Numerous trophozoites and shortened mucosal villi were observed in histologic sections of infected animals. Mucosal architecture was measured and compared from 3 areas of the small gut in 24 control, 16 pair fed and 16 infected mice. Villus height in pair fed and infected animals was altered, mostly in the duodenum and jejunum (fig.8). Alterations in crypt depth, although present, were less dramatic (fig.8). Features of small intestinal histopathology in primary *Giardia muris* infection can be summarized as follows:

Duodenum: Malnutrition did not alter duodenal villus

height in pair fed animals (fig.8). In infected animals however, duodenal villi were significantly shorter than in control mice throughout the 50 days of study (fig.8). When compared to pair fed animals, this reduction in villus height was significant at day 8 and 30 (fig.8). On day 8, duodenal villus atrophy in infected animals was most apparent (fig.8) yet no difference in crypt depth could be found between all three groups of study (fig.8). On day 30, pair feeding resulted in crypt shortening, and on day 50 in crypt hyperplasia when compared to control animals (fig.8). On day 30, crypt hyperplasia was also observed in infected mice when compared to pair fed animals (fig.8). Infected duodenal mucosa exhibited a significantly lower villus to crypt ratio than control mucosa throughout the 50 days of study (fig.9). Eight and thirty days post inoculation, villus to crypt ratio from infected duodenum was also lower than in pair fed animals (fig.9). When compared to control mice, duodenal villus to crypt ratio in pair fed animals was similar on day 8, significantly higher on day 30 and lower on day 50 (fig.9).

Jejunum: Eight days post inoculation, both infected and pair fed groups exhibited similar jejunal villus atrophy when compared to control villi (fig.8). On day 30, villus height in infected and control jejunum was similar, while pair feeding resulted in a significant increase in villus height (fig.8). On day 50, jejunal villi were shorter in

infected than in control and pair fed animals (fig.8). On day 8, jejunal crypt depth was similar in control and infected animals, while crypts were significantly shallower in pair fed mice (fig.8). On day 30, both pair fed and infected groups exhibited a significant decrease in jejunal crypt depth (fig.8). On day 50, infected animals demonstrated jejunal crypt hyperplasia when compared both to control and pair fed mice (fig.8). On day 8 and 50, jejunal villus to crypt ratios were significantly lower in infected mice than in control and pair fed animals (fig.9). On day 50, pair feeding resulted in an increased jejunal villus to crypt ratio when compared to control mucosa, while no difference was observed between these two groups on day 8 (fig.9). On day 30, while control and infected jejunum exhibited similar villus to crypt ratios, values were significantly higher for pair fed animals (fig.9).

Ileum: In this study, ileal villus height was only altered 50 days post inoculation, and the alteration consisted of a significant villus lengthening in pair fed and infected mice when compared to control animals (fig.8). On day 8 and 30, crypts were markedly shallower in infected ileum than in control and pair fed animals (fig.8). On day 30, a decrease was also observed in pair fed mice when compared to control animals (fig.8). On day 50, pair fed animals exhibited ileal crypt hyperplasia when compared to control and infected mice (fig.8). On day 8 and 30, ileal

villus to crypt ratios in infected mice were significantly higher than in control and pair fed mice (fig.9). On day 30, this ratio was also increased in pair fed animals when compared to control mice (fig.9). Ileal villus to crypt ratios were similar in all three groups on day 50 (fig.9).

3.5. DISACCHARIDASES

Mucosal enzyme activities are illustrated in figures 10, 11 and 12 and their alterations in giardiasis can be summarized as follows:

On day 2, sucrase activity in the distal small intestine of pair fed and infected animals was depressed when compared to control; maltase showed similar results but differences observed did not reach significance (fig.10 and 11). On day 2, jejunal and distal lactase levels were lower in infected animals than in control (fig.12). At the same time, impaired lactase activity could also be observed in the distal small intestine of pair fed animals when compared to control (fig.12). The results found for lactase activity in the duodenum on day 2 and throughout the small gut on day 24 showed no differences between control and infected animals, but a significant increase in activity in pair fed mice (fig.12). The latter findings are similar to the results obtained for sucrase and maltase (fig.10, 11, 12).

On day 8 of the infection, infected mucosa exhibited

deficient sucrase and maltase activity when compared to control throughout the small intestine while pair fed animals showed depressed sucrase and maltase activity in the jejunum and distal small gut only (fig.10, 11). At this time, infected animals also had lower sucrase and maltase activity levels than pair fed mice in the duodenum and jejunum (fig.10, 11). On day 8, changes in lactase activity could be observed in the jejunum only where this enzyme was depressed in infected animals when compared both to control and pair fed groups (fig.12).

On day 24, lactase, maltase and sucrase activities were identical in control and infected animals throughout the small gut, while pair feeding resulted in a significant increase of activity of all enzymes (although this increase did not reach significance in jejunal lactase) (fig.10, 11, 12).

On day 65, all enzyme activity levels were similar throughout the small gut of infected and control animals (fig.10, 11, 12).

3.6. JEJUNAL ULTRASTRUCTURE

Control and infected jejunal epithelia are shown in figure 13. In control mice, columnar absorptive cells typically exhibited a dense and even microvillous brush border, numerous mitochondria above and below the nucleus

and dense cytoplasm containing few lysosomes (fig.13). Abnormal ultrastructure in infected jejunum was characterized by occasional shortening of microvilli where the edges of a trophozoite's adhesive disk penetrated the brush border (fig.14), and, more significantly, swelling and destruction of mitochondria combined with proliferation of cytoplasmic lysosomes (fig.13). Other pathological signs (intercellular spacing, interruption of basement membrane, shortening of the columnar cells, enterocyte desquamation, etc.) could not be found and general appearance of infected epithelial cells was similar to that of a mature columnar enterocyte. Malnutrition in pair fed animals resulted in no apparent injury of the epithelium (with the exception of microvilli shortening as we will demonstrate further). A major finding of this study was that infection resulted in significant shortening of the microvillous brush border when compared to control and pair fed epithelia (fig.14). This diffuse injury was observed throughout the jejunum and in all villous regions although the mid-villus seemed to be the most severely affected (fig.14). There was a similar reduction in pair fed when compared to control, but at a lesser degree than in infected (fig.14).

4. DISCUSSION

This study correlates food intake, growth, histopathological, enzymatic and ultrastructural alterations with the course of a primary Giardia muris infection in growing mice. Weaned Swiss Webster mice infected with 1000 Giardia muris cysts developed a consistent and reproducible infection. Highest trophozoite populations and largest cyst outputs occurred between day 2 and 12 indicating a peak in infection during this period. The greatest numbers of trophozoites were found in the proximal small intestine although during the peak of infection substantial numbers were present in the distal small gut. Cyst output became undetectable in some animals 20 days post inoculation. Such findings are similar to those previously described in an animal model using adult mice (48,79). In these studies however, some animals harbored few or no trophozoites 8 weeks post inoculation (48). Despite an identical inoculum size (1000 cysts), in our model trophozoites were found throughout the time of study, even 65 days post inoculation. Whether this difference is related to counting techniques or to different defense mechanisms in young and adult animals has not been determined.

In the present study, we showed that giardiasis resulted in decreased food intake. It has been previously demonstrated that protein-calorie malnutrition alone causes

intestinal mucosal atrophy and alterations in brush border enzyme activities (90,91). Another study (81) showed that a low protein diet increased mucosal damage during parasitic infection suggesting that malnutrition can alter the host-parasite relationship to the detriment of the host. More recently, in a model of bacterial enteritis, it was found that part of the pathogenesis of Yersinia enterocolitica infection in rabbits was caused by reduced food intake associated with the disease (97). Therefore, in order to assess separately the pathological effects of malnutrition and infection in giardiasis, a pair fed control group was examined. The reduced food intake of pair fed animals during the first 8 days of experimentation resulted in retarded growth, but at a lesser degree than in infected animals. This suggests that impaired weight gain in infected animals is caused partially by malnutrition. As we will demonstrate further, malabsorption may account for the more severe growth retardation in infected animals. Interestingly, infected animals resumed normal growth quicker than pair fed animals did, via a mechanism which remains to be explained.

Giardiasis altered mucosal architecture, with the most obvious injury consisting of moderate villus shortening. Villus height and villus to crypt ratio were most significantly decreased in the duodenum, where trophozoites were most numerous. Interestingly, after about 4 weeks of infection, mucosal architecture in the jejunum returned to

normal in infected animals, while malnourished pair fed exhibited increased villus to crypt ratios throughout the small gut. Combined with such alterations in villus architecture, an increase in mucosal enzyme activity was also observed: On day 24, values for control and infected animals were identical while pair fed enzyme activities exhibited an increase throughout the small gut. These original findings suggest that later in infection, the malnourished animal tries to overcompensate for its decreased food intake and that infection actually prevents this adaptive mechanism. Therefore, although values for control and infected animals are identical at that time, we can postulate that giardiasis causes intestinal injury and mucosal enzyme deficiencies not only during the peak of the infection, but also up to about 4 weeks post inoculation. Some pathological aspects in giardiasis, including jejunal and duodenal villus atrophy, crypt hyperplasia and decreased villus to crypt ratios can still be observed 50 days post inoculation. Alterations of small bowel architecture caused by giardiasis were reversed in the ileum: ileal villous height was above normal on day 50, crypts were shallower on day 8 and 30, and villus to crypt ratios were supranormal on day 8 and 30. These observations indicate that the effects of the infection are largely confined to the upper small intestine, where trophozoites are most numerous, and this also demonstrates the remarkable adaptive potential of the

ileum.

Disaccharidase deficiencies were found in infected animals on days 2 to 24 of the infection. The extent of this damage cannot be explained by mucosal injury alone. The fact that villus height at the peak of the infection was not altered in infected jejunum when compared to pair fed, despite a simultaneous marked decrease in maltase and sucrase activities, further suggests that villus loss alone cannot account for the entire extent of disaccharidase deficiency. Furthermore, the spectacular increase in duodenal maltase and sucrase activity observed on day 24 in pair fed animals was not parallelled by villus hypertrophy. Clearly, another mechanism is involved in mucosal enzyme impairment associated with giardiasis. Migration of immature crypt-like enterocytes along the villi seems to explain disaccharidases impairment in viral enteritis (98). However, studies of epithelial cell kinetics during a primary Giardia muris infection (48) did not demonstrate any significant increase in crypt cell proliferation, thus ruling out the possibility that changes in disaccharidases during this infection would reflect the presence of an immature enterocyte population. As demonstrated in the present study, mature ultrastructure of epithelial cells in infected mucosa also tends to exclude the possibility of delayed cell maturation in this disease.

An alternate explanation for the mechanism of brush

border enzyme impairment could be direct damage to the microvilli by the parasite, a phenomenon that has been described in bacterial overgrowth (99,100) and bacterial enteritis (105). The only reported microvillous brush border injury associated with giardiasis was shortening and bending of microvilli where the edges of the trophozoite's adhesive disk penetrated the brush border (35,52,53,54,55). Alterations of the fuzzy coat covering the microvilli in giardiasis have also been described (44,55). Further investigation showed that the altered appearance of the brush border probably recovers with time (102). Such temporary deformities cannot reasonably explain the marked diffuse disaccharidase deficiencies observed in giardiasis. Therefore it was decided to examine, at same magnification, the jejunal microvilli of control, pair fed and infected animals during the peak of infection. At that time, jejunal maltase and sucrase of pair fed mice exhibited a marked decrease in activity when compared to controls. Values for lactase followed a similar pattern but failed to reach significance. In infected animals, all three enzymes were significantly further impaired. Ultrastructural observation of the jejunal mucosa revealed that the infected epithelium contained a high concentration of swollen and destroyed mitochondria combined with increased numbers of cytoplasmic lysosomes, which are clear signs of enterocyte injury (103). More significantly, microvillus height was decreased along

the entire villus when compared to control and pair fed animals. This diffuse injury could also be observed, but to a lesser extent, in pair fed. Thus, the loss of brush border surface area during the peak of infection can be directly related to the observed disaccharidase deficiencies and this mechanism is likely an important cause of malabsorption in giardiasis. It should be noted that the observed mitochondria damage did not result from poor fixation since normal healthy looking mitochondria could be found along the same epithelium where others were swollen or destroyed. Furthermore, care was taken to process all control, pair fed and infected tissues simultaneously until embedding.

While all injuries described above were diffuse and present throughout the gut, many seemed most obvious in areas where trophozoite population was highest. Direct effect of trophozoites in the pathogenesis of giardiasis can therefore be suggested. Whether this results from release of a toxin, presence of pathogenic endosymbionts within the Giardia organisms or other mechanisms remains to be determined.

The present study demonstrates that intestinal parasitic infection results in a similar response in the gut to that caused by protein-calorie malnutrition (90,91), bacterial enteritis (97,105) or bacterial overgrowth syndrome (99,100,104). This consistent response to intestinal insult involves at least decreased villus to

crypt ratio and brush border enzymes deficiencies caused by diffuse shortening of the microvilli. Other characteristic responses of the gut to various malabsorptive and diarrheal disorders deserve further investigation in order to improve our understanding of small bowel physiology in health and in disease.

5. CONCLUSION

When growing mice are inoculated with 1000 cysts, primary Giardia muris infection is characterized by a peak in cyst output and trophozoite numbers between day 2 and 12. Highest trophozoite population is found in the upper third of the small intestine. Infection results in decreased food intake and retarded growth and these alterations are most significant during the peak of the infection. At that time, lack of weight gain results from the combined effects of malnutrition and infection induced malabsorption. At the peak of the infection, malnutrition impairs brush border enzyme activity by shortening the epithelial microvilli along the entire jejunal epithelium. Injury becomes worse with infection and intestinal absorption is further impaired by markedly decreased villus to crypt ratios in the duodenal and jejunal mucosae. Later in the infection, enterocytes attempt to compensate for decreased food intake by increasing mucosal surface area in the jejunum and superactivating brush border enzymes throughout the small gut. But presence of Giardia infection prevents such an adaptive mechanism. Infection induced pathology is confined mostly to the upper small gut, where trophozoites are most numerous. Such observations indicate that trophozoites play a direct role in the pathogenesis of giardiasis. We conclude that infection and presence of numerous trophozoites, to a

greater extent than malnutrition, impairs small intestinal absorption in giardiasis by markedly decreasing mucosal and brush border surface areas, hence causing diffuse disaccharidase deficiencies, and that these mechanisms in part explain retarded growth associated with the disease.

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Figure 1: Light micrograph from infected duodenum demonstrating high numbers of Giardia muris trophozoites (arrows) in the intestinal lumen and on the surface epithelium 8 days post inoculation. (Methylene blue and basic fuchsin, 500 X, bar = 50 μm.)

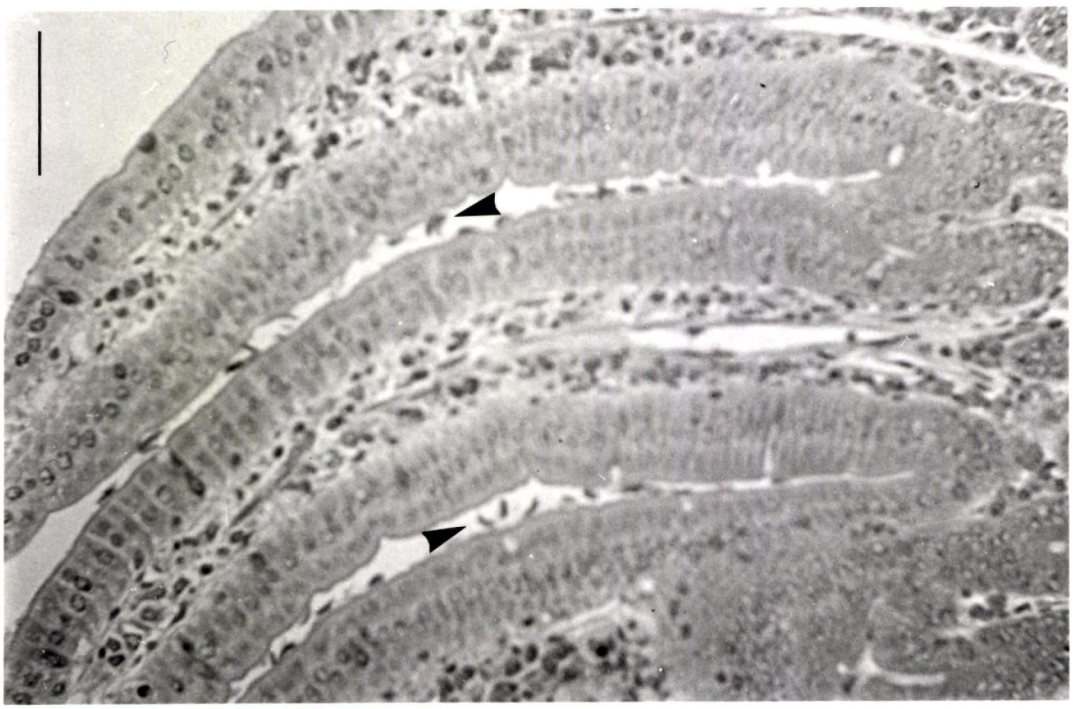


Figure 2: *Giardia muris* cyst as observed when isolated from murine feces by sucrose flotation (4600 X, bar = 10 µm.).

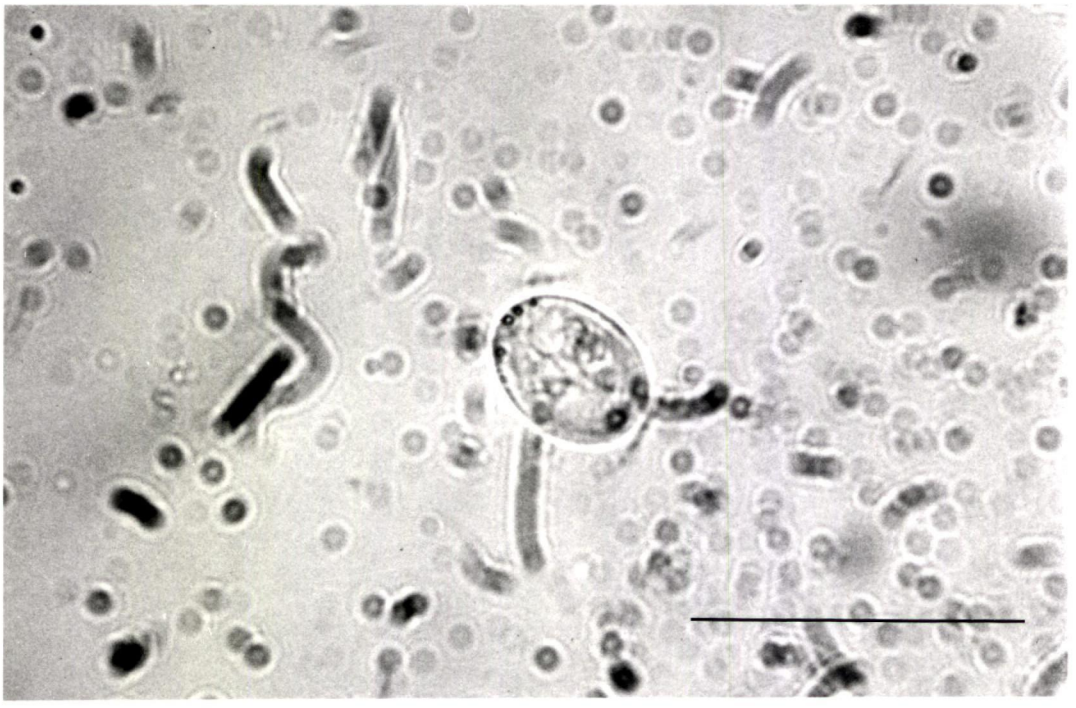


Figure 3: Time course of infection in mice orogastrically inoculated with 1000 *Giardia muris* cysts as measured by fecal cyst counts (log 10 No. / gm. feces) and total numbers of trophozoites (log 10 No.) in the small intestine. Values are mean ± SE.

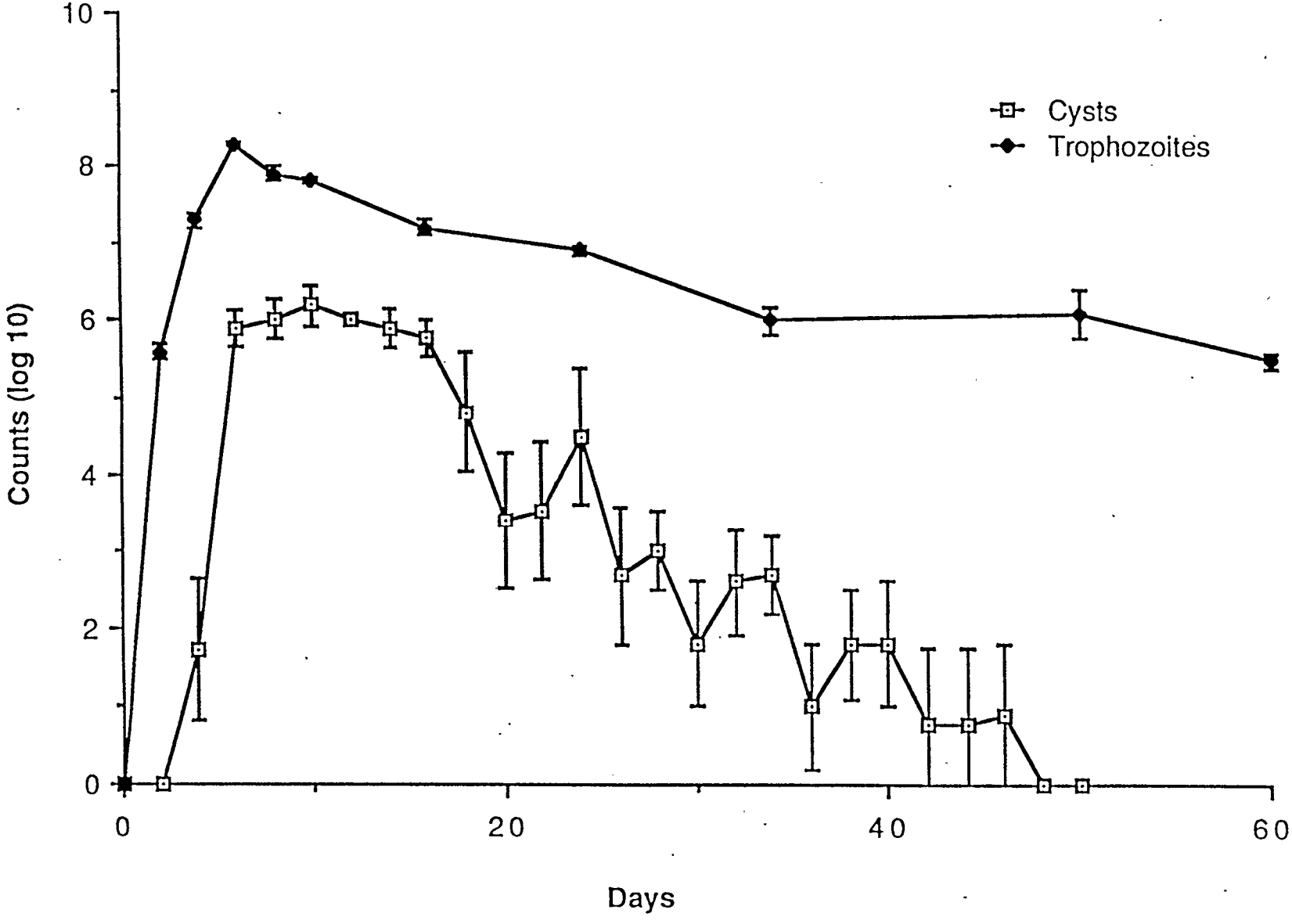

Cysts
Trophozoites
Counts (log 10)
10
8
6
4
2
0
0
20
40
60
Days

Figure 4: Sequential distribution of the trophozoite population along the small intestine of mice infected with 1000 *Giardia muris* cysts. Values are mean from 3 to 5 animals for each data point and are expressed as log 10 No. per cm. of gut.

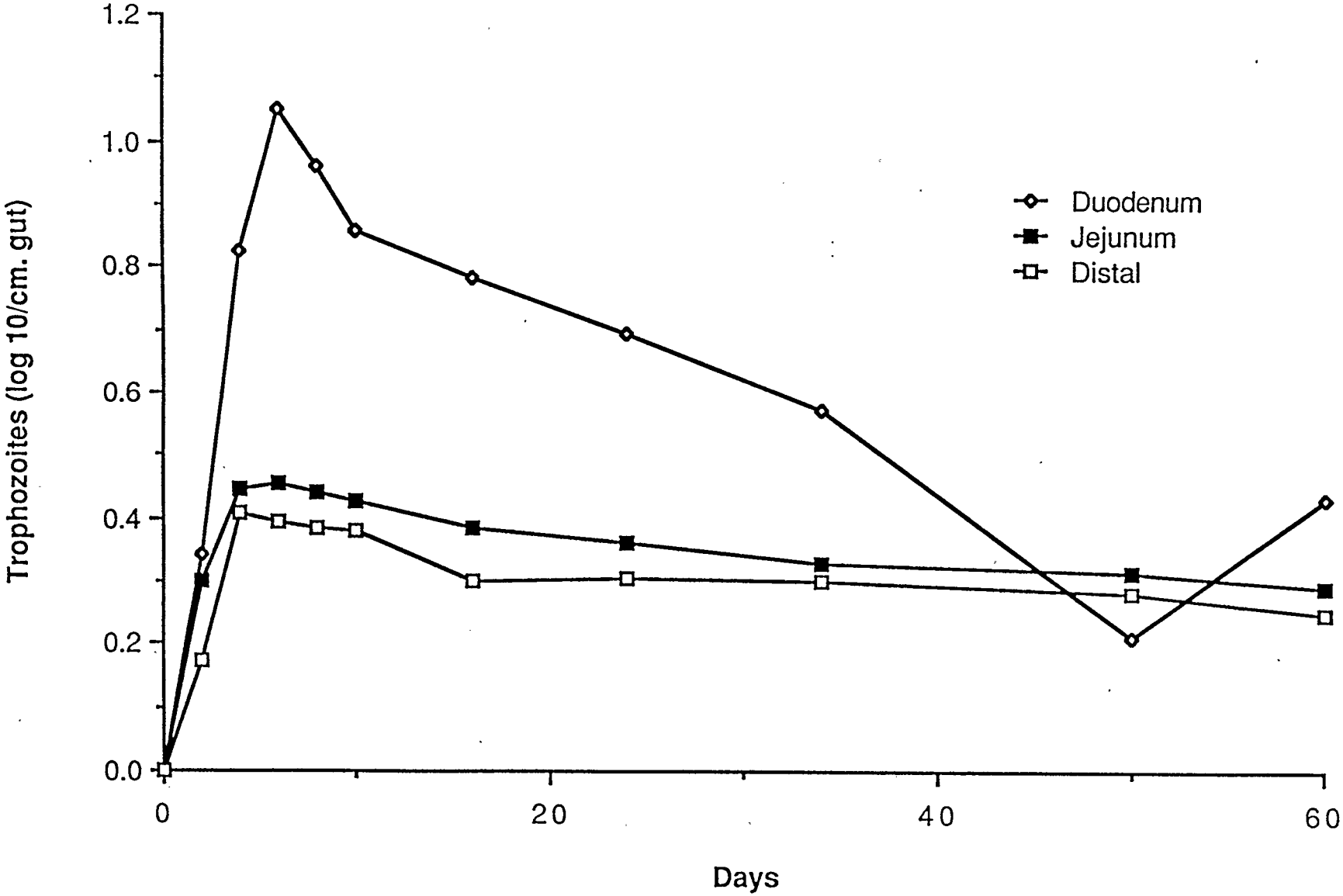

Trophozoites (log 10/cm. gut)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
Days
Duodenum
Jejunum
Distal

Figure 5: Food intake in control mice and in mice infected with 1000 *Giardia muris* cysts. Values are mean ± SE. ▲ $P < 0.05$ compared to control as analyzed by Student's *t* test and one-way analysis of variances (ANOVA) followed by Student-Newman-Keuls multiple comparison test.

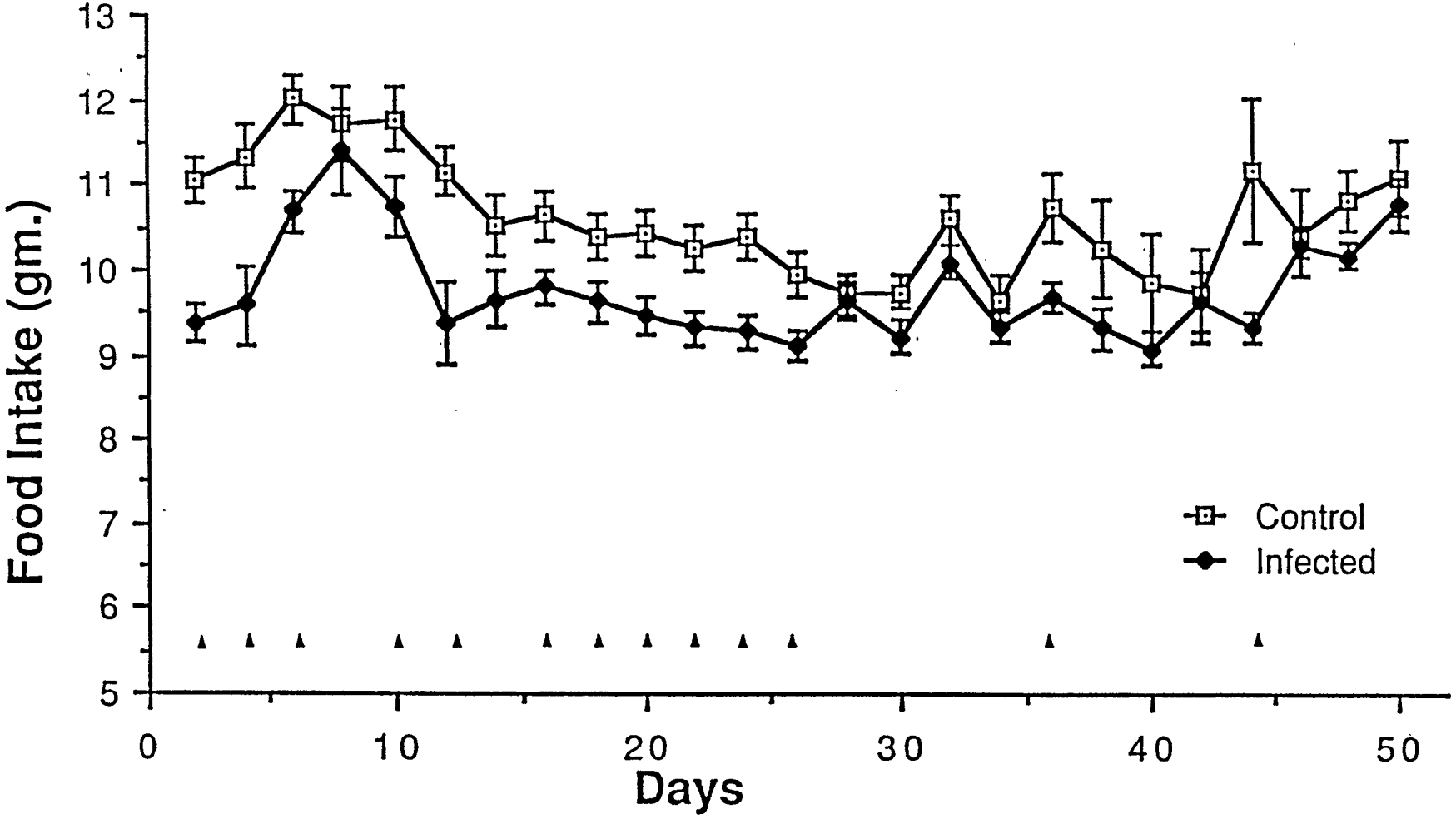

Food Intake (gm.)
13
12
11
10
9
8
7
6
5
0
10
20
30
40
50
Days
Control
Infected

Figure 6: Weight gain as a percentage of weight on day 0 (inoculation) in control mice and mice infected with 1000 Giardia muris cysts. Values are mean ± SE. * $P < 0.05$ when compared to control as analyzed by Student's t test and one-way analysis of variances (ANOVA) followed by Student-Newman-Keuls multiple comparison test.

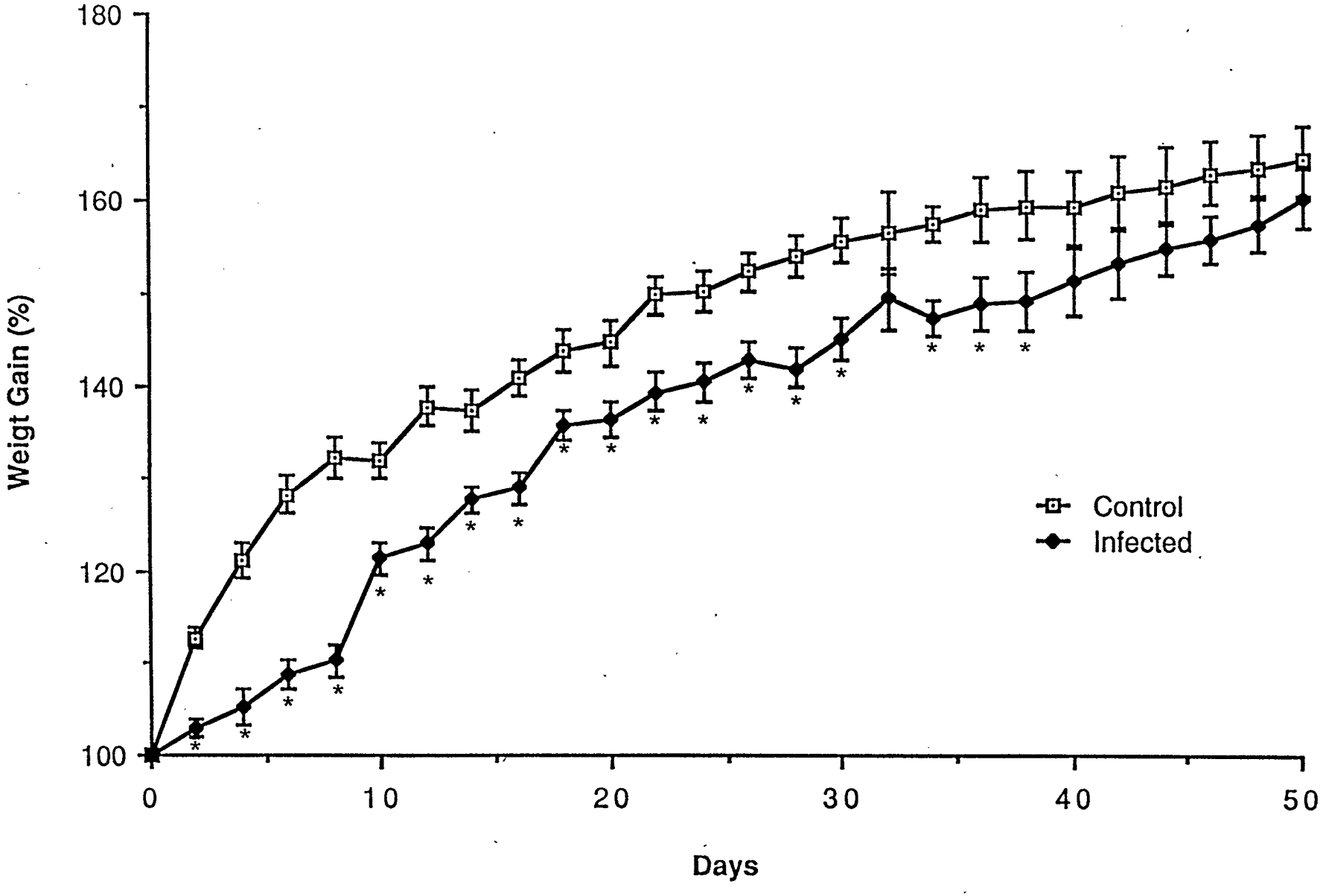

180
160
140
120
100
Weigt Gain (%)
0
10
20
30
40
50
Days
Control
Infected

Figure 7: Weight gain in control, pair fed and infected mice as a percentage of weight on day 0 (inoculation). Values are mean ± SE. ▲ $P < 0.05$ infected compared to control, • $P < 0.05$ infected compared to pair fed and ◄► $P < 0.05$ pair fed compared to control as analyzed by one-way analysis of variances (ANOVA) followed by Student-Newman-Keuls multiple comparison test.

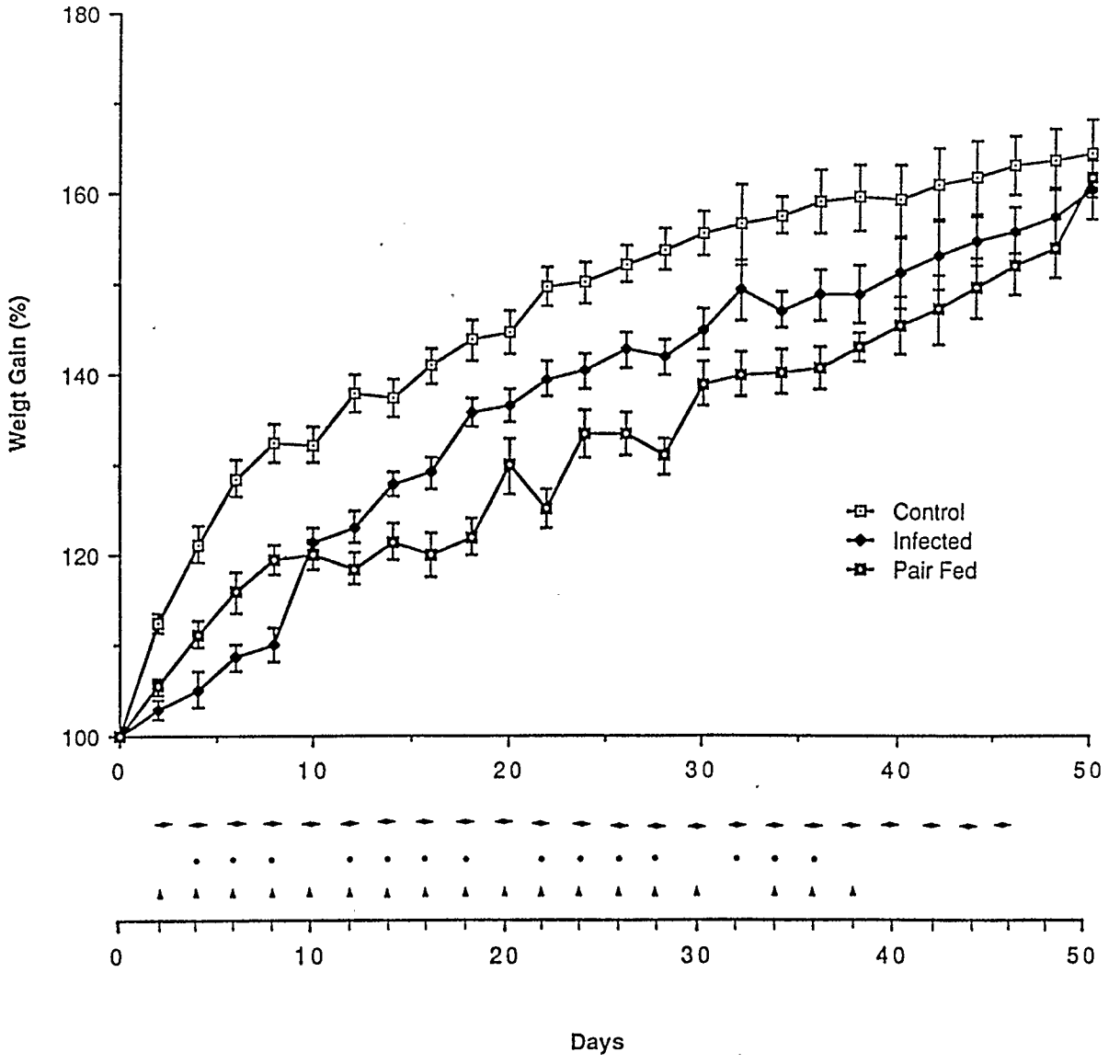

180
160
140
120
100
Weigt Gain (%)
0
10
20
30
40
50
Control
Infected
Pair Fed
Days

Figure 8: Villous height and crypt depth of duodenal, jejunal and ileal mucosae in control, pair fed and infected mice. Values are mean ± SE. * $P < 0.05$ compared to control, + $P < 0.05$ compared to pair fed when analyzed by Student's *t* test and one-way analysis of variances (ANOVA) followed by Student-Newman-Keuls multiple comparison test.

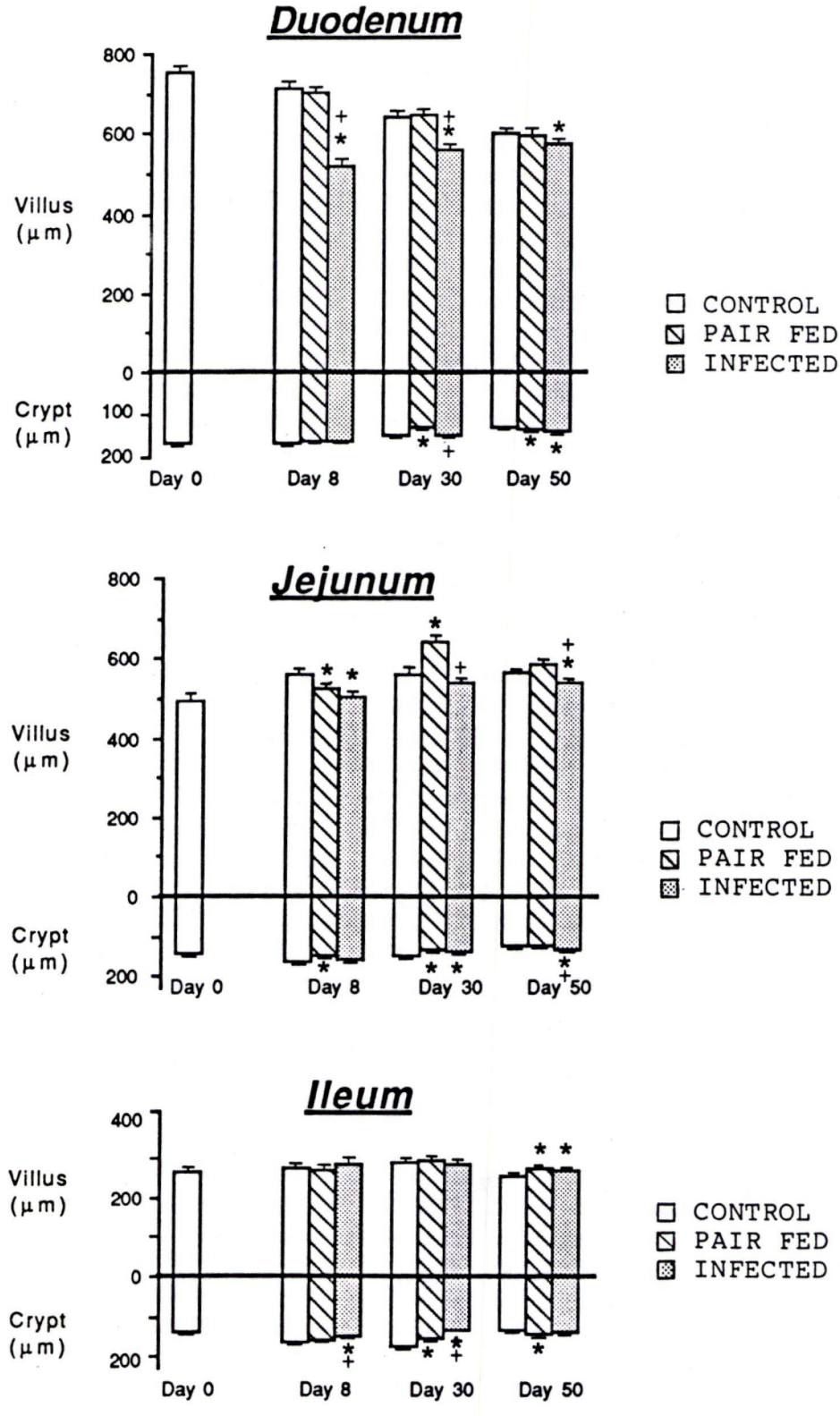
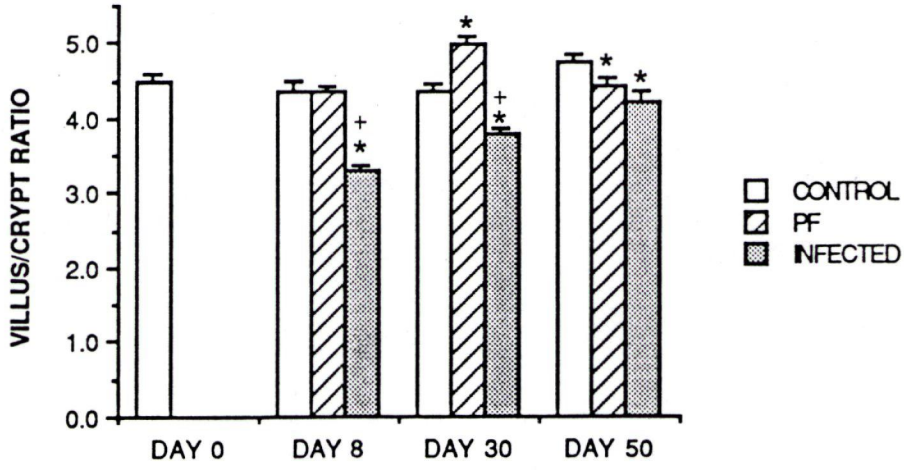
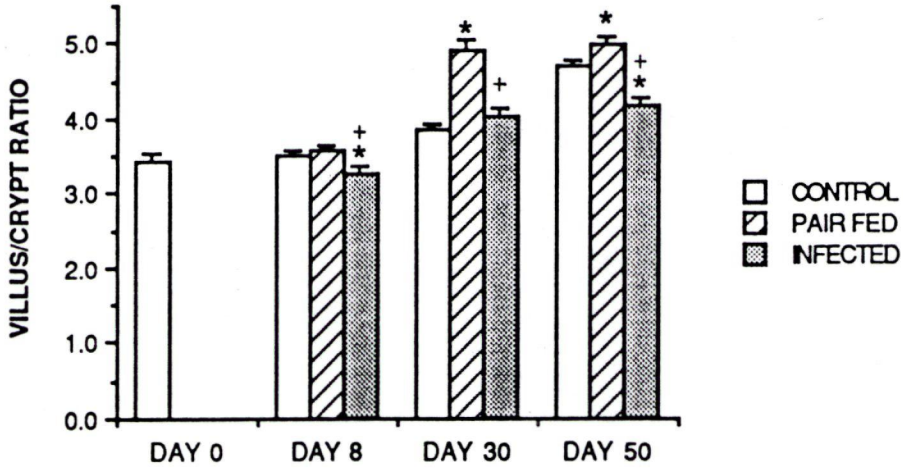

Duodenum
800
600
400
200
0
100
200
Villus (μm)
Crypt (μm)
Day 0
Day 8
Day 30
Day 50
CONTROL
PAIR FED
INFECTED
Jejunum
800
600
400
200
0
200
Villus (μm)
Crypt (μm)
Day 0
Day 8
Day 30
Day 50
CONTROL
PAIR FED
INFECTED
Ileum
400
200
0
200
Villus (μm)
Crypt (μm)
Day 0
Day 8
Day 30
Day 50
CONTROL
PAIR FED
INFECTED

Figure 9: Villus to crypt ratios of duodenal, jejunal and ileal mucosae in control, pair fed and infected mice. Values are mean ± SE. * P < 0.05 when compared to control, + P < 0.05 when compared to pair fed as analyzed by one-way analysis of variances (ANOVA) and Student-Newman-Keuls multiple comparison test.

DUODENUM



JEJUNUM



ILEUM

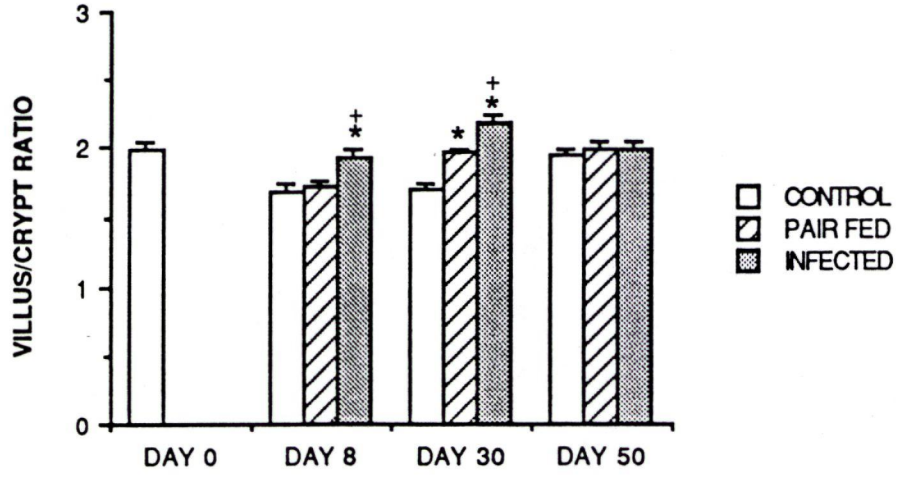


Figure 10: Mucosal maltase activity from duodenum, jejunum and distal small intestine, 0, 2, 8, 24 and 65 days post inoculation. Values are mean $\pm$ SE and are expressed as units per gram of protein. * $P < 0.05$ compared to control, + $P < 0.05$ compared to pair fed when analyzed by one-way analysis of variances (ANOVA) and Student-Newman-Keuls multiple comparison test.

MALTASE

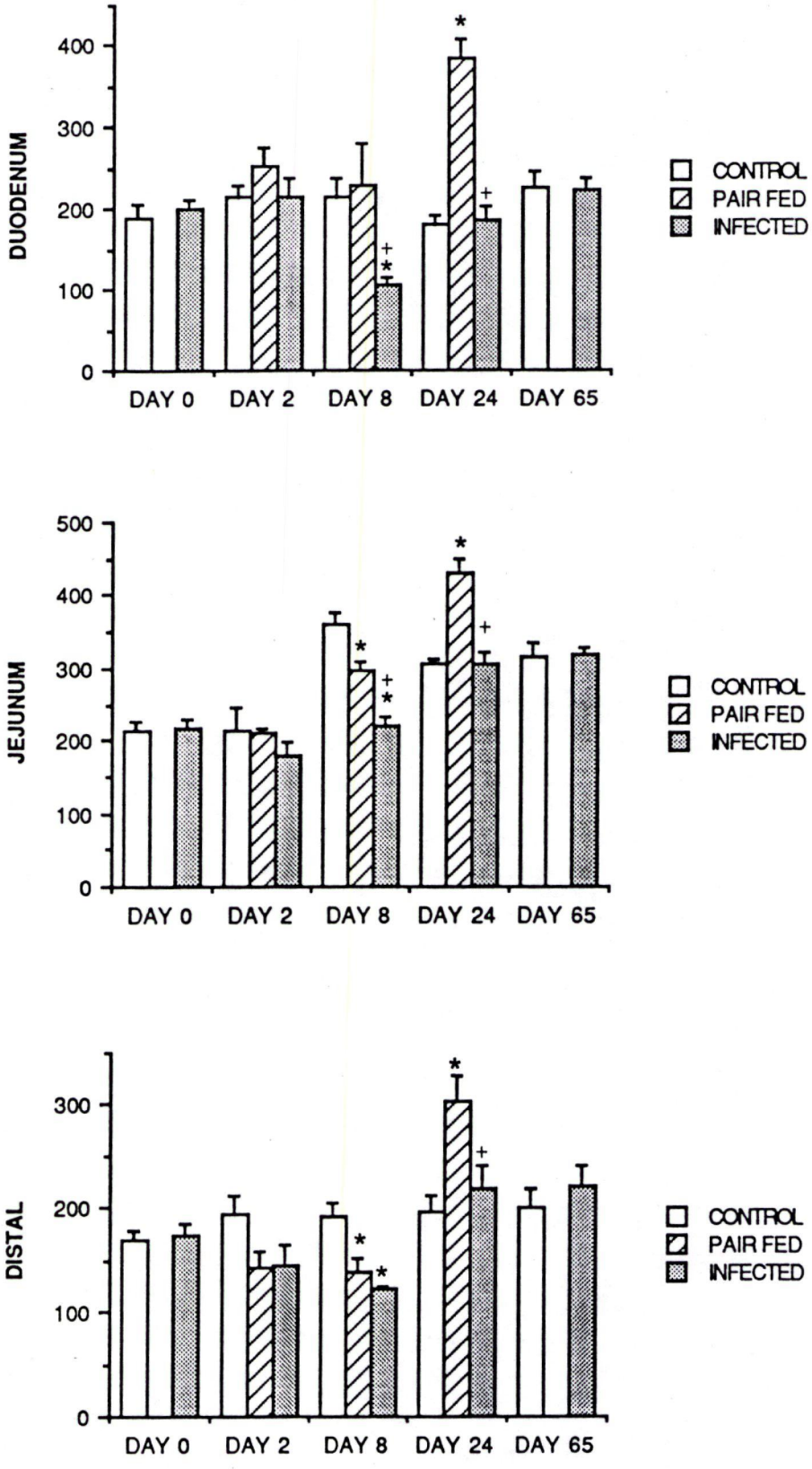

DUODENUM
400
300
200
100
0
DAY 0
DAY 2
DAY 8
DAY 24
DAY 65
CONTROL
PAIR FED
INFECTED
JEJUNUM
500
400
300
200
100
0
DISTAL
300
200
100
0

Figure 11: Mucosal sucrase activity from duodenum, jejunum and distal small intestine, 0, 2, 8, 24 and 65 days post inoculation. Values are mean $\pm$ SE and are expressed as units per gram of protein. * $P < 0.05$ compared to control, + $P < 0.05$ compared to pair fed when analyzed by one-way analysis of variances (ANOVA) and Student-Newman-Keuls multiple comparison test.

SUCRASE

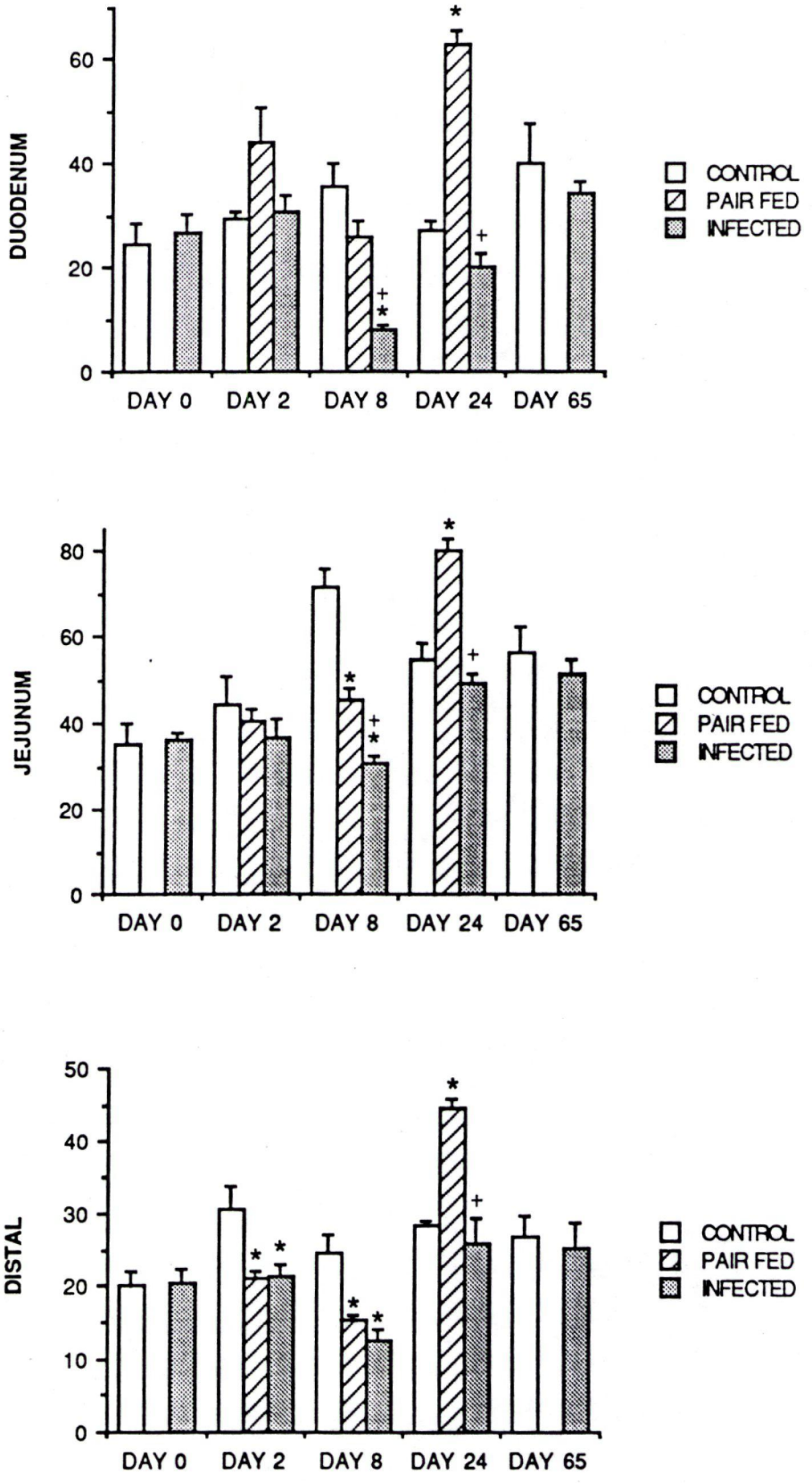

DUODENUM
60
40
20
0
DAY 0
DAY 2
DAY 8
DAY 24
DAY 65
CONTROL
PAIR FED
INFECTED
JEJUNUM
80
60
40
20
0
DAY 0
DAY 2
DAY 8
DAY 24
DAY 65
CONTROL
PAIR FED
INFECTED
DISTAL
50
40
30
20
10
0
DAY 0
DAY 2
DAY 8
DAY 24
DAY 65
CONTROL
PAIR FED
INFECTED

Figure 12: Mucosal lactase activity from duodenum, jejunum and distal small intestine, 0, 2, 8, 24 and 65 days post inoculation. Values are mean ± SE and are expressed as units per gram of protein. * $P < 0.05$ compared to control, + $P < 0.05$ compared to pair fed when analyzed by one-way analysis of variances (ANOVA) and Student-Newman-Keuls multiple comparison test.

LACTASE

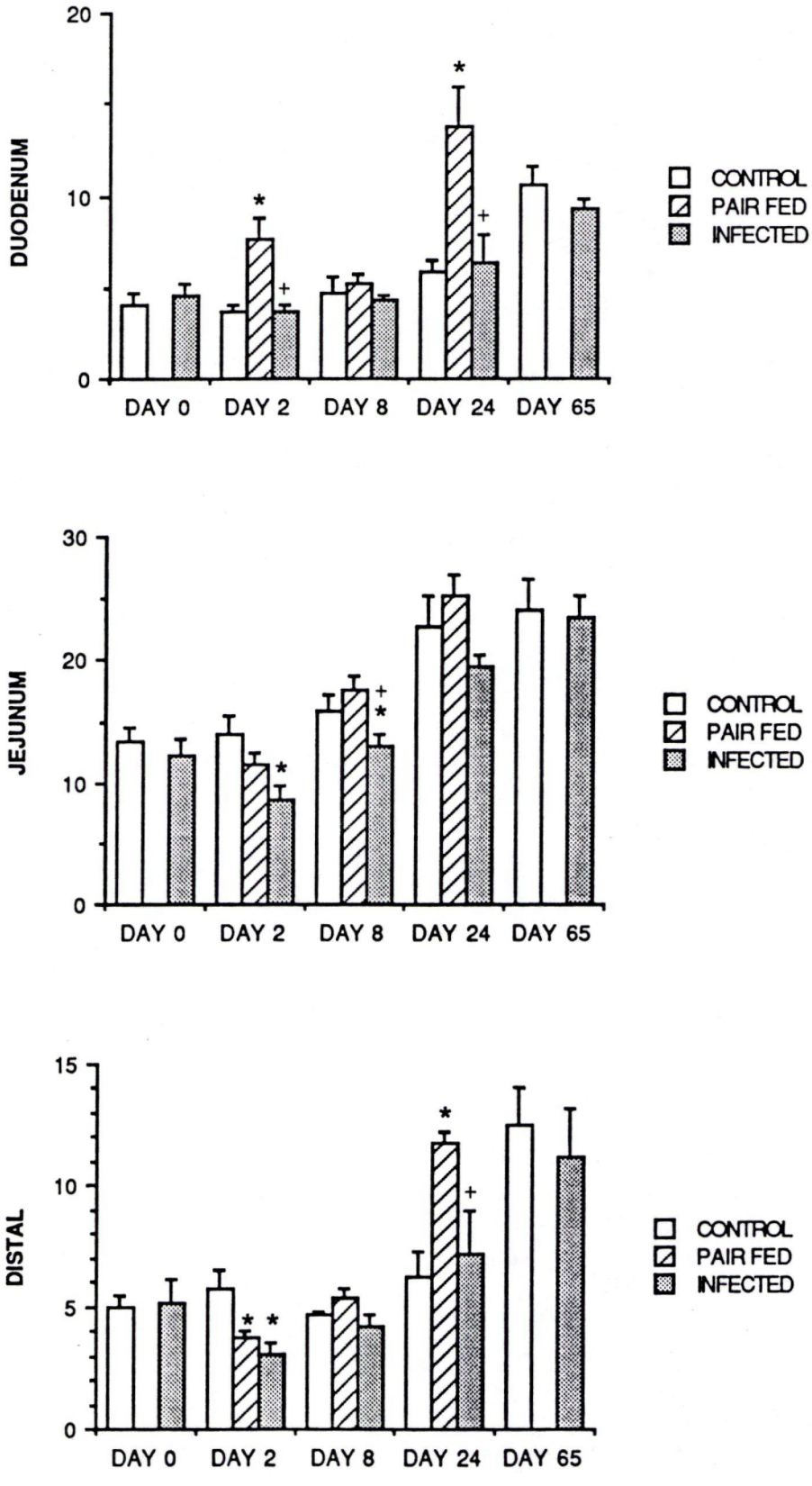

DUODENUM
20
10
0
DAY 0
DAY 2
DAY 8
DAY 24
DAY 65
CONTROL
PAIR FED
INFECTED
JEJUNUM
30
20
10
0
DISTAL
15
10
5
0

Figure 13: Transmission electron micrographs of epithelia from a control mouse (A) and a mouse infected with Giardia muris (B) at same magnification (8900 X). Infection resulted in swelling and destruction of mitochondria (arrows), proliferation of cytoplasmic lysosomes (L) and diffuse shortening of the microvilli (MV). In both epithelia, nuclei (N) are found towards the lower pole of columnar cells, intercellular membranes (IM) appear tight and tortuous, Golgi apparatus (G) is situated above the nucleus and its cisternae are collapsed and arranged in parallel fashion. In the micrograph illustrating infected epithelium, note the presence of an intraluminal trophozoite (T) and mucous droplets of a goblet cell (D). Bars = 5 μm.

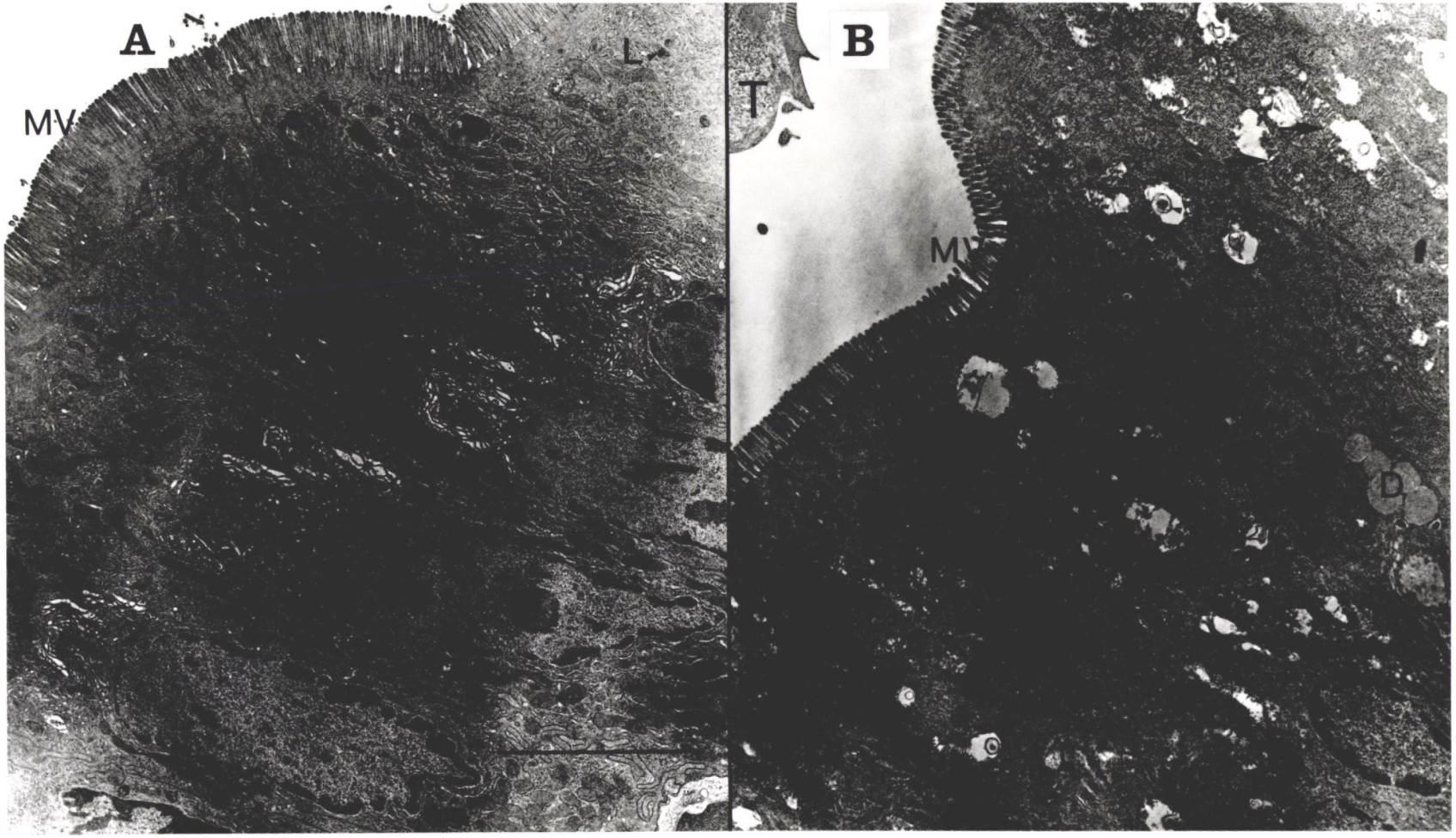
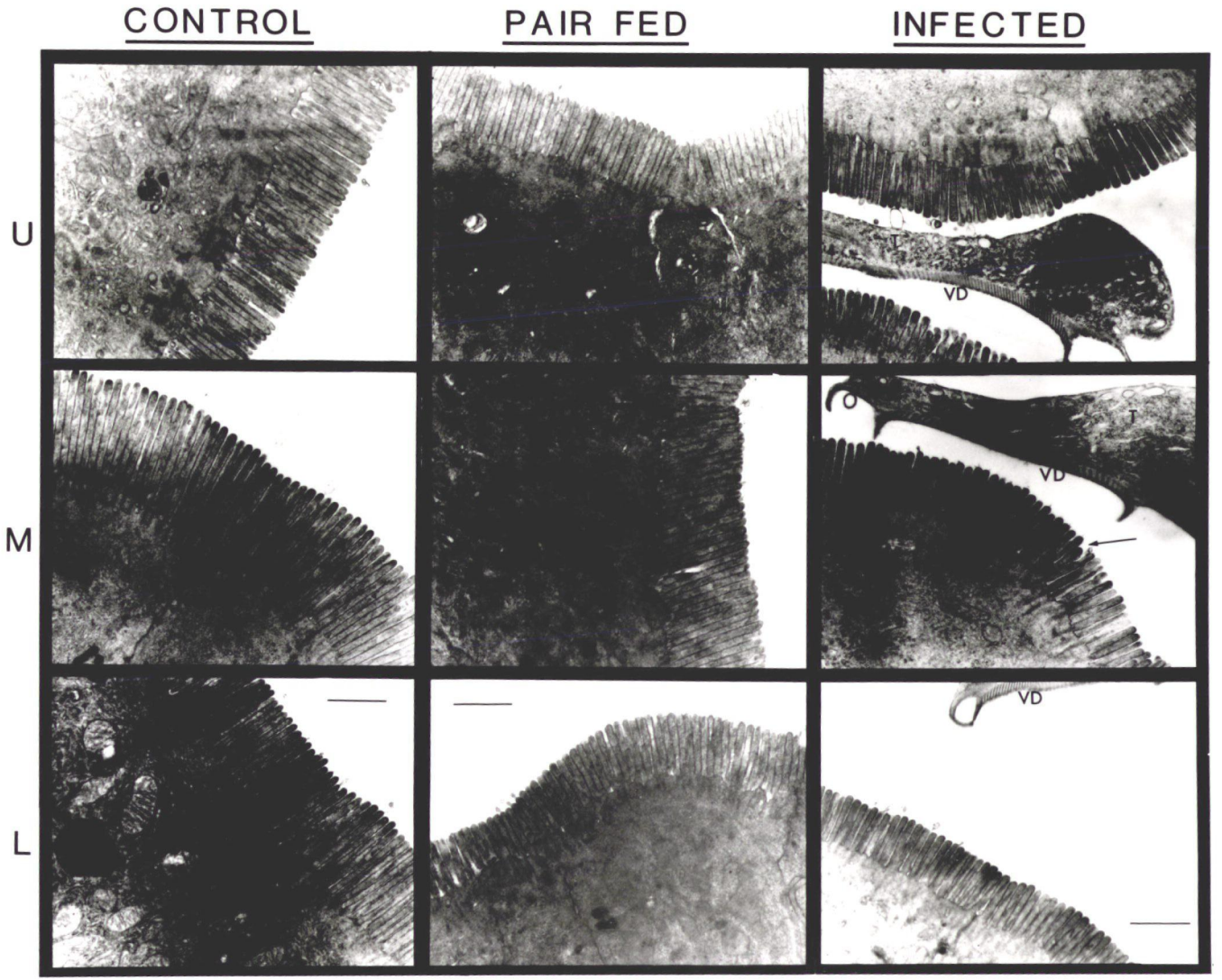

A
L
MV
B
T
MV
D
D

Figure 14: Transmission electron micrographs of jejunal microvillous brush border in upper (U), mid (M) and low (L) villus of control, pair fed and infected mice at same magnification (11000 X). In infected gut, note the presence of trophozoites (T) and localized shortening and bending (arrow) of microvilli where the edge of an adhesive ventral disk (VD) penetrated the brush border. Bars = 1 µm.


CONTROL
PAIR FED
INFECTED
U
M
L
T
VD
O
T
VD
VD