Aus dem Institut für Veterinär-Anatomie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Comparative macroscopic, microscopic and morphometric study of the stomach, intestine and liver of a dual-purpose and a broiler chicken line

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Zaher Alshamy
Tierarzt aus Hama, Syrien

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Meiner Familie

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List of abbreviations

ANOVA	One-way analysis of variance
BW	Body weight
d	Day
D+	Broiler chicken lines genetically selected for a high digestion efficiency
D−	Broiler chicken lines genetically selected for a low digestion efficiency
h	Hours
H&E	Meyer's Haematoxylin and Eosin
LCI	Lower confidence interval
LD	Lohmann Dual chickens
LSD	Least Significant Difference test
MA	Macula adhaerens
n	Animal number
r	Pearson's correlation coefficient
R^2	Coefficient of determination
Ross	Ross 308
SEM	Standard error of the mean
SD	Standard deviation
TEM	Transmission electron microscopy
UCI	Upper confidence interval

1 Introduction

1.1 Issues related to selection for high production in chickens

The use of specialized chicken lines for meat and egg production has resulted in undesired offspring in egg laying breeds. This problem affects a large number of male chicks of laying hens which are immediately killed after hatching, as they are not of use for meat production. The killing of healthy chicks poses a serious animal welfare problem and different ways to solve this problem are under discussion (Leenstra et al., 2011). This issue should be dealt with from a “humanitarian” point of view and killing should be avoided in such a way as to protect welfare of animals (Bruijnis et al., 2015). There are already ways to solve this problem, e.g., by gender identification before the chicks are even hatched. Different methods have been reported with regard to time and type of gender identification. With incubated eggs, gender identification is based on the differences in the hormone production of embryonic male and female gonads. The variations in gender steroid levels can be measured in the blood plasma from day 7 of incubation onward (Woods et al., 1975). Recently, an in ovo gender determination method was introduced with an accuracy above 90% on the fourth incubation day (Galli et al., 2018).

The increased integration of dual-purpose chicken lines into meat and egg production chains offers one possible solution to end the culling of one-day old male chicks. By crossing meat and laying hybrids, new dual-purpose lines have been developed, such as the Lohmann Dual chickens (LD), which yield good results in meat and egg production (Urselmans et al., 2015). LD chickens can be expected to have an egg yield of about 250 eggs per year and a marketable body weight of about 2.2 kg at the age of 8 weeks (Icken & Schmutz, 2013). In Germany, this type of chicken has already been tested under conventional husbandry conditions. Besides, a report of production costs is also already available (Damme, et al., 2015). However, there is a lack of systematic and comprehensive studies as to whether or not these chickens lend themselves to modern poultry farming. At this point, an interdisciplinary project was launched with the subject "The integration of fattening and egg production with the use of the dual-purpose chicken as an improvement in animal welfare (Integhof)”. In this cooperative project, researchers from different disciplines and from different institutions in Germany have come together to test the feasibility of using such a dual-purpose chicken from different points of view: meat and egg production, environmental protection as well as economic efficiency (Rautenschlein, 2015).

The overall project was divided into several task groups. In this frame, the aim of my doctoral thesis was to examine parameters concerning the anatomical robustness of the gastrointestinal tract of the dual-purpose chicken.

Rapid growth in broilers and the digestive tract

Broilers are a good example for understanding high growth rates that are related to certain physiological and anatomical parameters (Jackson & Diamond, 1996). Several studies have shown that weight gain and growth is correlated directly with the functional and morphological development of the gastrointestinal tract (Jackson & Diamond, 1996; Sell et al., 1991; Yamauchi et al., 1996). The growth rates of specific organs, i.e. intestine and liver of neonatal birds, are particularly high; these organs grow considerably faster than does the body weight, so as to provide enough nutrients for the increased growth of the muscles (Foye & Black, 2006).

The birds' digestive segments are relatively small. All the same they are highly efficient in rapidly supplying energy and nutrients so that their high metabolic rates may be sustained (Dyce et al., 2017). The digestive tract of modern broilers, in particular, has had to adapt to tremendous changes due to intensive breeding for a rapid growth rate (Svihus, 2014).

1.2 Essentials of bird anatomy and physiology

1.2.1 The gastrointestinal tract

The gastrointestinal tract begins with the beak and ends at the vent. Feed is gathered by the beak and travels on to the oesophagus. Feed storage and moistening take place in the crop. The glandular stomach produces enzymes that are mixed with the feed which subsequently is mechanically broken down in the gizzard (Svihus, 2014).

The small intestine specializes in enzymatic digestion of nutrients. Absorption of water from the fecal material takes place in the cecum. The cloaca serves as a terminal chamber receiving both fecal materials from the intestines and waste products from the urogenital tract. Finally, the cloacal vent serves as outlet (Schokker, 2012).

1.2.2 The stomach

The stomach of granivory birds is divided by an isthmus into two chambers: a glandular stomach (*proventriculus*) and a muscular stomach or gizzard (*ventriculus*).

The spindle-shaped proventriculus has a thin wall; it is about 4.5 cm long in adult fowls (Hodges, 1974) and weighs 7.48 ± 0.85 g in Ross 308 chicken (Mabelebele et al., 2014). It is lined with a mucosa; its wall mainly consists of superficial and deep gastric glands (Dyce et al., 2017).

The ventriculus is shaped like a biconvex lens and lies in the lower left quadrant of the body cavity, much of its surface covered by the left abdominal air sac. Part of the ventriculus is in contact with the body wall and, as such, is retroperitoneal. The wall of the ventriculus has a typical layered structure (tunica mucosa, tela submucosa, tunica muscularis and serosa) (König & Liebich, 2018). The ventriculus weighs about 38.42 ± 3.2 g in Ross 308 chickens (Mabelebele et al., 2014). Due to the muscular movements in the gizzard, the nutrients are mixed with hydrochloric acid and pepsinogen that are secreted by way of the proventriculus (Duke, 1992). The gizzard has an important additional function in grinding feed material, as this is not done in

the mouth. Therefore, the gizzard contains a strong tunica muscularis and, additionally, has a koilin layer, which supports the grinding process (Duke, 1992). It has been reported that the volume of the gizzard may increases substantially when certain additional components like wood shavings are included in the diet (Amerah, et al., 2008; 2009).

1.2.3 The intestines

The growth of the chicken´s digestive tract has been studied from different points of view (Tallentire et al., 2016). Growth and function of the gastrointestinal tract are associated with rapid body growth. Heavy chicken lines have longer intestines when compared to lighter chicken lines (Fan et al., 1997; Uni et al., 1995). Overall, the bird's intestines are shorter and the ingesta passage moves faster than in mammals (Denbow, 2000). The average retention time in the gastrointestinal tract of the broiler is only about 4 - 6.5 hours (Klis, et al., 1990). Therefore, the intestines must be very effective to enhance body growth (Denbow, 2000).

1.2.4 The small intestine

The small intestine which is divided into duodenum, jejunum and ileum, is only about 120 cm long in the adult bird.

The duodenum forms a loop (duodenum descendens and ascendens) following the caudal curvature of the gizzard; it ends at the height of the flexura duodenojejunalis (Vollmerhaus & Sinowatz, 2004). The pancreas lies between the loops and empties into the distal end of the ascending duodenum (Dyce et al., 2017). Sklan et al. reported that 95% of the fat is digested in the duodenum (Sklan et al., 1975). The adjacent jejunum - according to some authors - ends at the Meckel's diverticulum (Duke, 1986), which is often used as a boundary landmark to separate the jejunum and ileum (Denbow, 2000). According to histological studies of certain authors, this distinction is not justified; they use the terminus jejunoileum (König & Liebich, 2018).

The absorption of digestion products from starch (Riesenfeld et al., 1980; Uni et al., 1995) and protein (Uni et al., 1995) is to a large extent completed upon reaching the end of the jejunum. The ileum is the last segment of the small intestine. This segment plays a role in water and mineral absorption. The ileum terminates at the ileo-ceco-colic junction (Vollmerhaus & Sinowatz, 2004).

1.2.5 The large intestine

The large intestine consists of two ceca (except birds with only one or no cecum) and the colorectum (Dyce et al., 2017). The ceca begin at the ileo-ceco-colic junction. Immediately before this junction there is lymphoid tissue called the cecal tonsil. Important functions of the ceca comprise the bacterial breakdown of cellulose (Dyce et al., 2017) as well as electrolyte and water absorption. The ceca also have a role in the recycling of renal nitrogen (Thomas, 1982).

The colo-rectum is the last segment of the intestine and located between the ileo-cecal junction and the cloaca. In the colo-rectum water and electrolytes are reabsorbed (Dyce et al., 2017).

1.2.6 The cloaca

The cloaca is the terminal part of the digestive and urogenital system and opens into the vent. The cloaca of the chicken is about 2.5 cm long and 2.0-2.5 cm wide (Vollmerhaus & Sinowatz, 2004). The colo-rectum, ureters and deferent ducts in the male - or the left oviduct in the female - enter it at various levels (Dyce et al., 2017). It is divided into three chambers; the coprodeum, the urodeum, and the proctodeum (Hodges, 1974).

1.3 Histology of the intestine

The intestinal wall contains of three layers; mucosal tunic, muscle tunic and the serosal tunic. The mucosa consists of a simple, high prismatic, resorptive epithelium, a lamina propria and a lamina muscularis mucosa (Denbow, 2000). The total thickness of the tunica mucosa decreases along the length of the small intestine while at the same time the lamina propria mucosae increases in thickness (Hodges, 1974).

The large number of goblet cells increases in number aborally (Hodges, 1974). In both, small and large intestine of chickens, the mucosal layer forms intestinal villi. These are narrow and finger-shaped, decreasing in length from 1.5 mm in the duodenum to 0.4–0.6 mm in the ilium and the colo-rectum. The number of villi decreases between day 1 and 10 after hatching, and remains constant thereafter (Denbow, 2000). Genetic selection for rapid body growth has altered intestinal villous morphology (Yamauchi, 2002). It is assumed that an increased villus height is an indication of improved function (Awad et al., 2011). Ileal villi can be enlarged as a result of a dysfunctional jejunum (Yamauchi, 2007). Thus, an increased villous height may also be the result of an increased need for digestive capacity.

Compared to white leghorns, the villi of broilers are larger and their duodenal villi show more epithelial cell protrusions on the apical surface (Yamauchi, 2002). Nevertheless, the villi from both types of chickens form a zigzag-like arrangement, which is thought to slow down the ingesta flow. The cells are about 50 µm high and 8-10 µm wide and have a clear brush border of microvilli (Hodges, 1974). The crypts of Lieberkühn generate epithelial cells that cover the villi (Denbow, 2000). The intestinal crypt depth decreases from the duodenum towards the end of the ileum (Hodges, 1974).

1.4 The liver

The liver is the largest gland of the body. As in mammals, the liver plays an important role as to digestion and metabolism, regulating the production, storage, as well as release of lipids, carbohydrates and proteins (Denbow, 2000).

In contrast to mammals, the synthesis of fat in birds takes place predominantly in hepatic tissue and very limited in adipose tissue (Hermier, 1997). The liver plays a major role in the synthesis and metabolism of fat that is derived from three main sources, i.e., dietary fat, depot fat and fat from *de novo fatty* acid synthesis, i.e. from feed carbohydrates (Denbow, 2000).
The liver produces a great variety of proteins, including blood proteins, enzymes and hormones (Denbow, 2000). It is the main site of phagocytosis by the Kupffer cells, which take up older blood cells as well as pathogens that may penetrate via the hepatic portal blood (Akers & Denbow, 2013).

1.4.1 Anatomy and histology of the liver

The liver is located caudal to the lungs in the front end of the avian thoraco-abdominal body cavity. Its shape has modified to fit within the contour of the internal surfaces of the body wall, as well as to the adjacent and enclosed structures (Zaefarian et al., 2019). Apart from a small lobus intermedius, it is divided into two main lobes, the right and left lobe of which the right lobe is larger. The visceral peritoneum covers the liver and adheres closely to its surface. The triangular ligaments and lesser omentum hold the liver in place (Denbow, 2000).
The classic hexagonal hepatic lobule is the basic functional unit of the liver. (Hodges, 1974). It is formed by hepatocytes and sinusoids (Hodges, 1974). Hepatocytes make up almost 80% of the total liver volume and perform various liver functions. The hepatocyte is a complex cell with a large nucleus and many mitochondria (Ohata, et al., 1982), lysosomes, rough and smooth endoplasmic reticulum, Golgi apparatus and other organelles (Ohata et al., 1982). The hepatocyte is limited by a plasma membrane which makes contacts with the intercellular space, bile canaliculi, or the space of Disse associated with the sinusoids. Sinusoids of variable width surround the hepatic plates (Hodges, 1974). The walls of the sinusoids are composed of endothelial cells as well as scattered phagocytic Kupffer cells (Purton, 1969).

2 Aims and hypotheses

The overall goal of this doctoral thesis was to investigate the basic anatomical responsiveness and robustness of the gastrointestinal tract and the liver of two different genetic chicken lines when raised under intensive husbandry conditions and fed high-energy diets.

Therefore, morphology and microstructure of the gastrointestinal tract and liver of the new dual-purpose LD chicken line and the conventional broiler line Ross 308 were examined and compared. The following parameters were included in the study:

- The weight of the empty glandular stomachs, the gizzards, and the intestine segments were measured
- The normalized mass for each segment of the gastrointestinal tract was calculated as segment mass (g)/body weight (BW) (g)] x 100
- The allometric relationships between the segments and BW were determined
- The villus height, epithelium height, crypt depth, mucosal enlargement factor and thickness of the tunica muscularis were determined via histology in jejunum and ileum
- The length of microvilli as well as the length of junctional complexes of jejunal enterocytes was measured via transmission electron microscopy
- The liver mass was recorded
- The normalized liver mass was calculated as [liver mass (g)/body weight (g)] x 100
- The liver lipid content was determined histologically
- The number and area of the hepatic lymphatic aggregations were counted in histological sections
- The ultrastructure of the liver was examined using transmission electron microscopy

3 Comparison of the gastrointestinal tract of a dual-purpose to a broiler chicken line: A qualitative and quantitative macroscopic and microscopic study

Zaher Alshamy, Kenneth C. Richardson, Hana Hünigen, Hafez Mohamed Hafez, Johanna Plendl, Salah Al Masri

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Comparison of the gastrointestinal tract of a dual-purpose to a broiler chicken line: A qualitative and quantitative macroscopic and microscopic study

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Abstract

The transition to using dual-purpose chickens is an alternative to killing male hatchlings of high performance egg-laying chickens. This study aimed to compare the gastrointestinal tract of a recently developed genetic line of dual purpose male chicken, Lohmann Dual (LD), with that of a broiler line, Ross 308. Eighty birds from each line were grown until they reached an average body weight 2000 g (5 weeks for Ross and 9 for LD birds). Six birds of each line were sampled weekly. Body weight (BW), normalized mass of gastrointestinal segments and relative length of intestine were determined. Histologically the villus height, epithelium height, crypt depth, mucosal enlargement factor and the tunica muscularis thickness were measured in jejunum and ileum. Data were regressed against body weight and genetic line. Jejunal enterocyte microvilli and junctional complexes length were measured. Normalized mass and relative length of the gastrointestinal segments were greater in LD birds than in Ross birds at all ages. After day 7 these decreased steadily over the lifetime of the birds in both genetic lines. The growth curves of the gastrointestinal segments of the LD birds were similar to those of the Ross birds. In birds of the same BW, LD birds had a significantly heavier gizzard, shorter intestine, higher jejunal villi, thicker ileal tunica muscularis and smaller ileal mucosal enlargement factor than were found in Ross birds. The large gizzard in LD chickens presumably increases the degree of food processing and enhances availability of nutrients in the orad part of the intestine leading to a lower nutrient concentration and a smaller absorption surface area in the ileum of the LD compared to the Ross chickens. The anatomical differences between the two lines are important criteria for further selection and should be considered in their feeding management.

data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

The commercial chicken industry is based on the genetic development of highly productive breeds selected for either egg laying or meat production. Since males of laying breeds are slow growing, they are not profitable for meat production and are killed immediately after hatching [1]. This outcome has caused intense ethical debate [2]. One suggested approach to resolve this problem is to raise layer-type males to a live weight of 600 or 2000 g and market them as alternatives to quail or broiler chickens respectively. However, to reach a live weight of 2000+ g, the layer-type males require twice the quantity of feed and triple the time compared to specifically selected broiler breeds [1]. In a public survey conducted by Leenstra et al. (2011), of 5 potential alternatives to killing one-day-old chicks, "transillumination of the fresh eggs to determine the sex of the eggs and not incubate male eggs" and "using dual-purpose chickens" scored best (25% and 24%, respectively) out of the proposed alternatives" [1].

Investigations in the nineteen nineties of traditional dual-purpose breeds such as the Australorp, Bielefelder, New Hampshire and Rhode Island Red, reported that these breeds were inefficient in both meat and egg production in comparison to highly selected lines such as Ross and Lohmann Brown [3]. More recently developed dual-purpose chicken lines such as Walesby Specials, Dominant Red Barred, Novogen Dual and Lohmann Dual (LD) have a better body weight gain and feed efficiency than the traditional dual-purpose breeds [3]. Calculations based on current prices for eggs and meat indicate that the LD breed exceeds the performance of the other dual-purpose breeds, particularly in respect to meat production. The LD line has a better feed conversion efficiency compared to Walesby Special and Brown hybrids [3].

Functional anatomical and histological characteristics of the avian gastrointestinal tract are critical to their feed conversion efficiency [4]. The chicken's gastrointestinal tract consists of the glandular stomach (proventriculus), gizzard (ventriculus) and intestine (small and large). In the glandular stomach which is lined by glands secreting pepsin, hydrochloric acid and mucus, food is mixed with these digestive juices [5]. The gizzard is the second compartment of the stomach where powerful contractions crush the food [6]. In the small intestine, feed constituents are hydrolyzed into simple molecules, in particular; free small peptides, amino acids, free fatty acids, and monosaccharides. These molecules are absorbed in the duodenum and jejunoileum and transported via blood circulation to other tissues [7].

To facilitate maximal absorption of dietary components the intestinal mucosa is highly convoluted and specialized. The epithelium is folded into villi and the epithelial cells have an apical aspect covered by a dense matting of microvilli forming a brush border. This increases the small intestinal surface area for absorption by about 600 fold resulting in a higher capacity for nutrient absorption [7].

The aim of this study was to compare the gastrointestinal tracts of a recently bred genetic line of dual-purpose chicken, the Lohmann Dual, to a highly selected conventional broiler line, the Ross 308, to investigate specific morphological characteristics involved in intestinal digestion, resorption, secretion and barrier function. A better understanding of the gut development and anatomy in the two types of chickens provides new knowledge to improve feed efficiency and growth in the LD birds. Here, the allometric growth of the gastrointestinal segments were determined. The epithelium height, crypt depth, the height of intestinal villi, mucosal enlargement of the villus, and the thickness of the tunica muscularis of the intestine were measured morphometrically. The ultrastructure of the enterocytes and their interepithelial cell junctions were examined using transmission electron microscopy.

Material and methods

Birds and management

Two groups of 80 one day old male chicks, one a highly selected commercial broiler line (Ross 308) obtained from BWE-Brüterei-Weser-Ems GmbH & Co. KG, Visbek, Germany and the second a novel dual-purpose line (Lohmann dual, LD) were supplied by Lohmann Tierzucht, Cuxhaven, Germany. The rearing of the chickens was carried out in accordance with German animal welfare law. The study was approved by the Animal Welfare Committee: Landesamt für Gesundheit und Soziales, Berlin, Germany, ID: 0236/15.

The two groups were received at the same day in the Institute of Poultry Diseases of the Department of Veterinary Medicine, Freie Universität Berlin and housed under the same husbandry conditions. To reach the optimal body weight of 2000 g for slaughter the Ross chicks were kept for 5 weeks and the LD chicks for 9 weeks. Each group was kept in a separate 4.2 m^2 pen in the same room having the same floor litter material. Both pens had a width of 1.63 m and a length of 2.55 m with 2.55 m high walls. The pens were separated by wire mesh. The photoperiod program used was 24 hours (h) light for the first three days, followed by 20 h of light until day 7 and 16 h of light thereafter. At the beginning of the study, temperature was kept at 30˚C for three days, and was gradually reduced by 3˚C per week until reaching 24˚C. The desired temperature was regulated by a thermostat and the ventilation was controlled automatically, whereby the desired stable temperature was controlled by a thermostat. A mash diet was fed ad libitum to the two groups throughout the study and the birds were allowed free access to water. Birds were fed a starter diet from 1 to 14 days of age, then a grower diet from day 15 to the end of the study. The composition of the diet is shown in Table 1. On day one at the hatchery all birds received vaccinations against Newcastle disease and infectious bronchitis. Lohman dual birds also received vaccination against Marek's disease. The birds were checked daily by a veterinarian. Samples from each group were collected at the following ages: Ross = 1, 7, 14, 19, 21, 25, 28, 32 and 35 day(s), LD = 1, 7, 14, 21, 28, 32, 35, 42, 49, 56 and 63 day(s). On sampling days, six birds were selected at random from the genetic line being examined, the live body weights were recorded, then the birds were killed by decapitation.

Macroscopic examination

Directly after death, the abdominal cavity was opened by a ventral ceolomic incision, and the glandular stomach, the gizzard and the entire intestine of each bird were excised. The length of each intestinal segment was measured, i.e., duodenum (from the gizzard junction to the end of the pancreatic loop), jejunum (from the aboral pancreatic loop to Meckel's diverticulum), ileum (from Meckel's diverticulum to the ileocecal junction), and colorectum (from the ileocecal junction to the orad cloaca) as defined by De Verdal et al. (2010) [4]. Care was taken to avoid stretching the intestine. All adherent anatomical structures (i.e. mesentery, associated blood vessels, fat and pancreas) were removed from each organ at the time of collection. Organs were kept in Ringer's solution for transport and processing.

In the laboratory, the glandular stomach, gizzard and the entire intestine including the ceca for each bird were opened longitudinally along the antimesenteric side, flushed with tap water to remove the contents, and blotted dry using filter paper. The weights of the glandular stomach, gizzard, and intestine segments were determined using an electronic laboratory balance (AND HF-200G, Tokyo, Japan) with a measurement accuracy of 0.01g. The normalized mass of the glandular stomach, gizzard and intestinal segments was calculated as [mass (g)/total body weight (g)] × 100. The weekly weight gain of the glandular stomach, gizzard and the intestinal segments compared with their final mass was calculated using the following

Table 1. Composition of diet feed.

Ingredients		Starter	Grower
Maize meal	%	23.95	29.13
Soybean meal	%	36.04	30.60
Wheat meal	%	28.00	28.00
Limestone	%	1.54	1.60
Monocalcium phosphate	%	1.2	0.85
[1] Premix	%	1.2	1.2
DL-Methionine	%	0.27	0.25
L-Lysine HCl	%	0.15	0.17
Soybean oil	%	7.65	8.2
Analyzed nutrient content of the (g or mg/kg in feed)			
ME_N [2]	MJ/kg	12.60	13.00
Dry matter	g/kg Us	896.43	894.65
Crude ash	g/kg Us	55.57	50.16
Crude protein	g/kg Us	231.5	214.4
Crude fat	g/kg Us	98.83	97.08
Crude fibre	g/kg Us	55.94	53.12
Starch	g/kg Us	284.09	334.67
Potassium	g/kg Us	8.20	7.44
phosphorus	g/kg Us	2.33	2.16
Calium	g/kg Us	8.20	7.24
Sodium	g/kg Us	1.57	1.43
Magnesium	g/kg Us	2.36	2.29
Iron	mg/kg Us	348.05	320.69
Manganese	mg/kg Us	113.44	103.2
Copper	mg/kg Us	20.94	19.39
Zinc	mg/kg Us	98.63	92.55

[1]Contents per kg premix: 600000 I.U. Vit. A (acetate); 120000 I.U. Vit. D3; 6000 mg Vit. E (α-tocopherol acetate); 200 mg Vit. K (MSB); 250 mg Vit. B1 (mononitrate); 420 mg Vit. B2 (cryst. Riboflavin); 300 mg Vit. B6 (pyridoxin-HCL); 1500 μg Vit. B12; 3000 mg niacin (niacin amide); 12500 μg biotin (commercial, feed grade); 100 mg folic acid (cryst., commercial, feed grade); 1000 mg pantothenic acid (Ca d-pantothenate); 60000 mg choline (chloride); 5000 mg iron (iron carbonate); 5000 mg zinc (zinc sulfate); 6000 mg manganese (manganous oxide); 1000 mg copper (copper oxide); 45 mg iodine (calcium-iodate); 20 mg selenium (sodium-selenite); 140 g sodium (NaCl); 55 g magnesium (magnesium sulphate); carrier:calcium carbonate (calcium min38%).

[2]Estimated according to equation of WSPA 1984.

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relationship: [(the mean mass in the present week − the mean mass in the previous week)/ the final mean mass on the last day of the study) × 100]. The relative length of the intestine was calculated as [intestine length (cm)/total body weight (g)] × 100. The intestine mass per unit of length (g/cm) was defined as the "intestinal density" [8].

Sample collection and histological examination

Samples (size 1x1x1 cm) of jejunum (1 cm proximal to Meckel's diverticulum) and ileum (1 cm proximal to the ileocecal junction) were excised and fixed in neutral buffered formalin (4%, pH 7, 24 h, 20–24 ˚C). Then the tissues were dehydrated in an ascending graded series of ethanol and embedded in paraffin wax. Serial sections were cut at 5 μm with a microtome (Jung, Histoslide, 2000 Sliding, Wetzlar, Germany). Four cross-sections of jejunum and ileum

per bird were stained with Meyer's Hematoxylin and Eosin (H&E) according to standard histological protocol [9].

Specimen preparation for electron microscopic examination

Samples of the jejunum (2 cm proximal to Meckel's diverticulum) collected from two Ross 308 birds and two LD birds on days 1, 7, 21, and 35, and from the LD group on day 63, were fixed in Karnovsky solution (7.5% glutaraldehyde and 3% paraformaldehyde in phosphate buffered saline), washed in 0,1 M cacodylate buffer (cacodylic acid sodium salt trihydrate, Roth; Karlsruhe, Germany), incubated in 1% osmium tetroxide (Chempur; Karlsruhe, Germany) for 120 min., dehydrated in an ascending series of ethanol and washed in the intermedium propylene oxide (1, 2 Epoxypropan; VWR, Germany). Then the specimens were embedded in a mixture of agar 100 (epoxy resin), DDSA (softener), MNA (hardener) and DMP 30 (catalyst) (all: Agar Scientific; Stansted, GBR). Polymerization was done at 45˚C and 55˚C, each for 24 hours. Semi- and ultrathin sections were cut on an ultramicrotome Reichert Ultracut S (Leica; Wetzlar, Germany). Semi-thin sections (0.5 μm) were stained according to a modified Richardson protocol [9] for 45 seconds on an electric hotplate adjusted to 80˚C and checked under a light microscope (Olympus CX21, Olympus; Stuttgart, Germany) to determine the area of interest. Ultrathin (80 nm) sections were mounted on nickel-grids (Agar Scientific; Stansted, GBR) and examined with a transmission electron microscope (Zeiss EM 900; Oberkochen, Germany) (TEM).

Morphometric analysis

For each bird, the mucosal and muscular layers of the jejunum and ileum were examined using a light microscope (Axioskop, Carl Zeiss, Jena, Germany) and an analyzing system, NIS-Elements AR (Nikon Instruments Inc., U.S.A.).

The following parameters in H&E stained sections were measured on one cross-section per bird and intestinal segment:

- Villus height: Ten villi were measured from their base at the level of the crypt's entrance through to their distal tips. Only full finger-shaped and well-oriented villi were used (Fig 1A).
- Epithelium height: Ten jejunal epithelial cells of different villi were measured from the basement membrane to the tip of their microvilli (Fig 1B).
- Crypt depth: Ten crypts were measured from the crypt's base to the closest villus base. The ratio of villus height to crypt depth was calculated by dividing villus height by crypt depth (Fig 1C).
- Mucosal enlargement factor of the villus: Here the continuous length of the mucosal surface of ten adjacent villi was measured. The length of the corresponding underlying lamina muscularis mucosae was measured [10] (Fig 1D). Mucosal enlargement factor = total mucosal surface length divided by lamina muscularis mucosae length.
- Thickness of the tunica muscularis: This parameter was defined as the distance between the lamina muscularis mucosae internally and the tunica serosa externally. Ten measurements were performed per intestinal segment (Fig 1E).

The following parameters were measured in each jejunal sample using transmission electron micrographs.

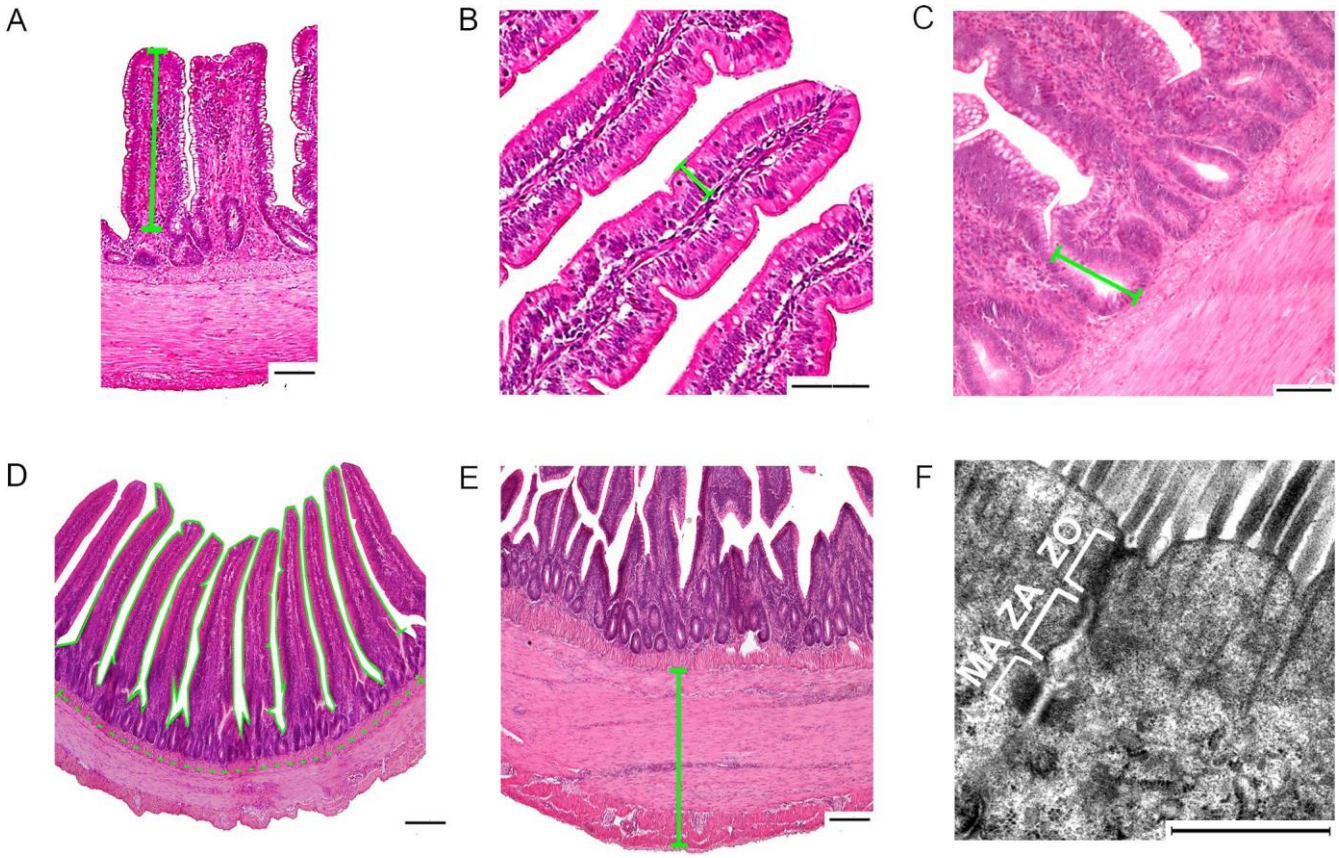

Fig 1. Morphometric measurements. (A) ileal villus height, (B) jejunal epithelium height, (C) crypt depth, (D) enlargement factor of the mucosal surface of the jejunum (The continuous line is the mucosal surface of the villi length and the interrupted line is the lamina muscularis mucosae length, (E) thickness of the tunica muscularis of the jejunum. Bar: 100 μm for A, B, C, D and E. (F) transmission electron micrograph of the enterocyte junctional complex. Here: ZO, zonula occludens; ZA: zonula adhaerens; MA, macula adhaerens (desmosome). Bar: 2000 nm.

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- Microvillus length: Forty individual microvilli were measured from the tip of the microvillus to its attachment to the enterocyte membrane.
- Junctional complex and tight junction length: Ten enterocyte junctional complexes were measured from the apex of the tight junction to the innermost part of the desmosome. The length of each tight junction was measured from its apex to the start of the zonula adherens on the electron micrographs for each bird (Fig 1F).

Statistical analysis

Data were analyzed using the statistical package program IBM SPSS Statistics 23 (IBM Corporation, New York, USA). The graphs were made by using the statistical package program JMP Pro 13 (SAS Institute Inc., Cary, USA). Continuous variables are presented as standard error of the mean ± (SEM). Comparisons of the two lines of the same age groups were performed using the Mann–Whitney U test. The relationship between the normalized mass for each part of the stomach, intestinal segments and the relative length of the intestinal segments and age was assessed using one-way analysis of variance (ANOVA) with the post hoc Dunnett's test. To explore the effect of chicken line and body weight on the glandular stomach and gizzard and intestinal segments, all data collected were regressed against the genetic line and body weight using the log-log regression model. Due to the non-linear relationship between

the glandular stomach mass, gizzard mass, and intestinal segment masses to body weight, the data were log_{10}-transformed prior to analysis. All statistical analyses were two-sided with significance defined as a p value of < 0.05.

To examine whether the relative changes of the glandular and gizzard and intestinal segments masses were proportionate or disproportionate to overall body weight relative change, the allometric relationships between the glandular and gizzard and intestinal segments and body weight were determined based on the following relationship [11]:

ln y = k ln x + ln b, where ln natural logarithm, y is the mass of the glandular stomach, gizzard or intestinal segments, x is the weight of the bird, b is a constant reflecting the relationship between the mass of the stomach parts and intestinal segments and the total body weight of the bird. The symbol k is the slope of the regression line relating y and x and represents the rate of change of the stomach parts and intestinal segments with changes in the total body weight. If the value of k is equal to 1 (isometry), the rate of change of the glandular and gizzard and intestinal segments with changes in the total body weight is proportionate during the growth. If k departs significantly from a value of 1, then the relationship is allometric (k >1 = positive allometry; k $<$ 1 = negative allometry).

Results

Total body weight

From d 1 to d 35 post hatching, the body weight of the Ross birds increased at a rate of 57.67 g/d, whereas that of the LD birds increased by 22.03 g/d. The LD birds' weight increased by a rate of 31.76 g/d from d 1 to d 63 post hatching. When compared with the final body weight of each genetic line the greatest body weight gain of 39.35% was between day 28 and 35 for Ross birds and of 19.48% between day 42 and 49 for LD birds.

The Ross birds were about 2.6 times heavier than the LD birds by d 35 (Ross = 2013.16 g, LD = 791.66 g) (Fig2).

The glandular stomach

The mass of the glandular stomach of Ross birds increased from d 1 to d 35 post-hatching at a rate of 0.19 g/d, whereas that of LD birds increased at a rate of 0.09 g/d. The glandular stomach

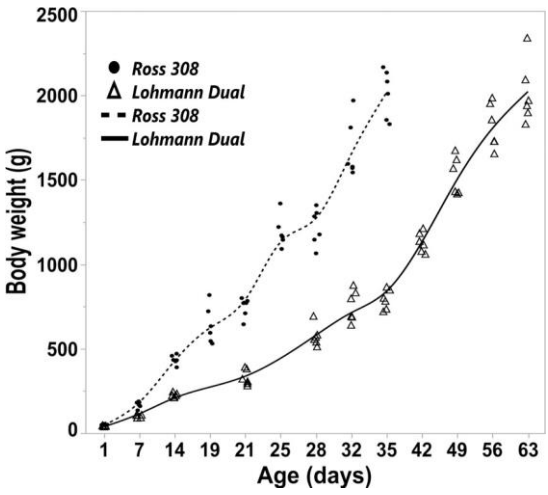

Fig 2. Trendlines of the changes in body weight versus day post hatching for Ross and LD chicken genetic lines. Symbols represent each individual value for each chicken line.

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mass of LD birds from d 1 to d 63 increased at a rate of 0.11 g/d (Fig 3A). The growth of the glandular stomach was nearly isometric until d 7 in both lines. From d 14 onward, the glandular stomach exhibited negative allometric growth relative to body weight (Table 2) (Fig 3C). Regression analysis showed that the genetic chicken line had no influence on the mass of the glandular stomach.

From d 1 to d 35 post-hatching, the normalized mass of the glandular stomach was numerically greater in the LD line than in the Ross line in all age groups. The difference was statistically significant from d 14 onward. A spike in the normalized mass of the glandular stomach was noted on d 1 for LD and Ross chicks at values of 1.09 and 1.00 g per 100 g BW, respectively (Fig 3E). Then, there was a decrease in the normalized glandular stomach mass with age until d 25 for Ross and d 35 for LD chicks. Subsequently the decrease was insignificant until the end of the study. Furthermore, the post hoc Dunnett's test indicated no differences between each age group from d 25 for Ross chicks and from d 32 for LD chicks compared with the last day of the study.

The gizzard

The gizzard grew gradually with age in both lines, with the mass of the gizzard increasing by 12.65 times in Ross birds and 15.15 times in LD birds by the end of the study (Fig 3B).

The gizzard of Ross chicks gained 0.91 g/d from d 1 to d 35 post-hatching ($R^2 = 0.91$), whereas the LD chick's gizzard gained 0.60 g/d over the same period. From d 1 to d 63, the gizzard mass of LD chicks increased at a rate of 0.55 g/d ($R^2 = 0.94$). Overall, the gizzard had negative allometric growth relative to body weight (Table 2) (Fig 3D).

Regression analysis showed that both the BW and the genetic line of the chickens had an influence on the mass of the gizzard, p ◆ 0.001, adjusted $R^2 = 0.97$. In birds of the same body weight, the gizzard mass of LD birds was heavier by 5.5% than that of Ross birds.

From d 1 to d 35 post hatching, the normalized mass of the gizzard was greater in LD birds than in Ross birds and the difference was statistically significant from the first day onwards (Fig 3F).

The normalized gizzard mass was greatest on the first day post-hatching, followed by a marked decrease until d 21 in both genetic lines. After this there was a gradual decrease until the end of the study. The post hoc Dunnett's test showed that there were no significant differences between each age group from day 21 for Ross birds and from d 42 for LD birds compared with the last day of the study.

The intestine

The length and mass of the entire intestine increased from d 1 to d 35 post-hatching, in Ross chicks at rates of 4.57 cm/d and 2.47 g/d, and in LD birds at rates of 2.98 cm/d and 1.08 g/d respectively. The entire intestine of Ross chicks grew faster than it did in LD birds over this period i.e.,1.53 times in length and 2.28 times in mass (Fig 4A and 4C). Between d 1 and d 63 the daily rate of increase in the intestinal length and mass in LD chicks were 2.13 cm and 1.01 g respectively. The greatest increase in intestinal length was in the first week post-hatching i.e. by 19.5% of the final mean length in both lines. The mean of the entire intestine mass on d 1 post-hatching was 3.08% (Ross birds) and 3.31% (LD birds) of the mean of the final intestinal mass. The greatest weight increase of the intestine was between day 21 and 35 in both lines. The allometric relationship between BW and intestinal mass was positive until day 7 in both genetic lines and became negative from day 14 onward (Table 2) (Fig 4F).

According to regression analysis, both the BW and the genetic line of the chickens had an influence on the entire intestine length, p ◆ 0.001, adjusted $R^2 = 0.95$. The entire intestine

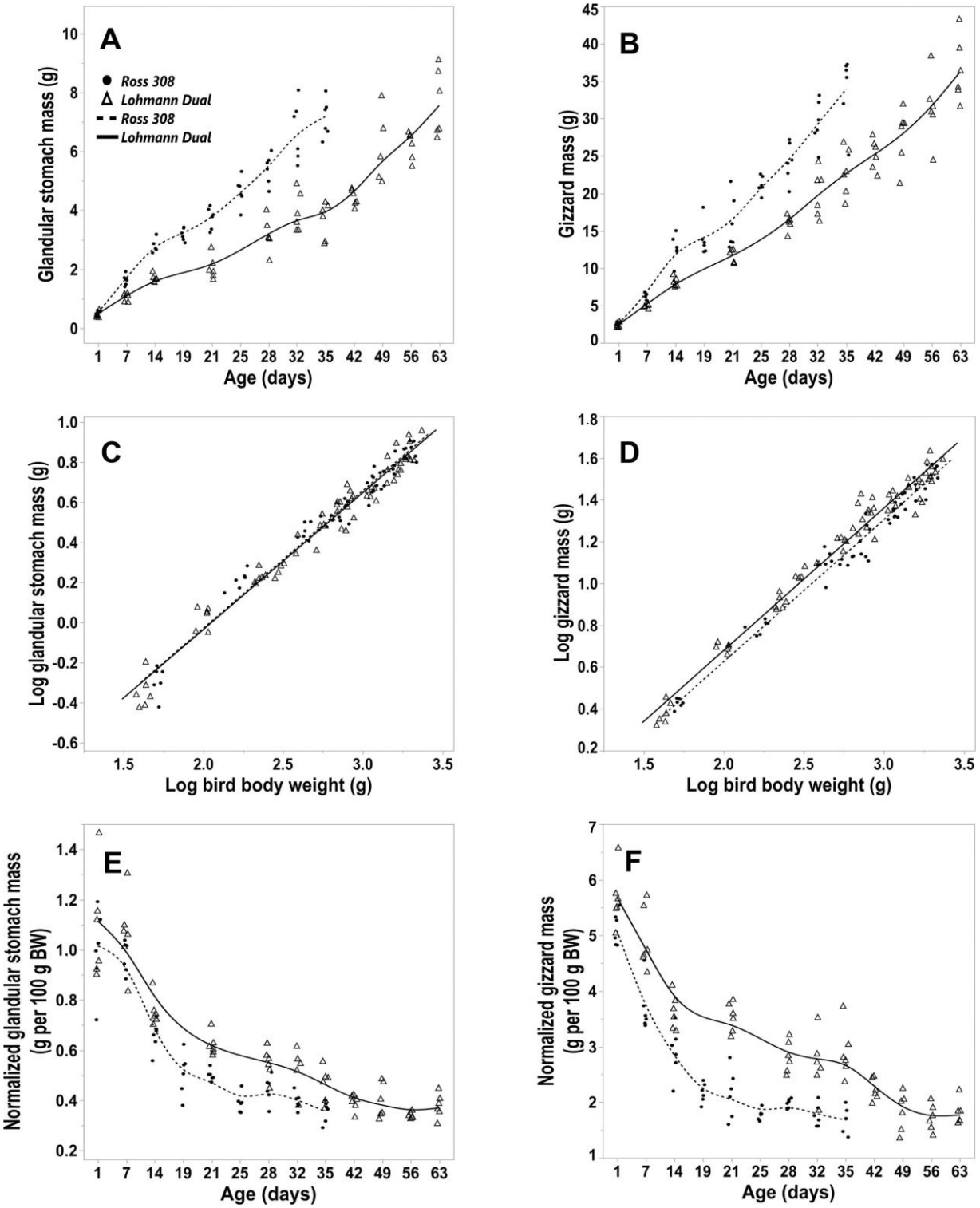

Fig 3. Morphometric parameters of the glandular stomach and gizzard for LD and Ross 308 chicken lines. (A, B) glandular stomach and gizzard mass versus day post hatching for Ross and LD lines. (C, D) allometric plot: logarithm (Log_{10}) of glandular and gizzard mass versus (Log_{10}) of total body weight for both genetic lines. (E, F) normalized glandular and gizzard mass versus day post hatching for both genetic lines. Symbols represent each individual value for each chicken line.

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Table 2. Allometric relationship between body weight and the mass of the glandular stomach, gizzard and entire intestine of both chicken lines for different post hatching growth periods.

	Period (days)	Line (n)	Unstandardized coefficients						
			Constant			Slope			R^2
			Mean	LCI	UCI	Mean	LCI	UCI	
Glandular stomach	**1 to 7**	**Ross (12)**	-1.97	-2.26	-1.68	0.98	0.84	1.12	0.96
		LD (12)	-1.92	-2.36	-1.48	0.97	0.73	1.21	0.89
	1 to 35	**Ross (54)**	-1.4	-1.5	-1.3	0.69	0.65	0.72	0.97
		LD (36)	-1.44	-1.55	-1.33	0.7	0.66	0.75	0.96
	1 to 63	**LD (66)**	-1.4	-1.48	-1.32	0.68	0.65	0.71	0.97
Gizzard	**1 to 7**	**Ross (12)**	-0.79	-0.94	-0.63	0.71	0.63	0.79	0.97
		LD (12)	-0.96	-1.21	-0.71	0.83	0.69	0.96	0.94
	1 to 35	**Ross (54)**	-0.73	-0.82	-0.65	0.68	0.65	0.71	0.98
		LD (36)	-0.8	0.89	0.71	-0.73	0.7	0.77	0.98
	1 to 63	**LD (66)**	-0.68	-0.76	-0.61	0.68	0.65	0.71	0.97
Entire intestine	**1 to 7**	**Ross (12)**	-1.52	-1.82	-1.22	1.13	0.98	1.29	0.97
		LD (12)	-1.99	-2.29	-1.69	1.43	1.27	1.59	0.97
	1 to 35	**Ross (54)**	-1.1	-1.18	-1.03	0.92	0.89	0.95	0.99
		LD (36)	-1.12	-1.23	-1.01	0.94	0.89	0.98	0.98
	1 to 63	**LD (66)**	-0.94	-1.03	-0.85	0.85	0.82	0.89	0.98

LCI: lower confidence interval; LD: Lohmann dual; n: animal number; R^2: coefficient of determination; Ross: Ross 308; slope: this value represents the allometric relationship between the dependent variables and body weight; UCI: upper confidence interval.

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length of Ross birds was longer on average by 4% than that of LD birds of the same BW. However, only the BW had a significant influence on the entire intestine mass, p < 0.001, adjusted $R^2 = 0.98$. The normalized mass of the entire intestine was significantly greater from day 21 onwards in the LD birds than in Ross birds. The normalized intestinal mass showed that the two genetic lines were similar, peaking at day 7 then decreasing to plateau between days 21–35 (Fig 4D), with levels in LD subsequently dropping noticeably between days 35 and 63. The post hoc Dunnett's test showed that there were no significant differences in the normalized entire intestine mass between the age groups from day 19 for Ross birds and from day 49 for LD birds compared with the last day of the study of each respective genetic line. From day 7 onwards the relative length of the entire intestine was significantly greater in LD birds than in Ross birds at the same age. The relative length of the entire intestine dropped sharply post-hatching until d 14 in both lines, where the mean values were 0.26 times and 0.38 times smaller than the value on d 1 for Ross birds and LD birds respectively. The relative intestinal length stabilised from d 25 onwards for Ross birds and from d 42 for LD birds to the last day of the study in each genetic line (Fig 4B). The post hoc Dunnett's test showed no differences in the relative entire intestine length between the age groups from d 25 for Ross birds and from d 42 for LD birds compared with values on the last day in each genetic line.

The intestinal density increased continually with age in both lines (Fig 4E). The intestinal density of Ross birds was significantly greater than that of LD birds from d 14 onward Regression analysis showed that both BW and the genetic line of the chickens had an influence on the intestinal density, p < 0.001, adjusted $R^2 = 0.97$. In birds of equivalent BW the intestinal density of LD birds was higher on average by 3% than that of Ross birds.

Intestinal segments. The analysis of the normalized mass and relative length of the individual intestinal segments mirrored those determined for the entire intestine. The density (g/cm) of the individual intestinal segments in both groups was highest in the duodenum and

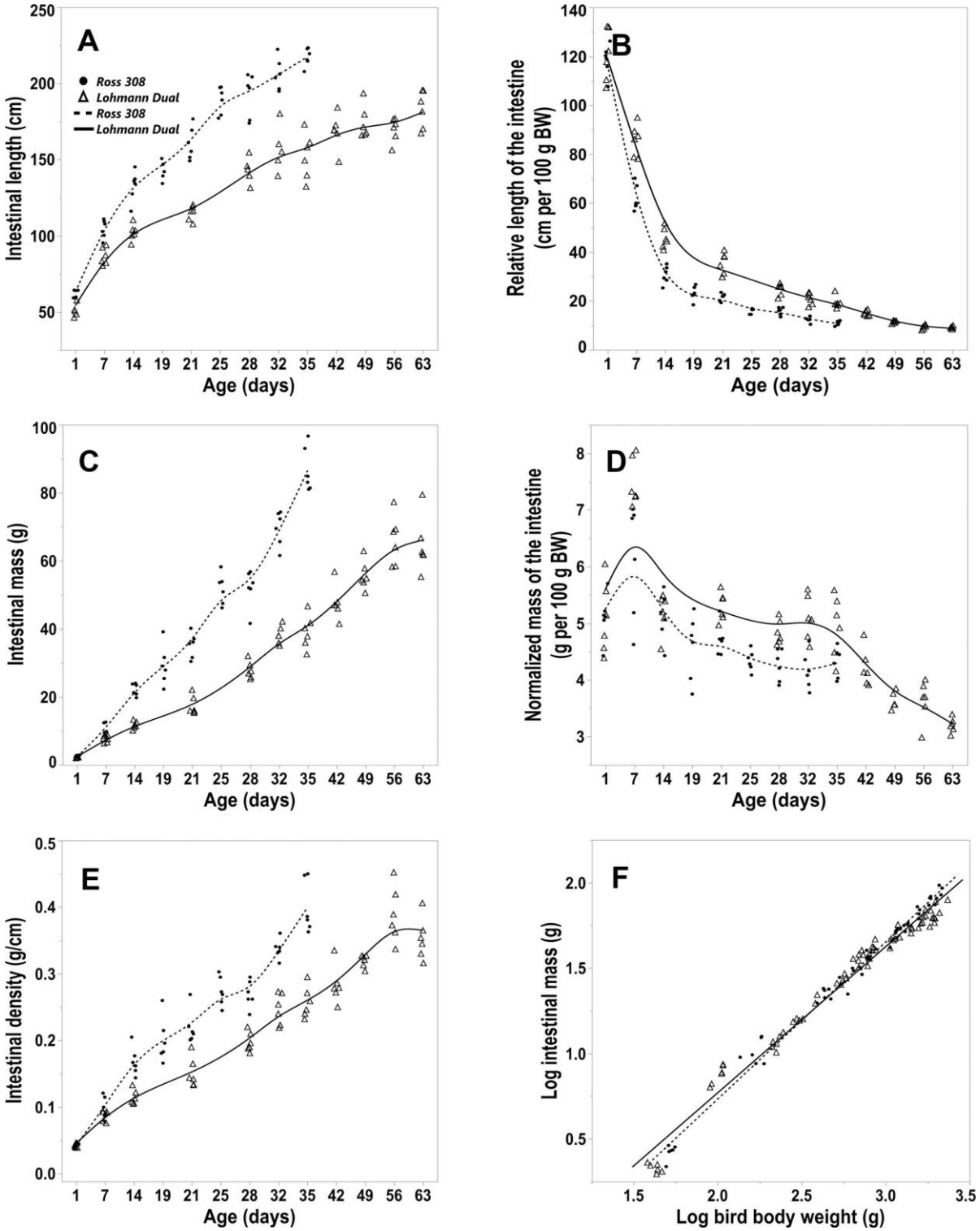

Fig 4. Morphometric parameters of the intestine for LD and Ross 308 chicken lines. (A) length of the entire intestine, (B) relative length of the entire intestine, (C) mass of the entire intestine, (D) normalized intestine mass, (E) intestinal density, all versus days post hatching. (F) allometric plot: logarithm (Log_{10}) of the entire intestine mass versus Log_{10} of total body weight. Symbols represent each individual value for each chicken line.

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Table 3. Intestinal density and length and mass of individual intestine segments as a percentage of the entire intestine of both chicken lines over the study period.

Intestinal segments	Line (n)	Intestinal density (g/cm)		Length (%)		Mass (%)	
		Mean	SEM	Mean	SEM	Mean	SEM
Duodenum	**Ross (54)**	0.29	0.02	15.04	0.3	19.2	0.32
	LD (66)	0.29	0.02	16.76	0.19	22.66	0.46
Jejunum	**Ross (54)**	0.24	0.02	33.17	0.3	33.88	0.45
	LD (66)	0.25	0.02	32.63	0.19	35.64	0.36
Ileum	**Ross (54)**	0.22	0.01	31.12	0.35	30.23	0.43
	LD (66)	0.2	0.01	28.59	0.22	25.78	0.32
Cecum	**Ross (54)**	0.16	0.01	15.9	0.17	11.77	0.28
	LD (66)	0.14	0.01	16.52	0.17	10.53	0.21
Colorectum	**Ross (54)**	0.06	0.003	4.76	0.07	4.92	0.19
	LD (66)	0.07	0.004	5.49	0.07	5.38	0.12

Intestinal density: intestine mass per unit of length; LD: Lohmann dual; Ross: Ross 308; length (%) and mass (%): the length and mass of individual intestinal segment as a percentage of the entire intestine; n: animal number; SEM: standard error of the mean.

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gradually decreased aborally to be least in the colorectum (Table 3). The length and mass of each of the intestinal segments is reported as a percentage of the entire intestine in Table 3. Regression analysis for each intestinal segment showed that the genetic line of the chickens had a significant influence only on the jejunoileum and colorectum. The jejunoileum of Ross birds was 6.2%longer (p ≤ 0.001) and 2.8% heavier (p = 0.015) than LD birds of equal BW adjusted $R^2 = 0.93$ and $R^2 = 0.98$, respectively. In contrast, the colorectum was 3.1% heavier in LD birds than in Ross birds of the same body weight, p = 0.015, adjusted $R^2 = 0.97$.

Intestinal histology

Jejunal villi had a finger-like appearance in birds of all ages in the study (Fig 1D). Ileal villi were shorter and had a more blunt appearance than those of the jejunum (Fig 1A). The height of the jejunal villus increased gradually with age in both genetic lines (Fig 5A). At d 35, the jejunal villus heights in Ross birds were 4 times higher than those at day 1. Whilst the jejunal villus heights in LD birds at day 63 were about 3 times higher than those of the one day old hatchlings. The greatest increase in jejunal villus height was from d 1 to d 7 in both genetic lines. On d 1, the jejunal villus heights were greater in LD birds than in Ross birds, however, by day 14 they were greater in Ross birds than in LD birds. Between d 21 and d 35 there was no difference between the two genetic lines (Fig 5A). The ileal villi were shorter than the jejunal villi in both genetic lines (Fig 5A and 5B). On the last day of the study, the ileal villus height was about 2 times greater than on the first day in both genetic lines. There were no significant differences in the ileal villus height between the age groups in both genetic lines (Fig 5B).

Regression analysis showed that the BW had a significant influence on the villus height of specific intestinal segments and that the genetic line of the chickens had an influence only on the jejunal villus height, p = 0.012, adjusted $R^2 = 0.76$. The jejunal villus height of LD birds was, on average, 4% larger than in Ross birds of the same BW.

In the first week of life, there was a marked increase in the jejunal and ileal crypt depth in both genetic lines that continued until day 14 in the jejunum but not in the ileum (Fig 5C and 5D). From then until the end of the study changes in crypt depths were insignificant. The crypt depth of the LD birds was smaller than those of the Ross birds at day 1 and 7 in the ileum and at day 32 in the jejunum.

The villus to crypt ratio increased gradually with age in both genetic lines and was greater in the jejunum than in the ileum (Fig 5E and 5F). At d 1 the jejunal ratio was significantly greater in LD birds than in Ross birds, whereas by d 28 this ratio was greater ($p < 0.05$) in Ross chickens. There was no significant line difference in the ileal ratio of both genetic lines between the age matched groups.

The jejunal epithelial height was significantly higher in LD birds (24.58 μm ± 0.87) than in Ross birds (20.81 μm ± 1.3) at day 1 but not thereafter (Fig 6E).

The mucosal enlargement factor of the jejunum was greater than that of the ileum in both genetic lines over the study period (Fig 6A and 6B). There was no difference in the mucosal enlargement factor of both genetic lines between matched age groups. Regression analysis showed that BW had a significant influence on the mucosal enlargement factor in jejunal and ileal segments and that the genetic line of the chickens had an influence only on the villus enlargement factor of the ileum, $p = 0.006$, adjusted $R^2 = 0.22$. In birds of the same BW, the ileal enlargement factor of Ross birds was, on average, 5.6% greater than that of LD birds.

Independent of genetic line, the tela submucosa of the intestinal wall was weakly developed and appeared to be almost non existent (Fig 1A, 1C and 1E). The tunica muscularis consisted of a thick inner circular layer and a thin outer longitudinal layer. The thickness of the tunica muscularis was greater in the ileum than in the jejunum in all birds sampled throughout the study (Fig 6C and 6D). Regression analysis showed that the BW had a significant influence on the thickness of the tunica muscularis in both intestinal segments and that the genetic line of the chickens had an influence only on the thickness of the tunica muscularis of the ileum, $p = 0.001$, adjusted $R^2 = 0.45$. The thickness of the ileal tunica muscularis in LD birds was on average 7.8% greater than in Ross birds of equal BW.

Transmission electron microscopy

The transmission electron micrographs of jejunal samples of both genetic lines showed that the enterocytes interspersed with goblet cells were attached to the basement membrane. The enterocytes were rectangular in shape and were characterized by a dense layer of regularly arranged microvilli on their apical surface. The large euchromatic nuclei of the enterocytes were located basally and their organelles were arranged primarily in the apical compartment (Fig 7A).

The microvilli of the enterocytes extended from the apical part of the cell as finger-like shapes containing filamentous and glycocalyx formations in birds from both genetic lines at all ages (Fig 7B). From d 1 to d 35 the average microvillus length ranged from 2.04 μm to 2.58 μm. In the LD birds the microvillus length averaged 4.03 μm by day 63 (Table 4).

The junctional complexes in both genetic lines had a similar form and electron density. They occurred close to the lumen along the lateral surfaces of adjacent epithelial cells. Each consisted of three sequentially arranged structures; a tight junction (zonula occludens), an intermediate junction (zonula adherens) and a desmosome (macula adherens) (Fig 1F). The tight junction resides adjacent to where the plasma membrane reflects from the apical to the lateral surfaces of the enterocyte. It was characterized by fusion of the adjacent cell membranes over varying distances resulting in obliteration of the intercellular space. The intermediate junction linked the tight junction to the desmosome below. It was characterized by the presence of an intercellular space, of low density material. The desmosomes were characterized by a intercellular space having an electron dense core plus the presence of a surrounding electron-dense disc (Fig 1F; Fig 8B and 8C). Table 4 reports the mean length of the junctional complex and the tight junction. At all ages and in both genetic lines, the tight junction involved about 48% of the junctional complex length (Table 4).

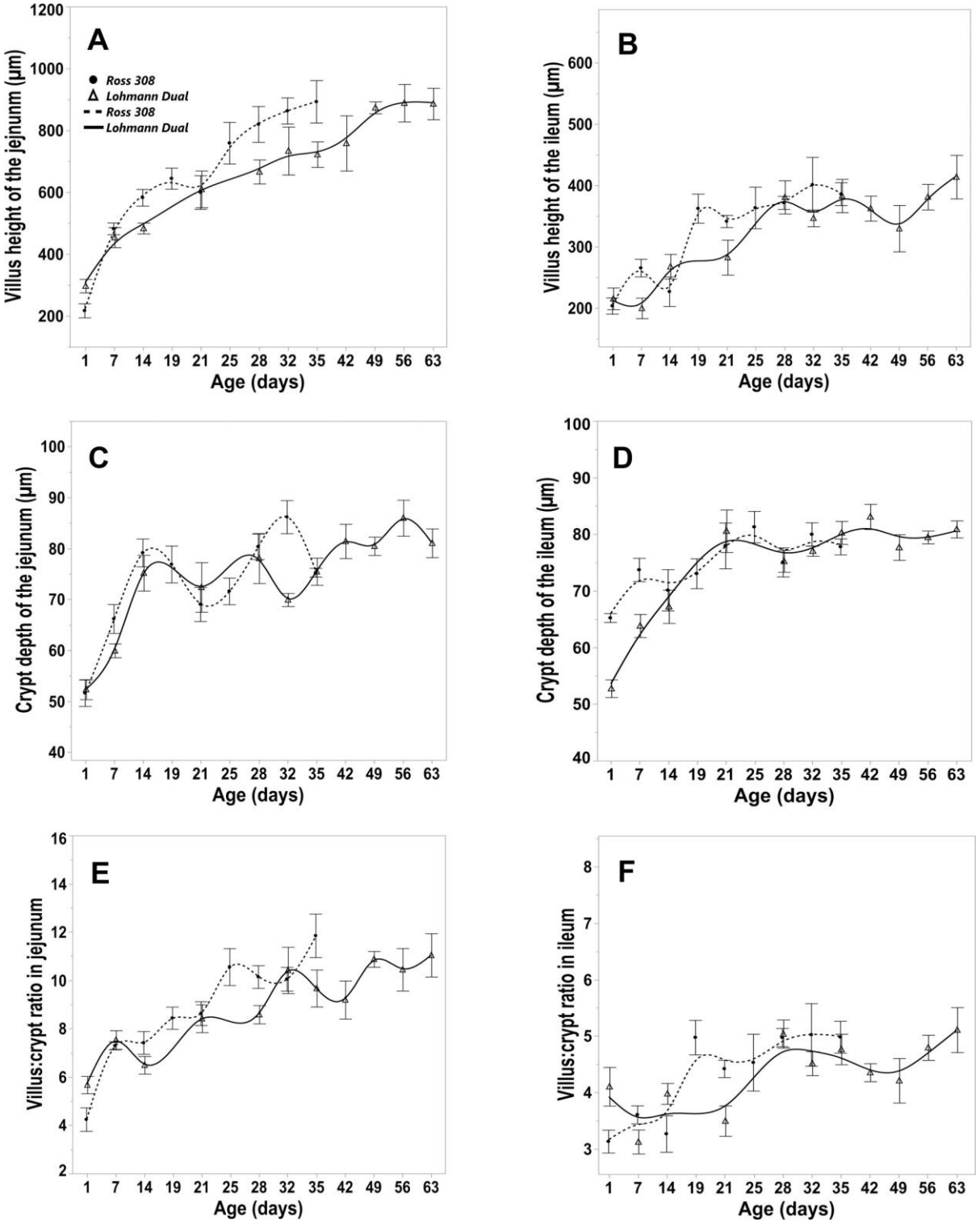

Fig 5. Morphometric parameters of the intestinal tunica mucosa for LD and Ross 308 chicken lines. Trendlines of changes in the villus height of the jejunum (A) and ileum (B), crypt depth of the jejunum (C), crypt depth of the ileum (D), villus crypt ratio in jejunum (E), villus crypt ratio in ileum (F). Bars refer to mean ± standard error of the mean of the sampled chicken at each time interval.

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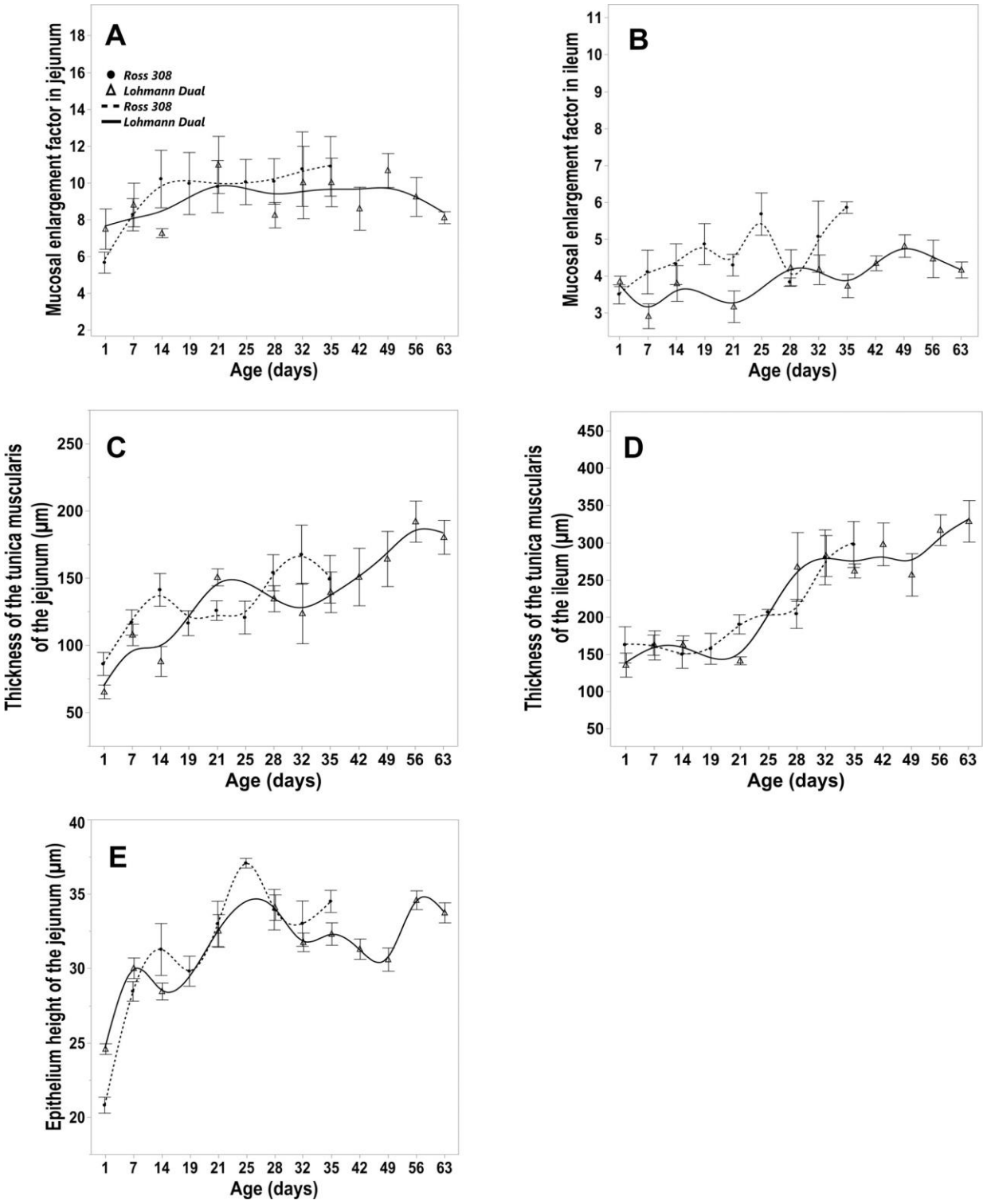

Fig 6. Morphometric parameters of the intestinal tunica mucosa and tunica muscularis for LD and Ross 308 chicken lines. Trendlines of changes in (A, B) mucosal enlargement factor in jejunum (A) and ileum (B), thickness of the tunica muscularis of the jejunum (C) and ileum (D), epithelium height of the jejunum (E). Bars refer to mean ± standard error of the mean of the sampled chicken at each time interval.

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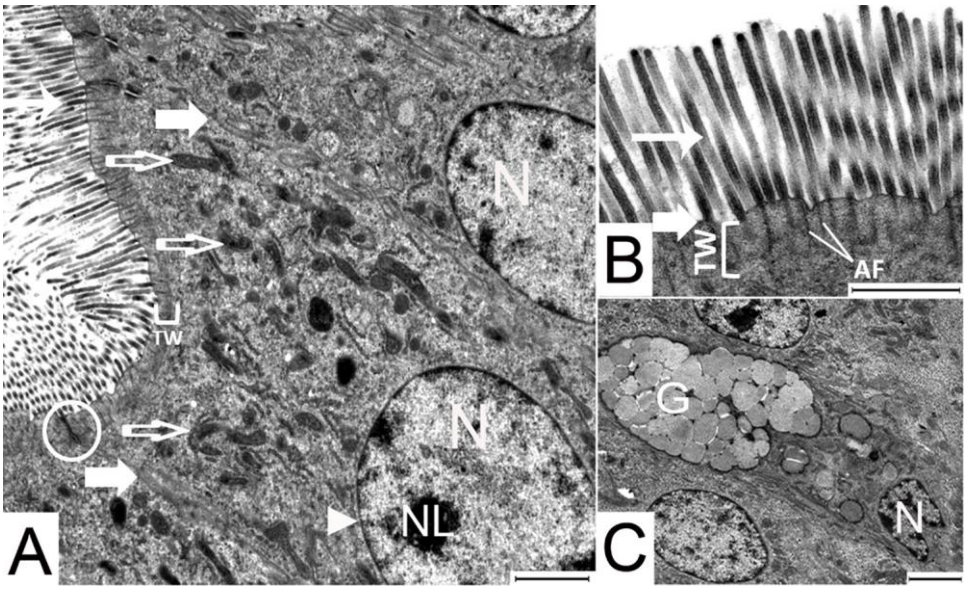

Fig 7. Transmission electron micrographs of jejunal enterocytes of 1 day old LD birds. (A) the organelles are arranged towards the apical end of the enterocyte, (B) microvilli, (C) goblet cell flanked on both sides by enterocytes. Arrow head: nuclear membrane; broad arrow, cellular membrane; circle, junctional complex; empty arrow, mitochondria; narrow arrow, microvilli; AF, actin filaments; G, secretory vesicles containing mucin; N, nucleus; NL, nucleolus; TW, terminal web. Bar: 2000 nm.

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The observations of the electron micrographs showed that, the enterocyte's cytoplasm on day 1 contained many lysosomes and free ribosomes in both genetic lines. The enterocytes of the Ross birds appeared to have greater concentration of lysosomes (Fig 8A and 8B) and free ribosomes (Fig 8C and 8D) than those of LD birds. By day 7 the enterocyte's lysosomes had disappeared and there were fewer free ribosomes. Mitochondria having different forms such as; rod-like, oval-shaped, and tadpole-shaped, were present throughout the time-line of the study in both genetic lines of the chicken. On day 1, the mitochondria were scattered throughout the cytoplasm. Subsequently by day 7 they were seen to be aggregated in the perinuclear

Table 4. Length of the microvillus, the tight junction and the junctional complex in the jejunum of both chicken lines versus day post hatching.

Age (days)	Line	Microvilli Length (μm)		Tight junction length (μm)		Junctional complex length (μm)		TJ:JC %	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	Ross	2.42	0.03	0.64	0.27	1.33	0.55	48.12	1.77
	LD	2.45	0.02	0.54	0.15	1.10	0.28	48.88	1.59
7	Ross	2.04	0.08	0.59	0.26	1.21	0.54	48.77	0.93
	LD	2.58	0.03	0.54	0.17	1.13	0.34	47.90	1.62
21	Ross	2.22	0.05	0.55	0.24	1.15	0.51	48.04	1.46
	LD	2.43	0.02	0.47	0.16	0.97	0.31	47.81	2.31
35	Ross	2.37	0.02	0.59	0.15	1.22	0.32	48.89	0.85
	LD	2.18	0.03	0.46	0.13	1.00	0.36	47.31	3.78
63	LD	4.03	0.04	0.53	0.15	1.09	0.29	48.04	1.83

JC: junctional complex; LD: Lohmann dual; Ross: Ross 308; SEM: standard error of the mean; TJ: tight junction; TJ:JC %: the tight junctions length as a percentage of the junctional complex length.

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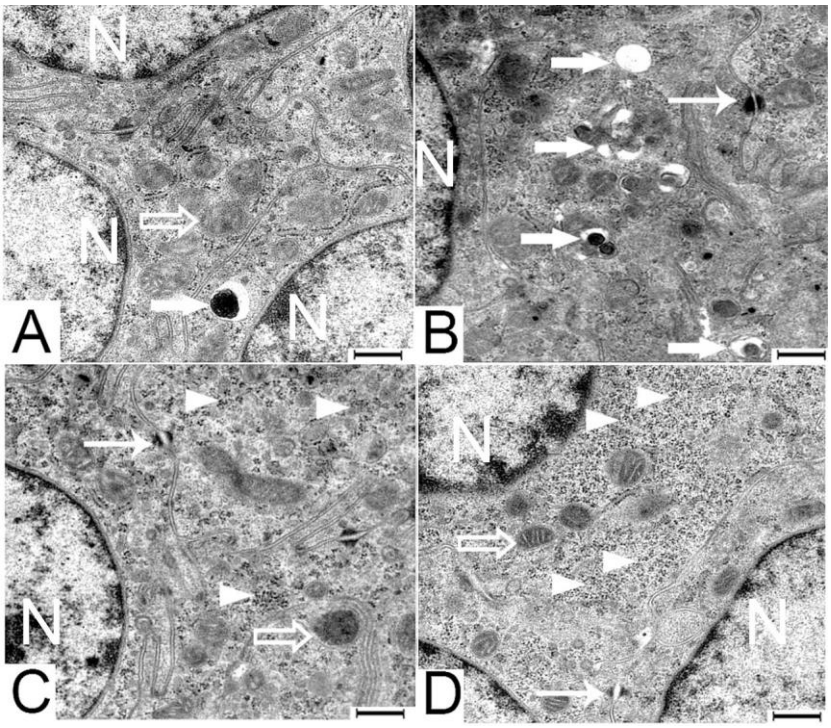

Fig 8. Transmission electron micrographs of jejunal enterocytes of 1 day old LD [A and C] and Ross [B and D] chickens. Arrow head, free ribosomes; broad arrow, lysosomes; empty arrow, mitochondria; narrow arrow, desmosomes; N, nucleus. Bar: 500 nm.

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region as well as in the apical part of the epithelial cells. Over time the number of mitochondria in the enterocytes increased in both chicken genetic lines.

The goblet cells are a simple columnar epithelial cell that were similar in size to their coexisting enterocytes. Their narrow bases were attached to the basement membrane and their cell bodies extended through to the lumen (Fig 7C). The cup shaped apical part of the goblet cells were filled with mucus granules of differing electron densities. Their ovoid nuclei were located basally in the narrow stem-like portion. Most other organelles lay between the nucleus and the mucus granules.

Discussion

The breeding of dual production chicken lines with high performance females for laying eggs and males being raised for meat production is an alternative to the culling of one-day-old male chickens of laying lines. Lohmann Dual, a recent commercial dual-purpose breed developed by crossing meat and layer lines and using the sex-linked dwarf gene, has acceptable performances in both meat and egg production [3]. Over the 70 days of their life their feed conversion ratio is 1:2.5 which is better compared to that of Lohmann Brown cockerels 1:4 [12].

The highly selected broiler line (Ross 308) and the Lohmann Dual line (LD) reported in the present study had markedly different growth patterns in their body weight and in gastrointestinal segmental morphology over the time frame of the study. The mean BW of Ross 308 chicks was significantly higher than that of the LD chicks at all ages since Ross's genetic makeup made it more prone to an increased BW compared to LD over a short time frame.

In 2015 Damme et al. examined the meat production of three commercial dual-purpose chicken lines; Lohmann Dual, Walesby Specials and Dominant Red Barred. After 84 days, the

male chickens of the LD had a BW of 3165 g while the Walesby Specials and Dominant Red Barred had BWs of 2423 g and 1818 g, respectively [3]. However Habig et al. (2016) reported that the BW of the LD males reached only 1803 g after 77 days [13]. This contrasts dramatically with our finding that LD birds reached 2012 g by 63 days. These disparities may be a result of either different feed and husbandry conditions or the effect of individual differences in the growth rate in this chicken line as reported by Urban et al. (2018) [14].

A significant side effect of the intensive genetic selection of high performance meat production chicken lines has been a disruption in the balance between the relative slow growth of their organs and their extremely rapid increase in muscle mass [15–18]. Our results confirm this side effect where the normalized mass and relative length of the gastrointestinal of the slow growing LD birds were greater than those of the fast growing Ross chicken. The high normalized mass and great relative length of the gastrointestinal tract reported in this study of LD birds corresponds to those observed in slow growing chicken lines [4, 19, 20].

In this study the intestinal mass had positive allometric growth over the first week post-hatching but subsequently negative allometric growth for the remainder of the study period in both genetic lines. Similarly the greatest increase in jejunal villus height occurred over the same period. This reflects the chicken's conversion from a reliance on yolk sac constituents to the components of their solid diet over the first week of life [21]. Subsequently throughout their life, the relative weight and length of their intestine decreased whilst overall villus heights increased. This most likely enables the chicken to absorb nutrients efficiently as their metabolic demands rise with increasing body mass over time [7]. When chickens get older, the small intestinal absorptive capacity depends more on increasing overall villus surface areas rather than increases in the intestinal length and mass [7].

From day 1 the relative intestinal length and from day 7 the normalized mass of the gastrointestinal segments decreased significantly in both lines until a specific time, after which the decrease became insignificant. This suggests that a balance between the growth rates of gastrointestinal segments to the body weight had been established at this time point. This balance occurred earlier in Ross chicks than in LD chicks where; normalized glandular stomach mass was 7 d later, normalized gizzard mass was 21 d later and intestinal relative length was 17 d later. Similar findings for gastrointestinal tract segments have been reported in four different genetic lines of ducks, where the maximal growth rate of the gastrointestinal segments was earlier in the fast-growing genetic lines than in the slow-growing genetic lines [22]. This suggests there is a temporal discrepancy between the development of the organs and the final body weight. The same phenomenon occurred between LD and Ross. Here the time-lines to reach the balance time of each gastrointestinal tract segment and reach the final body weight of 2000 g was different in each genetic line. To reach a BW of 2000 g LD birds needed 4 more weeks than Ross birds. In general, the different growth rates are due to a complex interaction of genetic, physiological and environmental factors including; diet composition, diet form, feeding strategy [23]. In this study, the genetic influence appears more relevant than others since both lines were maintained under similar conditions. Most likely these differences in body growth rates in our study may have anatomical reasons, since the normalized, stomach and intestinal segment masses as well as relative lengths of LD birds took 1–3 weeks longer in LD birds than in Ross birds to reach a stable level.

In this study regression analysis indicated that LD birds had a larger gizzard, shorter intestine and smaller mucosal enlargement factor than did Ross birds of the same BW. Recently a morphometric study on the gastrointestinal tract of two broiler chicken lines genetically selected for a high digestion (D+) or a low digestion (D−) efficiency was reported [4]. Although the D- birds consumed more feed and had a greater villous surface area than the D + birds, the D+ birds had faster growth rates than the D− birds. However, the ability to digest

starch, protein and lipids was shown to be lower in the D− compared with D+ line. The glandular stomach and gizzard were significantly lighter and the length and weight of the small intestine, villus width and surface area and the tunica muscularis thickness of the D- birds were significantly greater than in D+ birds. The authors suggested that the intestinal adaptations of the D− birds may be an attempt to compensate for the low functionality of their gastric area [4].

Taylor and Jones (2004) fed Ross 308 chickens for 42 days on a pelleted diet, of wheat or barley that was either whole grain or ground. They found that the whole grain diet reduced the relative mass of the glandular stomach but increased the relative mass of the gizzard. The birds fed whole grain diet had a highly viscous alkaline ileal digesta together with a decreased relative mass of each intestinal segment [24]. The authors suggested that an active gizzard increased starch availability in the intestine and that this is associated with enhanced peristaltic movement and influenced the mass of each intestinal segment [24].

The villus height in chickens decreases gradually from orad to aborad along the small intestine [25]. This is due to the greater absorptive capacity of the duodenum followed by that of the jejunum and then the ileal villi [25]. The intestinal histological modifications are mostly related to the availability and nature of the nutrients within the intestine, for example, the small villi can undergo compensatory enlargement due to the intestine having elevated nutrient levels over a period of two to five days [26, 27].

Our study supports the idea that the more muscular gizzard in the LD birds increases the degree of food processing and enhances the availability of nutrients in the proximal small intestine where gut plasticity is more pronounced [24, 28]. This is mirrored histologically by more elongated jejunal villi that in turn lead to the ileum receiving digesta having lower nutrient levels resulting in a decreased ileal absorptive surface area in LD birds compared to Ross birds of the same BW.

Because the tunica muscularis determines the rate and power of intestinal motility it governs the progression of a bolus and that in turn affects the absorption processes by increasing or decreasing the contact between the mucosa and the intestinal contents [4]. A thick tunica muscularis and a shorter intestine in LD birds could lead to a more rapid intestinal passage time and a lower uptake of available nutrients.

Whilst in our study the husbandry and nutrition were the same for both chicken lines there are many factors that can influence the growth rate of the gastrointestinal tract and its morphological characteristics. The differences between the two genetic lines in intestinal length and gizzard weight are probably due to genetic differences and are important criteria for ongoing selection for improved performance of the LD male birds.

In the current study epithelial cells of day old Ross chicks had greater concentration of lysosomes than did LD chicks. Yamauchi et al (1992) presumed that these lysosomes were residual bodies of lipid digestion derived from the yolk sac [29]. Noble and Cocchi 1990 reported that during the incubation period of the chick, 80% of the yolk lipid content is mobilized and absorbed by embryonic tissues. The metabolism of the yolk lipids continues after hatching and is sufficient for the chick's metabolic maintenance for several days post-hatching [30]. Our study corroborates these observations as we also noted that the lysosomes present at hatching had disappeared entirely within 7 days. It is believed that the growth of the small intestine of birds after hatching occurs without the formation of additional villi [31]. This indicates that the villi that exist at hatching only change in size over time [31]. With age, the increase in villus size is limited. This is compensated for by increases in the microvillus length that continue with age thus enlarging the mucosal surface area [32]. Bohórquez et al. (2011) have reported a similar tendency in the growth of microvillus length of the turkey's intestine from 1.90 μm at d 1 to 3.11 μm at 12 d post hatching [33]. In our study, this phenomenon was observed only in

LD chicken between day 35 and day 63. The change in the microvilli length was limited when the increase in the villi height was permanent i.e. until day 35 in both chicken lines. Where the increase in the microvilli was obvious when the increase in the villi height was limited i.e. between day 35 and 63 in the LD chicken.

Tight junctions form a selective permeability barrier across the epithelial cell layer that are crucial for normal epithelial functions. The barrier functions of tight junctions are largely a consequence of their complex molecular composition [34, 35]. In our study of hatchlings through to 35 d or 63 d for Ross 308 and LD chickens respectively, the junctional complexes were fully developed throughout the entire study. This suggest that the intestine of the LD birds have the same barrier function on the level of the junctional complex compared to those observed in Ross chicken.

Conclusion

The gastrointestinal tract of the LD birds was not affected by the breeding and selection criteria used in the development of this dual purpose chicken line. At gross anatomical, histological and ultrastructural levels the gastrointestinal tract of the LD birds grew proportionately to the increase in body weight without any abnormalities or deformations being observed. However, there were several anatomical differences between the LD birds and the more rapidly growing birds Ross 308 birds that may contribute to the slower growth rate of LD birds. We suggest that LD chickens have a lower nutrient absorption capacity due to their shorter intestine and smaller intestinal mucosal surface area that result in a slower body growth rate than found in Ross chickens. Moreover, the earlier establishment of the time of a balance between the growth of gastrointestinal tract segments and the overall increase in body weight in Ross chickens compared to LD chickens is noteworthy. The earlier balance time-point in the Ross birds is possibly an indicator of their better growth and performance. Thus anatomical characteristics of the gastrointestinal tract such as the time of body weight-organ balance, intestinal length and intestinal mucosal surface area could be considered as criteria for the ongoing selection of the LD birds to improve their performance and to optimize feeding strategies. However, the interactions between feeding regimens to gizzard and intestine development needs further investigation.

Supporting information

S1 Table. Mean and standard error of the mean (SEM) of body weight, mass and normalized mass of the glandular stomach and gizzard in Ross and LD chickens. BW: body weight; LD: Lohmann Dual; Ross: Ross 308; n: animal number.
(DOCX)

S2 Table. Mean and standard error of the mean (SEM) of body weight and entire intestinal length, mass, normalized mass and relative length in LD and Ross chickens. BW: body weight; LD: Lohmann Dual; Ross: Ross 308; n: animal number.
(DOCX)

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4 Structure and age-dependent growth of the chicken liver together with liver fat quantification: A comparison between a dual- purpose and a broiler chicken line

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RESEARCH ARTICLE

Structure and age-dependent growth of the chicken liver together with liver fat quantification: A comparison between a dual-purpose and a broiler chicken line

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Abstract

Rearing dual-purpose chickens is a practicable approach to avoid culling one-day-old male layer chicks. The present study examined the impact of a conventional fattening diet on the liver of a novel dual-purpose chicken line (Lohmann Dual, LD) in comparison to a broiler (Ross 308) chicken line. Age-related changes of structure and lipid content of the liver were assessed. One hundred twenty and newly hatched chicks (LD = 66, Ross = 54) were kept under the same husbandry conditions and fed a commercial diet for 5 weeks for Ross and 9 weeks for LD. Six birds of each line were examined weekly. Their body weight (BW) and liver mass were recorded. Microscopic structure and ultrastructure of the liver were investigated and the liver lipid content was measured using a pre-validated method. During the study period, liver mass increased with age, while normalized liver mass decreased. Furthermore, liver mass of Ross birds was greater than that of LD birds of the same BW. Overall, no significant differences were observed in the hepatic structure or ultrastructure between the two chicken lines. The hepatic lymphatic aggregations were without fibrous capsules and their number and area increased throughout the first week, then the values began to fluctuate with age in both chicken lines. The changes in the liver lipid content of the two chicken lines were within the normal physiological range over the term of the study. The liver lipid content correlated negatively with age and body weight in both lines. It was the highest on the first day then decreased until day 7 and thereafter did not change in both chicken lines. However, given the same body weight, the Ross chickens had a 9% greater liver lipid content than LD chickens. It is concluded that there is no apparent adverse effect of a high-energy diet on the liver of LD chickens.

role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

An alternative to avoid killing male hatchlings of high-performance egg-laying chickens is to use dual-purpose chickens for both meat and egg production [1]. Efforts already underway in developing such dual-purpose lines include a new hybrid chicken line, the Lohmann Dual, developed by crossing highly specialized meat and egg chicken lines [2]. The Lohmann Dual chickens have excellent growth and feed efficiency parameters when compared with other dual-purpose chicken breeds [2]. However, for further advances utilizing these intensive genetic selection processes to succeed, there is a great need for reliable data on the baseline anatomical and physiological responsiveness of the gastrointestinal tract and the liver of LD chickens when fed high-energy diets. In a recent study, we showed that LD chickens have a heavier gizzard, shorter intestine and smaller intestinal absorptive surface area than the highly specialized broiler breed, Ross 308, chickens [3]. These morphological features mirror the dynamic interface of the feed and its utilization within the gastrointestinal tract. In a similar manner, it is important to consider the role of the liver in the growth and development of highly specialized genetic lines of production birds. The intensive production conditions for rapid growth rate associated with increased metabolism have resulted in an increased workload by the liver [4, 5].

The liver is the largest accessory gland of the avian digestive system and lies immediately caudal to the heart and lung complex. The liver is encapsulated by a thin tough capsule. Internally the liver is arranged into a series of interlinked hexagonal hepatic lobules. Each hepatic lobule has a portal canal (hepatic portal vein, proper hepatic artery, bile ductule, lymphatic vessels and vagus nerve branch) at each corner. Radially arranged linear cords of hepatocytes link the portal canals to a central vein. The basal surface of adjacent hepatocyte cords abuts elongate sinusoids that drain into the central vein. Between the hepatocytes and the sinusoidal endothelial cells are perisinusoidal spaces. The apical face of adjacent hepatic cords formed slender bile canaliculi that drain centrifugally to the nearby portal canal. A sparse collagen III fiber meshwork supports hepatocytes and sinusoids [6]. Sparse irregularly shaped lymphatic aggregations containing mainly lymphocytes are scattered throughout the liver parenchyma. In turkeys, both encapsulated and non-encapsulated lymphatic aggregations are present [7].

The liver has numerous key functions in the storage and conversion of metabolites [4]. In birds, the liver plays a major role in the synthesis and metabolism of lipids, notably because lipogenesis takes place primarily in the liver, unlike in mammals, where the adipose tissues are the site for lipogenesis [5]. *De novo* lipogenesis comprises those metabolic pathways that are involved in the synthesis of triglycerides from non-lipid precursors most commonly from dietary carbohydrate. [5]. Unfortunately, the high-energy diets used in the commercial poultry industry such as high carbohydrate diets stimulate hepatic lipogenesis [8]. Under some of these feeding regimes, pathological conditions such as the fatty liver and kidney syndrome in broilers arise [4].

This study aimed to investigate the structure of the liver in relation to age in a dual-purpose chicken line (Lohman Dual) and to compare it to that of a highly selected broiler chicken line (Ross 308). Histologically, the overall fat content of the liver was determined. The number of lymphatic aggregations and their area were measured. Ultrastructural details of the hepatocytes and sinusoids, as well as endothelial cells, hepatic stellate cells and Kupffer cells were examined using transmission electron microscopy. In addition, the area and diameters of lipid droplets were measured.

Material and methods

Birds and management

Two groups of eighty male chicks, the first a commercial broiler line (Ross 308) was obtained from BWE-Brüterei-Weser-Ems GmbH & Co. KG, Visbek, Germany and the second a novel dual-purpose line (Lohmann Dual, LD) was supplied by Lohmann Tierzucht, Cuxhaven, Germany. The chickens were reared in accordance with German animal welfare law. The study was approved by the Animal Welfare Committee "Landesamt für Gesundheit und Soziales", Berlin, Germany, ID: 0236/15.

The chickens of both lines were kept under the same husbandry conditions until they reached a body weight (BW) of 2000 g, i. e. 35 days for Ross chickens and 63 days for LD chickens. The husbandry conditions and the composition of their diet were published previously [3]. The chickens had ad libitum access to a mash diet and water for the duration of the study. A starter diet (231.5 g protein and 12.6 MJ ME/kg) was fed from hatching to day 14 and then they were fed a grower diet (214.4 g protein and 13 MJ/kg) from day 15 to the end of the study. Six birds were sampled from each group in a post hatching time series: Ross = 1, 7, 14, 19, 21, 25, 28, 32 and 35 day(s), LD = 1, 7, 14, 21, 28, 32, 35, 42, 49, 56 and 63 day(s). On the sampling day, the birds were selected at random from the genetic line being examined, their live BWs were recorded, and then the birds were killed by decapitation.

Macroscopic examination

Immediately after a bird's death, its abdominal cavity was opened by a mid ventral abdominal incision, and the liver excised. The liver was dissected free of ligaments and associated blood vessels and weighed to an accuracy of 0.01 g on an electronic laboratory balance (AND HF-200G, Tokyo, Japan).

The normalized mass of the liver was calculated as [liver mass (g)/total BW (g)] × 100. The weekly weight gain of liver was calculated using the following relationship: (the mean liver mass in the present week − the mean liver mass in the previous week)/ the mean liver mass in the previous week) × 100.

Specimen preparation for light microscopic examination

For light microscopy, a sample (1x1x1 cm) was excised from each bird from the most caudal part of the right liver lobe (Fig 1A), and then immersed in neutral buffered formalin (4%, pH 7, 20–24˚C) for 24 hours. Subsequently samples were dehydrated in an ascending graded series of ethanol, embedded in paraffin, serial sectioned at 5 μm and stained by Meyer's Haematoxylin and Eosin (H &E).

Basic microscopic examinations of the overall liver tissue components, including lymphatic aggregations were undertaken.

Using the imaging software, NIS-Elements AR (Nikon Instruments Inc., U.S.A.), the number and the surface area of lymphatic aggregation per section per bird were estimated. At first, the surface area of each section of liver was determined and then the number of the lymphatic aggregation per 1 mm^2 and the percentage of the surface area of the lymphatic aggregations in regard to the whole liver section was calculated. (Fig 1B).

Lipid content measurements

Validation of the histological method used in this study. Here we determined the lipid content in the livers of ten 15-week-old New Hampshire × White Leghorn chickens collected, on the day of slaughter using, both chemical and histological methodologies.

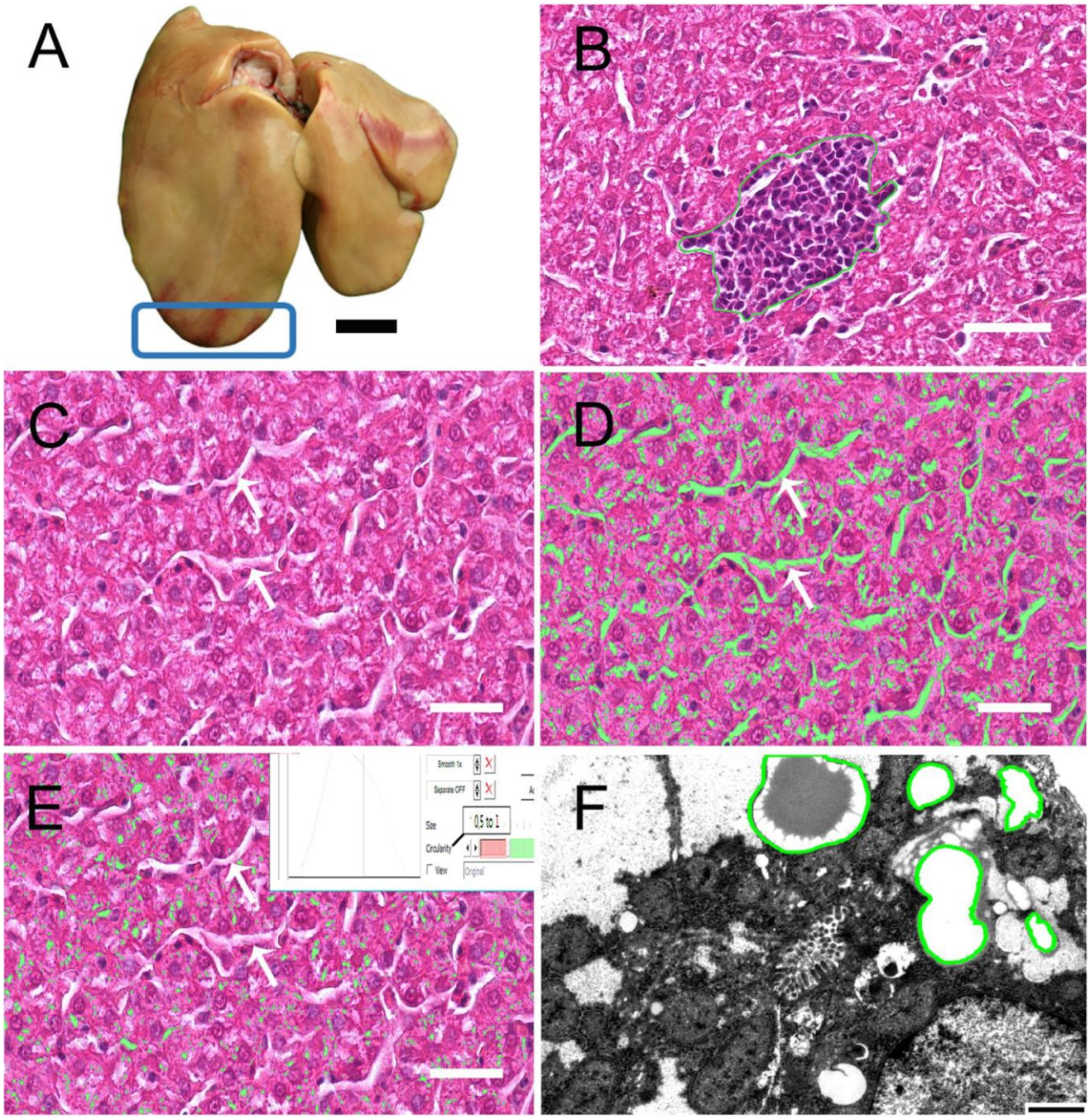

Fig 1. Determination of the lipid content and area and number of lymphatic aggregations in the liver. (A) parietal surface of the liver of LD chickens with the sampling site boxed for light and transmission electron microscope. (B) lymphatic aggregation outlined in green. (C) cross section of liver stained with Haematoxylin and Eosin, where arrows are sinusoids. (D) lipid content determination using a colour thresholding filter: fat accumulations and sinusoids highlighted (green) areas. (E) unspecific selection of the sinusoids (arrows) was eliminated by using shape filter with circularity degree of 90%. Bar: 1 cm for A and 25 μm for B, C, D, E, light microscopy, H&E stained. (F) liver lipid droplets outlined in green. Bar: 1000 nm for F, transmission electron microscopy.

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For the chemical analyses the lipid content of the left liver lobe of each bird was extracted using petroleum ether according to Weende Analysis [9]. The analyzed lipid content of the liver was reported as a percentage of wet liver mass.

For the histological evaluation of lipid content, samples (1x1x1 cm) of the most caudal part of the right liver lobes were fixed in neutral buffered formalin (4%, pH 7, 20–24˚C) for 24 hours. Then they were dehydrated in an ascending graded series of ethanol and embedded in paraffin wax. During the routine paraffin embedding process, lipids are removed from the liver tissue leaving behind empty circular spaces.

Serial sections were cut at 5 μm with a microtome (Jung, Histoslide, 2000 Sliding, Wetzlar, Germany). Cross-sections of liver were stained with Meyer's Haematoxylin and Eosin (H&E) according to standard histological protocols [10].

For quantitative histological analyses of lipid content in the liver, an analyzing system (NIS-Elements AR, Nikon Instruments Inc., U.S.A.) was used. Ten fields of view (area of each field = 33333.19 μm^2) at a magnification of 400× from around the portal canal area and distant to the large blood vessels of each sample were selected using a light microscope (Axioskop, Carl Zeiss, Jena, Germany).

Fat content was assessed by evaluating the percentage of the area occupied by lipid droplets inside the liver parenchyma. A lipid droplet was considered to be any roughly circular shaped, non-staining area (bright empty spaces) of the section (Fig 1C).

To determine the percentage of lipid droplets a chromatic followed by a morphological analysis was conducted on each field of view according to Liguori et al. (2009) [11]. In the first step a colour thresholding filter was used that highlighted all white-coloured spaces (Fig 1D) such as fat accumulations and sinusoids. In the second step a series of circular shape filters ranging from 30–70% circularity were employed to eliminate artifacts such as sinusoids.

The mean ± SD of the liver lipid content (%) derived by chemical analysis was 3.63 ± 0.79. The liver lipid content (%) estimated histologically, decreased with increasing circularity of the shape filters (Table 1). This ranged from 6.37 ± 1.01 to 2.18 ± 0.38 using 30% to 70% circularity shape filters (Table 1). To determine at which degree of circularity the closest match between the chemical and the histological analyses occurred, statistical analysis was done using Spearman's rank correlation and one-way ANOVA with Dunnett t-test for multiple comparisons. P ≤ 0.05 was considered significant. The best match between the chemical and the histological analyses was obtained with filters having a degree of circularity ≤ 50% (Fig 1E). Subsequently this filter was used to measure the lipid liver content in the LD and Ross chicken liver samples.

Specimen preparation for electron microscopic examination

Samples of the most caudal part of the right lobe (0.5×0.5×0.5 cm) from two LD and two Ross birds on days 1, 7, 14, 21, 28 and 35, as well as on day 63 for the LD line were taken. The preparation of samples for electron microscopic examination was described previously [3]. Briefly, samples were fixed in Karnovsky solution, then washed in 0.1 M cacodylate buffer and incubated in 1% osmium tetroxide for 120 min. After dehydration in ethanol, the tissues were embedded in a mixture of epoxy resin, DDSA (softener), MNA (hardener) and DMP 30 (catalyst). Semi-thin sections were cut on an ultramicrotome and stained according to a modified Richardson protocol [10] for determination of the area of interest under a light microscope. The ultrathin (80 nm) sections were mounted on nickel-grids and examined with a transmission electron microscope (TEM, Zeiss EM 900; Oberkochen, Germany).

In the assessment of the TEM images, we considered a primary lysosome to be a membrane-bounded vesicle that is produced by the Golgi apparatus. Secondary lysosomes are formed when primary lysosomes fuse with phagosomes. Secondary lysosomes are larger than primary lysosomes and capable of releasing their content.

For each bird, the diameters of 40 lipid droplets as well as the number and area of the lipid droplets in 10 randomly selected fields of view (The area for each field = 244 μm^2) (Fig 1F)

Table 1. Histological analysis of the liver lipid content using shape filters compared to the lipid content derived from chemical analysis.

Method	Lipid content (%)		Dunnett t-test	Correlation	
	Mean	SD	p-value	r	p-value
Chemical analysis♦	3.63	0.79	n/a	n/a	n/a
No shape filter	26.47	7.27	◆ 0.001	0.19	0.603
30% circularity filter	6.37	1.01	◆ 0.001	0.56	0.090
40% circularity filter	4.91	0.79	0.001	0.65	0.043
50% circularity filter	3.78	0.61	0.990	0.64	0.048
60% circularity filter	2.90	0.47	0.093	0.62	0.054
70% circularity filter	2.18	0.38	◆ 0.001	0.53	0.111

n/a, not applicable; r, Pearson's correlation coefficient; SD: standard deviation

♦ lipid content (%) in wet liver.

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were measured manually using a NIS-Elements AR analyzing system (Nikon Instruments Inc., U.S.A.). The number of lipid droplets in 1000 μm^2 of the liver section and the area of the lipid droplets (%) were calculated.

Statistical analysis

Data were analyzed using the statistical package program IBM SPSS Statistics 23 (IBM Corporation, New York, USA). The graphs presented were made by using the statistical package program JMP Pro 13 (SAS Institute Inc., Cary, USA). Comparisons of the two lines of the same age groups were performed using the Mann–Whitney U test. The relationship between the liver mass or lipid liver content and age was assessed using one-way analysis of variance (ANOVA) with the post hoc test Least Significant Difference (LSD). To explore the effect of chicken line and BW on the liver mass and lipid liver content, all data collected were regressed against the genetic line and BW using the log-log regression model. To test the relation between fat deposition in the liver and the body weight as well as the age, Pearson's correlation coefficient was used. A p value of < 0.05 was considered significant.

Results

Gross anatomy

Once the abdominal cavity was opened and the sternum and ribs were elevated, the liver was visible lying ventrally in the viscus. The liver's colour varied depending on the bird's age. The liver was pale-brown on the day of hatching due to the abundant yolk pigment but by the end of the first week, it darkened to a light brown. Thereafter, the colour ranged from light brown to reddish brown.

The liver mass of the LD chickens increased by 0.51 g/day (d) from d 1 to 35 post-hatching, whereas the liver of Ross chickens increased by 1.14 g/d over the same period. From d 1 to 63 the LD liver mass increased by a rate 0.52 g/d. The liver grew gradually with age in both lines (Fig 2A), with the mass of the liver increasing by 19.1 times in LD birds and 20.87 times in Ross birds by the end of the study. The greatest weight increase of the liver (196.66% for Ross and 107.73% for LD) was over the first week of life for Ross and over the second week for LD chickens. After day one, the liver masses of LD chickens were lighter than those of Ross chickens ($p < 0.01$). On the first day of life the normalized liver mass peaked at 4.2 ± 0.53% for LD and 3.7 ± 0.4% for Ross chickens, after which there was a gradual decrease in the contribution

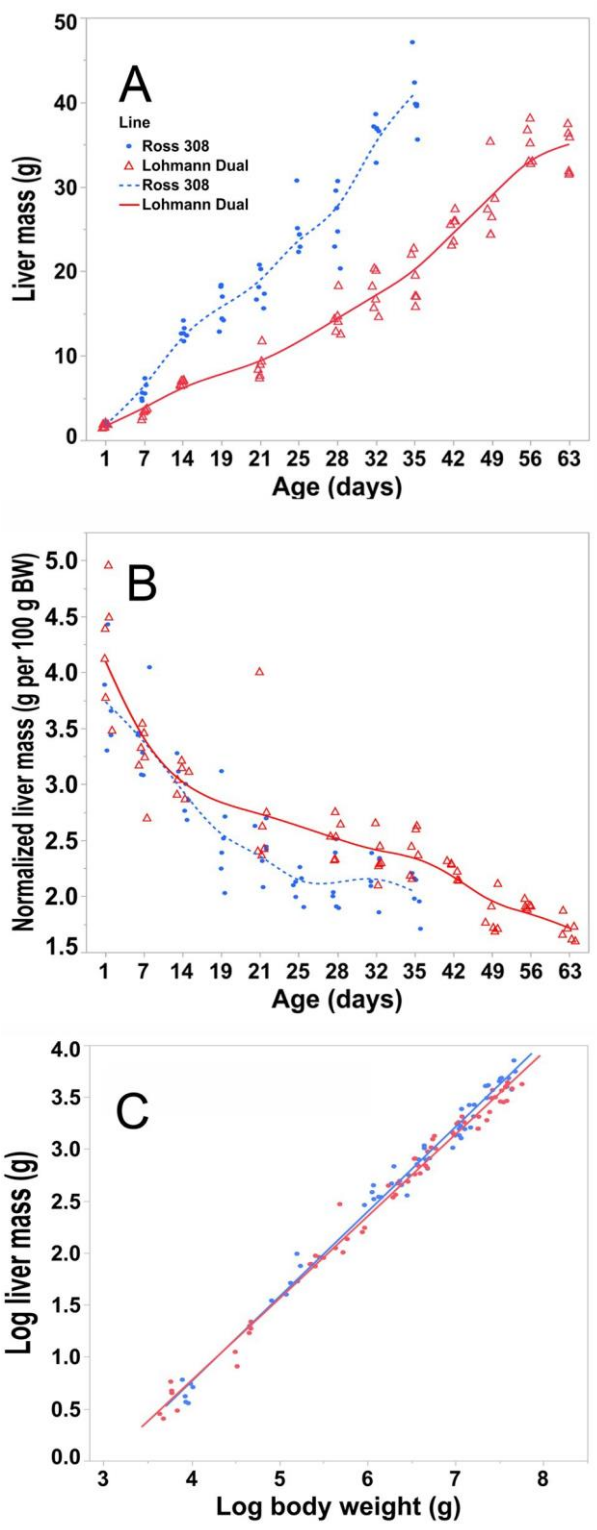

Fig 2. Liver mass and normalised mass of both chicken lines. Trendlines of the changes in mass (A) and normalised mass (B) of livers versus days post hatching for Ross and LD chicken lines. (C) linear regression line of logarithm of liver mass versus logarithm of total body weight for both chicken lines. Symbols represent each individual value for each chicken line. BW, body weight.

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of the liver to overall body weight (Fig 2B). The liver of LD chickens contributed a greater percentage of total body weight on days 28, 35 than that of Ross chickens ($p < 0.05$).

According to regression analysis, both body weight and chicken line had an influence on the liver mass, p ◆ 0.01, adjusted $R^2 = 0.99$. The liver mass of Ross birds was heavier on average by 2.3% than that of LD birds of the same BW (Fig 2C).

Microscopic examination of the liver

Light microscope. The overall histological structure and appearance of the liver of both genetic lines was similar. No pathological changes were observed in the chickens' livers.

The liver surface was covered by a thin loose connective tissue layer. Below this layer was a thin capsule of dense connective tissue that extended into the liver lobes and divided the liver into lobules. However, interlobular connective tissue was scant and difficult to distinguish. The hepatocytes were grouped into hepatic lobules. At the periphery of each lobule portal canals were found that consisted of the interlobular branch of the hepatic artery, interlobular branch of the portal vein, bile ductules as well as less visible lymphatic vessels and nerve branches. In the centre of each lobule, a large central vein was present. The hepatic parenchyma primarily consisted of rows of conically shaped hepatocytes flanking elongate sinusoids (Fig 3A). The hepatocytes were attached to each other in a hexagonal arrangement forming hepatic plates. The hepatic plates were arranged irregularly inward from the periphery edge of each liver lobe towards the central vein.

Lymphatic cells in the liver were formed into aggregations that varied in number (0.17 to 4.5 per mm^2) and in area (0.41 to 3.42% of the histological section).

In both chicken lines these aggregations primarily had a heterogenous distribution throughout the liver parenchyma but some aggregations were associated with portal canals. The aggregations characteristically consisted of a solitary population of tightly packed small lymphocytes. The lymphocytes were characterized by large, dark, round nuclei, and very little cytoplasm. These aggregations did not have capsules, sinuses, cortices, medullae, or germinal centers. Their peripheral borders were irregularly shaped and varied considerably in size (Fig 1B).

The absolute number and area of the lymphatic aggregations per 1 mm^2 of histological section are presented in Table 2. These values varied in the different age groups. The number and area of lymphatic aggregations increased sharply over the first week of life in both lines. After day 7, the number and area of the lymphatic aggregations varied without any discernable pattern.

Liver lipid content in LD and Ross chickens. The percentage of lipid in the liver of both lines correlated negatively with age as well as with body weight ($r = -0.56$ for LD chickens and $r = -0.52$ for Ross chickens, p 0.001 for body weight; $r = -0.97$ for LD chickens and $r = -0.96$ for Ross chickens, p 0.001 for age).

The lipid percentage was highest in one-day-old chickens in both lines (Table 3). From day 7 until day 35 for Ross chickens and until day 49 for LD chickens, there were little differences between the age groups. The lipid percentage in LD chickens decreased from day 49 to the end of the study (Fig 3B).

According to regression analysis, both BW and chicken line, had an influence on the lipid percentage, p 0.001, adjusted $R^2 = 0.54$. The lipid percentage in the liver of Ross chickens was greater on average by 9.1% than that of LD chickens of the same BW (Fig 3C).

Electron microscopy. The overall appearance and structure of the liver of LD chickens were similar to that observed in Ross chickens. The hepatocytes of LD and Ross chickens were conical in shape and their spherically shaped nuclei were located basally adjacent the sinusoid.

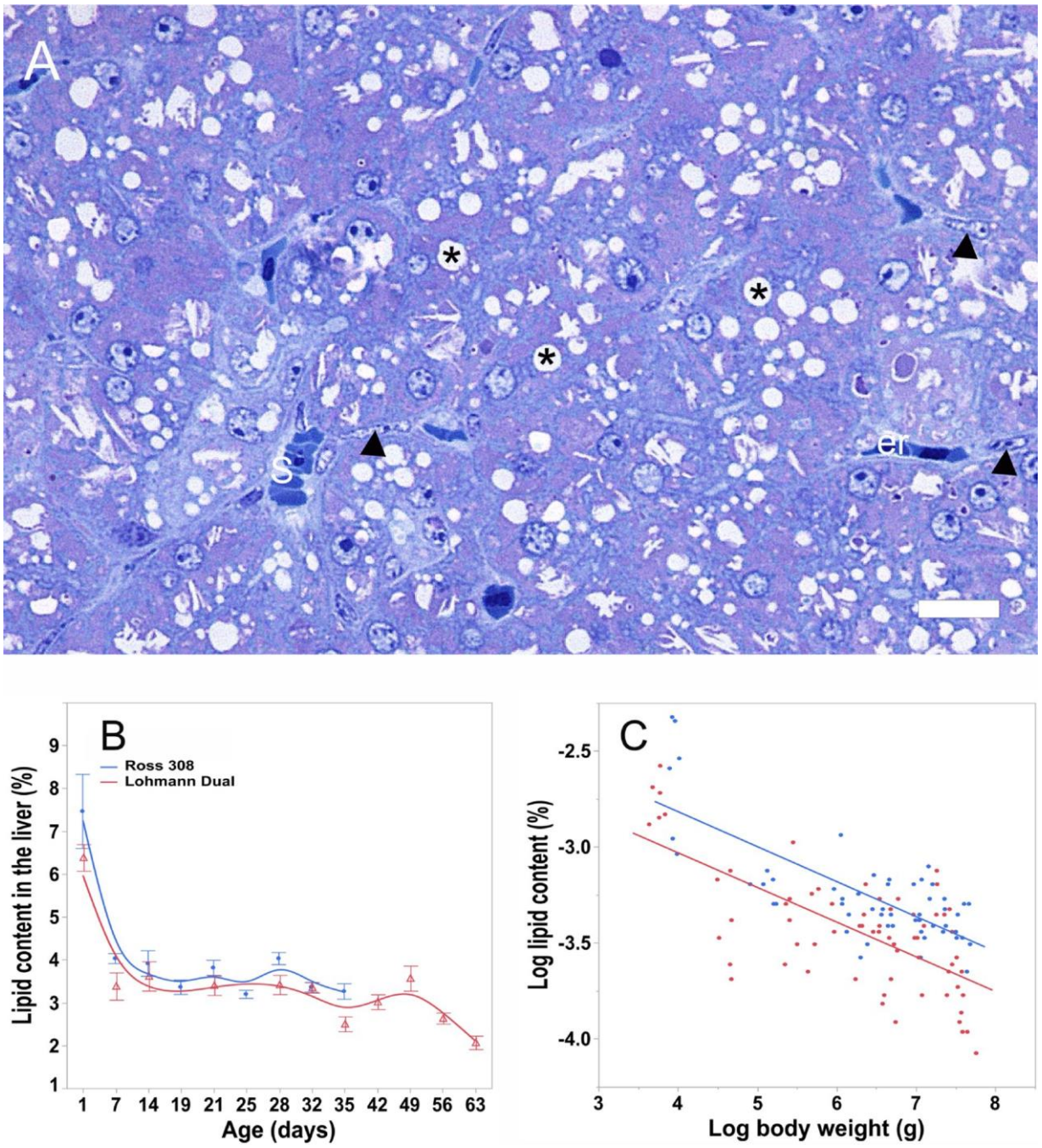

Fig 3. Liver lipid content (%) estimated via histological method. (A) Semi-thin section of a 1-day-old LD chick liver stained with Richardson blue. Where, (er) erythrocyte, (s) sinusoid, (asterisks) lipid droplets, (arrow) endothelial cell. Bar: 10 μm. (B) lipid liver content % versus age, bars refer to mean ± standard error of the mean of the sampled chicken at each time interval. (C) linear regression line of liver lipid content versus body weight. Symbols represent each individual value for each chicken line.

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On day 1, the hepatocytes of Ross chicks appeared to have the dense heterochromatin (Fig 4A) in the peripheral region of the nucleus, more densely packed than that of LD chicks (Fig 4B). By day 7, the heterochromatin was less densely packed than that observed on day 1 in both chicken lines. The nucleolus was irregular in shape in both lines.

Table 2. Area and number of the lymphatic aggregations in histological sections of LD and Ross chickens.

Age (days)	Line♦	Lymphatic aggregation area %		Number of lymphatic aggregations in mm²	
		Mean	SD	Mean	SD
1	LD	0.18	0.28	0.50	0.55
	Ross 308	0.41	0.93	0.17	0.41
7	LD	0.99	0.56	4.50	1.64
	Ross 308	1.02	0.58	3.33	1.86
14	LD	0.90	0.54	4.50	2.51
	Ross 308	0.74	0.45	3.17	1.33
19	Ross 308	1.01	0.83	3.00	2.00
21	LD	0.83	0.46	2.50	1.05
	Ross 308	0.63	0.54	2.00	1.10
25	Ross 308	2.37	1.77	3.83	1.83
28	LD	1.02	1.62	2.00	1.67
	Ross 308	0.43	0.35	0.83	0.75
32	LD	1.75	1.97	2.67	0.52
	Ross 308	1.42	1.12	3.00	1.67
35	LD	1.12	0.95	1.83	0.98
	Ross 308	1.82	1.17	2.67	1.63
42	LD	3.42	4.19	2.67	1.97
49	LD	1.19	0.90	2.33	1.75
56	LD	1.48	0.79	4.17	3.13
63	LD	1.32	0.72	3.67	1.75

LD: Lohmann Dual; Ross: Ross 308; SD: standard deviation; %, percentage of the lymphatic aggregations in the field of view area.

♦6 birds in each group for each chicken line.

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The hepatocytes were arranged radially around the bile canaliculi. Their narrow apical poles had short finger-like projections (microvilli) that protruded into the lumina of the adjacent bile canaliculi (Fig 4C). Tight junctions were seen between adjacent hepatocytes near the bile canaliculi. The wide basal poles of the hepatocytes were oriented towards the liver sinusoids. They had microvilli that projected into the perisinusoidal space (Fig 4E).

In both chicken lines the hepatocytes' cytoplasmic organelles were less numerous and densely packed in one-day-old chicks than in seven-day-old chicks (Fig 4B and 4D). The hepatocyte mitochondria were scattered throughout the cytoplasm with the greatest concentrations being perinuclear (Fig 4B). The mitochondria varied greatly in size and ranged from oval to rod-shape. Ovoid mitochondria were dominant in day old chickens of both genetic lines (Fig 4A and 4B). The rod mitochondria were first seen on day 7 (Fig 4C and 4D). From day 7 onwards there were greater concentrations of mitochondria consisting equally of ovoid and rod forms (Fig 4C and 4D). The mitochondria were associated closely with the endoplasmic reticulum, forming mitochondria-associated endoplasmic reticulum membranes (MAM) (Fig 4D). Here, the endoplasmic reticulum was aligned parallel to the mitochondrion periphery. The rough endoplasmic reticulum (RER) of the hepatocytes appeared abundant in the cytoplasm. It was arranged also beneath the plasma membrane (Fig 5A).

Lysosomes were distributed randomly within the cytoplasm and were variable in size and in the nature of their internal content (Fig 5B). Lysosomes were seen in their primary state as

Table 3. Percentage of lipid storage in the liver of LD and Ross chickens assessed by light microscopy.

Age (days)	Line♦			
	Lohmann Dual (%)		Ross 308 (%)	
	Mean	SEM	Mean	SEM
1	6.4	0.3	7.5	0.9
7	3.4	0.3	4.0	0.1
14	3.6	0.3	3.9	0.3
19	n/a	n/a	3.4	0.2
21	3.4	0.2	3.8	0.2
25	n/a	n/a	3.2	0.1
28	3.4	0.2	4.0	0.1
32	3.4	0.1	3.4	0.1
35	2.5	0.2	3.3	0.2
42	3.0	0.2	n/a	n/a
49	3.6	0.3	n/a	n/a
56	2.6	0.1	n/a	n/a
63	2.1	0.2	n/a	n/a

LD: Lohmann Dual; Ross: Ross 308; SEM: standard error of the mean; n/a: not applicable, %, percentage of the lipid areas in the field of view area.

♦6 birds in each group for each chicken line.

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vesicles without electron dense content and in their secondary state as vesicles with electron dense content. The largest number of lysosomes occurred on day 14 (Fig 5B).

Glycogen accumulations were seen as dark spots scattered throughout the cytoplasm. In both chicken lines the largest accumulations of glycogen were seen on day 14 (Fig 5B).

Within the hepatocytes lipid droplets were seen to be concentrated near the bile canaliculi. The droplets were spherical in shape and showed variation in electron density in both lines (Fig 1F). Lipid droplets were observed in different sizes and did not have a membrane (Fig 1F). Some small droplets fused together forming larger droplets (Fig 1F). The diameter and area of the lipid droplets of the liver were similar in both lines (Table 4). In both chicken lines the number of the lipid droplets peaked on the first day of life and then decreased steadily such that by day 14 only about 50% remaine (Table 4). From day 14 onward, the number of the lipid droplets did not differ greatly (Table 4). The greatest, percentage of liver fat and diameter of lipid droplets were observed on the first day of life in both lines (Table 4). By day 7, the fat percentage and droplet diameter had decreased sharply. After day 14 the changes in the fat percentage and diameter were slight in both chicken lines (Table 4).

The walls of the liver sinusoids were lined by a single layer of endothelial cells. The endothelial cells were elongate in shape and had an elongate oval nucleus. The endothelial cell cytoplasm was rich in organelles.

Hepatic stellate cells (Ito cells) were localized in the perisinusoidal space. They were irregular in shape (between oval to more or less elongated), had oval nuclei and were rich in endoplasmic reticulum (Fig 5C). They were smaller in size and had fewer mitochondria than did the hepatocytes. In both chicken lines the hepatic stellate cells were seen to be associated frequently with the nuclear region of endothelial cells (Fig 5C). The lipid droplets in hepatic stellate cells appeared to be smaller than those seen in the hepatocyte cytoplasm.

Stellate macrophages (Kupffer cells) were observed in the sinusoidal lumen, in close contact with the endothelial cells. The shape of Kupffer cells varied from spherical to spindle-shaped and they had large triangular to oval-shaped nuclei. Their cytoplasmic membrane possessed

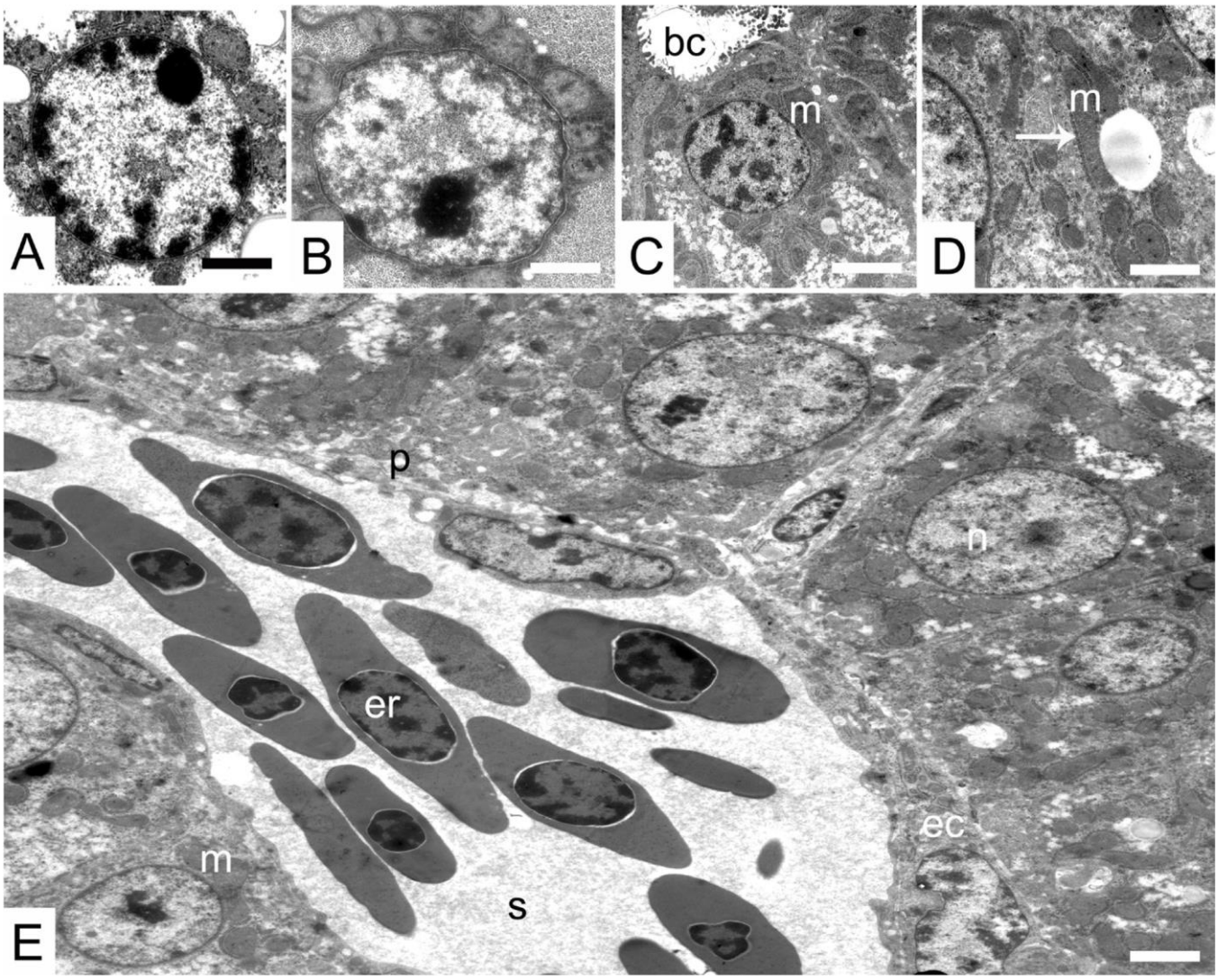

Fig 4. Transmission electron micrographs of the liver of 1 and 7 day old chicken of both lines. (A and B) hepatocyte nuclei of 1-day-old Ross (A) and LD chicks (B). (C and D) concentrations of mitochondria in 7-day-old LD (C) and Ross chicks (D). (E) a panorama micrograph of hepatocytes and sinusoid of 7- day-old LD chicks. Where, (bc) bile canaliculus, (ec) endothelial cells, (er) erythrocyte, (m) mitochondria, (n) nucleus, (s) sinusoid lumen. (p) perisinusoidal space. Symbols: (arrow) mitochondria associated membranes. Bar 1000 μm.

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conspicuous pseudopodia that protruded into the sinusoidal lumen (Fig 5D). Erythrophagocytosis by Kupffer cells was observed (Fig 5D).

Discussion

The essential requirement for rapid growth in young chickens is the rapid development of their digestive tract and liver relative to their pectoral muscle mass [12]. Scheele (1997) reported that during the early stages of development, growth is most marked in the alimentary tract and liver whilst that of the pectoral muscle mass lags behind. However, in the later stages of development, massive increases in pectoral muscle mass coupled with feather growth dominates [13]. Both of these are metabolically demanding and are based on the effective efficiency of the digestive system. In our previous paper [3] we reported that the length and mass of the gastrointestinal tract in relation to body weight were highest throughout the first week of life

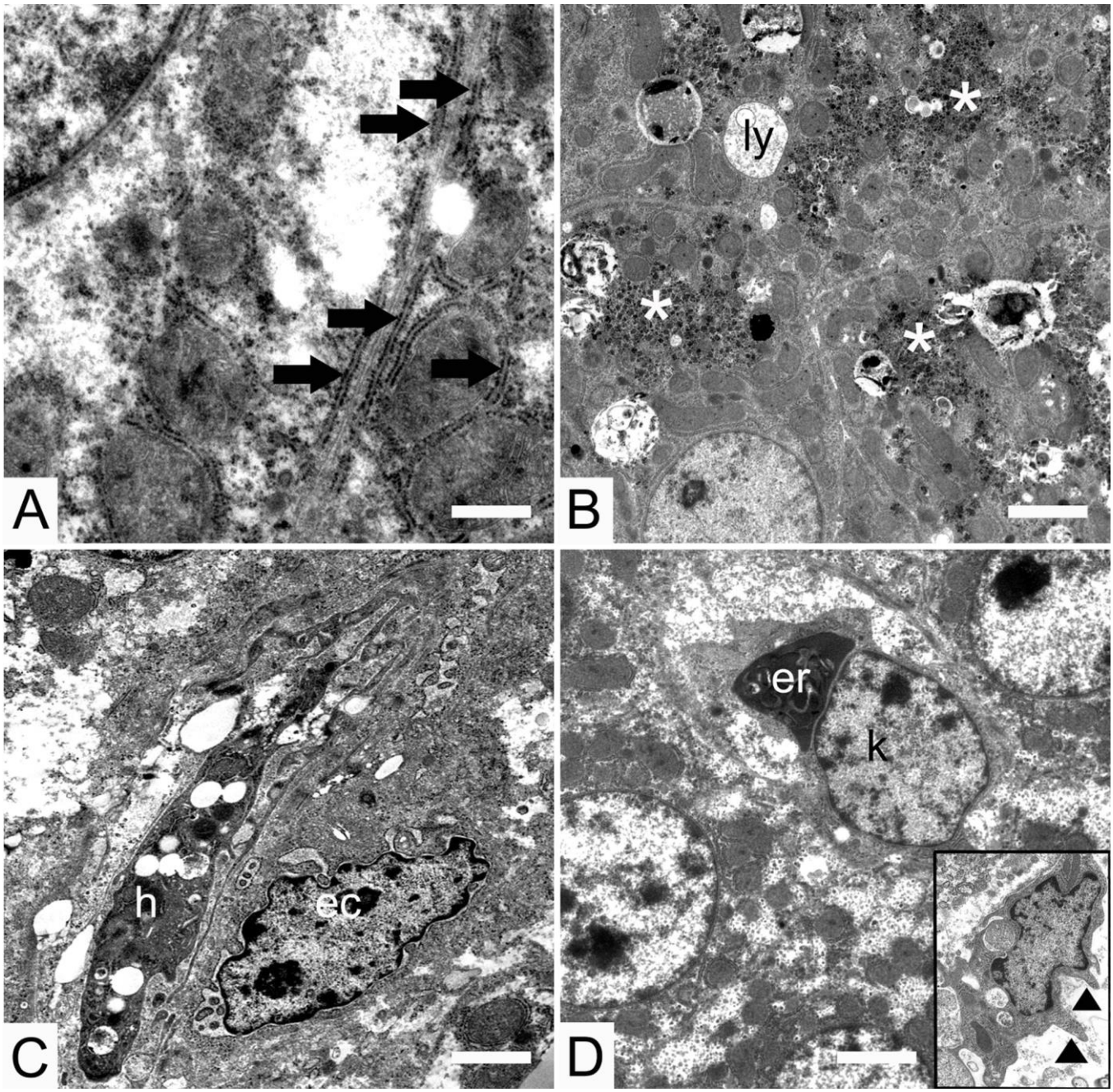

Fig 5. Transmission electron micrographs of the livers of both chicken lines. (A) neighbouring hepatocytes in a 7-day-old LD chick, where the rough endoplasmic reticulum is arranged beneath the plasma membrane (broad arrows), mitochondria associated membranes (thin arrows). (B) glycogen accumulations in the liver of 14-day-old Ross chicks where (ly) lysosomes, (asterisk) glycogen accumulations. (C) hepatic stellate cell (Ito cell) (h) of a 1-day-old LD chick with a neighbouring (ec) endothelial cell. (D) erythrophagocytosis by Kupffer cells in a 1 day Ross chick where (er) erythrocyte, (k) nucleus of Kupffer cell, (arrowhead) pseudopod. Bar 1000 nm.

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in both LD and Ross chickens. In the current study we found that the proportional mass of the liver was highest on the first day of life in both chicken lines.

As would be expected, the peak normalized liver mass found in one-day-old chickens of both lines corresponds to the necessary essential liver capacity required for their subsequent

Table 4. Lipid droplets percentage, number and diameter in the liver of LD and Ross chickens versus days post hatching under TEM.

Age	Line	Lipid content (%)		Lipid droplets number in 1000 μm² of the liver surface		Lipid droplets diameter (μm)	
		Mean	SD	Mean	SD	Mean	SD
1	**LD**	12.26	7.91	43.42	40.58	3.93	1.34
	Ross	18.71	8.44	33.31	15.25	4.67	1.10
7	**LD**	1.47	1.43	31.16	30.67	0.83	0.22
	Ross	4.34	3.87	27.65	16.44	1.30	0.27
14	**LD**	1.23	1.34	15.11	7.15	1.54	0.81
	Ross	2.31	1.77	17.35	11.07	1.57	0.61
21	**LD**	1.62	1.11	12.25	7.13	1.31	0.45
	Ross	0.46	0.56	11.23	4.93	1.27	0.78
28	**LD**	2.33	1.37	18.78	6.68	1.16	0.37
	Ross	1.61	1.17	13.06	6.16	1.24	0.45
35	**LD**	1.84	1.43	20.25	14.69	1.07	0.36
	Ross	1.93	1.22	34.86	23.79	1.03	0.29
63	**LD**	1.32	0.88	26.54	12.39	0.91	0.44

LD: Lohmann Dual; Ross: Ross 308; SD: standard deviation; %, percentage of the lipid to the field of view area.

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development. When limited hepatic capacity occurs, it has large-scale consequences such as suboptimal growth as well as non-specific clinical symptoms [14, 15]. After day one the liver growth in both LD and Ross chickens followed a similar pattern of slow decrease. This is in agreement with the previous findings of a reduction in the relative liver mass of broilers with age [16–18].

The microscopic structure of the liver of both chicken lines was similar to that reported previously [6, 19]. In chickens, there were no significant collagen septae between hepatic lobules [20]. This may explain why the connective tissue between liver lobules of LD and Ross chickens was difficult to distinguish.

The scattered lymphatic aggregations in both chicken lines were a normal feature of their liver parenchyma. Olah et al. (2014) reported that lymphoid aggregations develop in non-lymphoid organs such as the liver, pancreas, kidney, endocrine glands, gonads, brain and spinal cord [21]. Hünigen et al. (2016) reported that non-encapsulated lymphatic aggregations were more common than encapsulated lymphatic aggregations in the liver of the turkey [7]. However, in this study all lymphatic aggregations were non-encapsulated. In general, diffuse lymphatic aggregations are sites where the lymphocytes come into contact with antigens that subsequently stimulate ongoing proliferation of lymphocytes and promote B lymphocytes to become plasma cells, that produce antibodies [22]. This is supported by Younus et al. (2017) who reported that broiler chickens affected by viral hepatitis had diffuse inflammatory foci composed primarily of lymphocytes and macrophages in their liver parenchyma [23]. Increased periportal infiltrates of mixed lymphocytes and phagocytic cells were also observed in the avian liver affected by Borrelia bacteria [24].

The hepatic ultrastructure of LD and Ross chickens is comparable to previous observations of birds' liver [6, 25–27]. In our study only mononucleated hepatocytes were observed in both chicken lines. This contrasts to mammals where binuclear hepatocytes predominate [28]. The hepatocytes of both LD and Ross chickens had abundant, closely packed, large mitochondria confirming similar observations reported by Tanuma and Ito (1978) who concluded that the abundant large mitochondria in bat hepatocytes were necessary for the production of the large amount of energy required for flying [29].

Historically the standard semi-quantitative evaluation of lipid droplets in the liver was based on a grading scale from 0 to 3 and expressed as a percentage where 0 is < 5%; 1 is 5%–33%; 2 is 34%–66%; and 3 is > 66%) [30]. However this protocol has been claimed to "overestimate" the fat deposition [31] as well as to be "inaccurate and subjective" [11]. Liguori et al. (2009) applied automated image analysis software using chromatic and shape filters to examine the liver fat content on histological sections in rats. They found that using only a colour filter to select areas of lipid accumulation overestimated the percentage fat content due to the automatic inclusion of sinusoids that had the same colour profile. They circumvented this problem by using a shape filter that excluded the sinusoids [11]. Our study improved upon earlier studies because it used both colour and circularity filters and validated these results against those derived from more accurate chemical analyses of the same samples. However, 2D measurements may do not mirror the actual parameters and the distribution of lipid droplets in the liver may vary according to the sampling locations. Moreover, maintaining a constant cite of investigation may introduce a constant error. Therefore, the significance of sampling should be considered when analysing tissues. Standard sampling designs and procedures are available for stereological and histological tissue analysis for various organs and species [32]. In the literature, there is no preferred or specific sampling site from the liver in chicken. However, a recent study by Gerspach et al., (2017) reported that the lipid distribution in 10 different locations of the liver in 30 cows had only minor variation in the distribution of lipids at the histological level [33].

To overcome these issues, the same procedure to assess the liver lipid content, which we validated previously, was applied on both chicken lines; Ross 308 and Lohmann Dual. We believe that maintaining a constant sampling site makes our data reliable to compare the liver lipid content between Ross 308 and Lohmann Dual chickens.

The lipid content curves of each chicken line were nearly identical over the study period whether measured using images from light microscope or TEM. Our finding that the percentage of lipid in the liver of both lines is correlated negatively with age and body weight is in line with previous results [34]. In both chicken lines the liver lipid content was highest on the day of hatching in both histological and ultrastructural sections. Over the next few days post-hatching, the chicks were dependent on yolk lipid as they transitioned to their independently obtaining adequate nutrients and energy from their oral feed intake. Noble and Cocchi (1990) reported that during the incubation period of the chick, 80% of the yolk lipid content is mobilised and absorbed by embryonic tissues [35]. Then over several days post-hatching the chicks utilised the remaining yolk lipid as their main energy source. The decrease in the liver lipid content over the first week corresponds to their consuming the yolk lipid and their eating their starter formula [35]. Our study supports this by our noting that the normalized liver mass was greatest over the first week. This suggests that the liver is relatively functionally mature at hatching and that the final functional maturation is rapid as the chickens' transition from fat-rich yolk stores to a predominantly carbohydrate diet [18]. For the remainder of their lives the liver lipid content in both chicken lines ranged between 2.1–4.0%. This is in agreeance with previous values for total liver lipid percentages ranging between 1.6–3.2% of the wet weight of the liver from day 22 to 36 [36]. The changes in liver lipid content from the early days post hatching and onwards are attributed to the changing role of the liver from being mainly a depository organ during the first days post hatching to one of synthesizing fat for both structural and functional purpose afterwards [34].

A strain or breed of chickens is, more or less, sensitive to dietary or hormonal effects on hepatic lipid accumulation [37]. Genotype is one of the main factors affecting fat deposition in the broiler chickens in addition to sex, age and the nutrition. The origin of fats in a broiler chicken's body is by means of exogenous (from the diet) or endogenous (synthesized in the

liver) fat [38]. This suggests that Ross chickens could be genetically more prone to deposit fat in the liver than LD chickens of the same body weight. Commercially, feeding chickens high-energy diets based on a high percentage of carbohydrate stimulates fat deposition in the liver [8]. In addition to this, the rapid growth pattern of modern broiler chicken lines has been accompanied by an increase in voluntary feed intake that has led to increased fat deposition in the body [39]. This is a partial explanation of the higher liver lipid content of Ross chickens than that of LD chickens, in birds of the same body weight.

In our study, as there were no pathological changes in the structure and ultrastructure of the liver and no significant difference in the lipid content of the liver in the comparable age groups of Ross and LD chickens, it can be assumed that the liver lipid content of both genetic lines is within the normal physiological range. Our results suggest that the livers of LD chickens were able to metabolise high-energy diet used without side effects.

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5 Discussion

The overall aim of this study was to investigate the basic anatomical responsiveness and robustness of the gastrointestinal tract including the liver of two different genetic chicken lines when raised under intensive husbandry conditions. Morphology and microstructure of the gastrointestinal tract and liver of the new dual-purpose LD chicken line and the conventional broiler line Ross 308 was examined and compared. Body weight was an important parameter studied.

5.1 Body weight

Studies on the growth potential and body weight of LD chickens have already been carried out by Habig et al. (2016) and Urban et al. (2018). In the study of Habig et al. (2016) the body weight of LD chickens reached only 1803 g after 77 days (Habig et al., 2016). Urban et al. reported a final weight of 2000 g in the same time (Urban et al., 2018). The final body weight in our study was about 2000 g after only 63 days.

The broiler industry has been characterized as an “age-for-weight” market industry. The goal is to reach a marketable weight in the shortest possible time (Emmerson, 1997). In 1950, broilers took about 16 weeks to reach a marketable weight, then by 1990, this period was shortened to 6 or 7 weeks (Griffin & Goddard, 1994; Konarzewski et al., 2000; Thomas et al., 1958; Warren, 1958). In our study, the Ross chickens reached an average body weight of 2000 g within only 5 weeks, whereas the LD chickens needed 9 weeks (63 days).

Long-term genetic selection plays an important role in higher productivity of broilers (Schmidt et al., 2009). Their digestive system (including the liver as a closely connected organ) has been modified by genetic selection. An increased nutrient intake is essential towards achieving high productivity (Schmidt et al., 2009). The growth and functional development of the gastrointestinal tract seem to have been changed with special selection of domesticated poultry for a more rapid body growth (Jackson & Diamond, 1996; Mitchell & Smith, 1991; Sell et al., 1991; Uni et al., 1995; Yamauchi et al., 1996).

5.2 The gastrointestinal tract

5.2.1 Stomach and intestines

The general morphology of the proventriculus and ventriculus of the two genotypes examined was similar. The basic structure described for the stomach of granivorous birds (Hodges, 1974) was observed in both chicken lines. The gizzard reacts to variations of composition and size of the diet that can lead to changes in muscle performance. It has previously been shown that the intake of rough grit particles leads to a definite enlargement of the gastric muscles (Hetland et al., 2003; Jones & Taylor, 2001; Nir et al., 1993; Taylor & Jones, 2004).

The regression analysis done in this thesis, showed that the gizzard of LD chickens is significantly heavier ($p < 0.001$) than those of Ross chickens suggesting that the more muscular gizzard in the LD enhances peristaltic movement leading to a more rapid passage time.
Based on this result, I propose to further investigate how the feeding regimens influences the musculature of the gizzard and the subsequent intestinal passage time (Alshamy et al., 2018).

The genetic chicken lines showed a continuous intestinal growth at all age stages. The intestines of both chicken lines showed considerable post-hatching growth. The relative growth curves allow for a comparison of the growth rates of different organs regardless of any absolute differences. The normalized intestinal mass curves were similar. The LD chicken line, however, had a greater normalized intestinal mass than the Ross line. The intestines showed positive allometric growth over the first week. The peak of the normalized intestinal mass was on day 7 after hatching. This corresponds to the time point when the yolk sac and subdermal lipid deposits are exhausted. At this very moment, the chick must begin to rely on the intestines to digest and transport feed-derived nutrients (Schmidt et al., 2009). Thereafter, the intestines switched to negative allometric growth in both lines.
Several studies have reported that heavy chicken lines have longer intestines than lighter chicken lines (Fan et al., 1997; Mitchell & Smith, 1991; Uni et al., 1995). The present results showed that the intestine was shorter in LD than in Ross birds ($P < 0.001$). However, the LD chickens showed a greater normalized intestinal mass and a prolonged growth period that resulted from their larger organ dimensions and slower overall growth.

5.2.2 Histology of the small intestine

Fast-growing birds have a greater growth rate and consume more feed than slow growing birds (Marks, 1979; 1980). The selection towards a high growth rate is positively related to an increase of feed intake and to efficiency of feed utilization. It may be assumed that such an increased feed intake and efficient feed utilization have also altered the intestinal function, which in turn could influence intestinal morphology (Proudman et al., 1970; Marks, 1979;1980;). LD chickens have been selected for their increase in growth rate and egg production, respectively. Therefore, histological differences in the small intestinal wall between Ross and LD chickens are of considerable interest.
The jejunum and ileum of both chicken lines showed no fundamental histological differences in comparison to results described by other researchers (Bradley & Grahame, 1950; Calhoun, 1954; Demke, 1954; Malewitz & Calhoun, 1958).
Both height of the villi and mucosal surface area increased rapidly after hatching in both chicken lines. The microvillus length followed a gradual decrease from the jejunum to the ileum in both lines (Yasar, 1999). Villous height increases to at least 7 weeks of age (Gabriel et al., 2008; Miles et al., 2006). Birds intestine grows without developing additional villi. The increase in villi size is their strategy to increase intestinal absorptive surface during growth (Moon & Skartvedt, 1975).

The ileal villi were found to be shorter than the jejunal villi in both chicken lines. However, the mucosal surface area of the ileum was greater in Ross than in LD chickens, suggesting a higher absorptive capacity of the former ones.

It has been reported that epithelial cell turnover and nutrient absorption processes in chickens correlated with the villus height:crypt depth ratio (Fan et al., 1997; Wang & Peng, 2008; Wu, et al., 2004). In the present study, this ratio decreases from the jejunum to the ileum, indicating a higher rate of epithelium turnover in the proximal part of the small intestine. The ratio was not significantly different between the two chicken lines. Results indicate that cellular proliferation was constant and adequate.

In both chicken lines, the tunica muscularis in the jejunum was thinner than in the ileum. The main purpose of these layers is to move the ingesta through the digestive tract and mix the ingesta with the glandular and intestinal secretions (Smollich, 1992). This fact is of particular importance in birds due to the shorter length of the intestines. The average retention time in the gastrointestinal tract of the broiler is only about 4 - 6.5 hours (Klis et al., 1990).

The thick tunica muscularis and short intestine in LD chickens may lead to shorter retention times in the gastrointestinal tract, and hence consequently to less efficiency in feed utilization.

Out of day one, the epithelial height of the jejunum varied only slightly with age in both types of chickens. The epithelial height did not differ between the two chicken lines. The epithelial cells of both lines had few organelles on the first day of life. This seems to be more common with immature epithelial cells with just few organelles (Trahair & Robinson, 1986). The epithelial cells of 7 day old animals were more numerous in organelles in both chicken lines. In conclusion, the epithelial cells on day 7 seem to appear more active than those of day one. This suggests that the enterocytes could reach a morphological and physiological maturation around day 7.

5.3 The liver

5.3.1 Liver mass

Morphology, gross anatomy as well as microscopic structure and ultrastructure of the liver of both chicken lines at different age stages were fundamentally similar and were generally in agreement with what has already been described by Gille et al., 1999; Hodges, 1974; Ohata et al., 1982; and Purton, 1969.

My results indicate that there is little difference in the overall growth characteristics of Ross and LD chicken livers. The liver masses were lighter in Ross than LD chickens. The shapes of the growth curves were similar, and the allometric exponents did not differ significantly. However, the normalized liver mass peak took place on the first day post hatching. The peak corresponds to the essential liver capacity required post hatching, in order to accommodate the exhaustion of lipid supplies and switching to a carbohydrate-rich diet (Schmidt et al., 2009). As with the intestine, this dramatic decrease in the relationship between the growth of the liver and total body weight may reflect maturation of the digestive tract around d 7 post-hatching. Similar results were obtained

for the liver and the intestine weights of chickens by Govaerts et al., 2000; Lilja, 1982; and Matsuzawa, 1980. The observed morphometric changes probably play an important role in maturation of the intestine.

5.3.2 Avian hepatic lymphatic aggregations

Lymphatic aggregations are a normal feature of the avian liver parenchyma (Hünigen et al., 2016) as the avian liver possesses no lymphatic nodes (Rautenfeld & Budras, 1983). Hünigen et al. described encapsulated as well as non-encapsulated lymphatic aggregations as a normal feature of their liver parenchyma in turkeys (Hünigen et al., 2016). In the present study, groups of aggregated lymphocytes with no distinct feature were part of the liver parenchyma in both chicken lines. The hepatic lymphatic aggregations of the chickens examined have no sinuses, cortices, medullae, or germinal centers (Alshamy et al., 2019). The lymphatic aggregations were non-encapsulated and distributed irregularly in the histological cross-section. The number and areas of the hepatic lymphatic aggregations increased rapidly throughout the first week. Then the measured levels began to fluctuate with age in both chicken lines.

5.3.3 Liver lipids

Hepatocytes can store triglycerides from portomicrons as well as metabolise fatty acids to ATP. They can also synthesise lipoproteins and phospholipids or store the released energy as fat deposits (Scott et al., 1982).

In contrast to mammals, the synthesis of fat in birds is greater in the hepatic tissue and very limited in the adipose tissue (Hermier, 1997). This unique characteristic predisposes the birds to fat accumulation in the liver (Cherian, 2002).

The lipid content curves of each chicken line were similar over the study period no matter if they were measured by using images from light microscopy or by transmission electron microscopy. The percentage of lipids in the liver of both lines correlates negatively with age and body weight and is in line with previous findings (Mutayoba et al., 2013). It was highest on the first day and decreased until day 7. Afterwards there was no change in both chicken lines. These results suggest that the liver is functionally relatively mature at hatching and that the final functional maturation corresponds to the chickens' transition from the fat rich yolk stores to predominantly carbohydrate diets (Schmidt et al., 2009).

The changes in the liver lipid contents of the two chicken lines were within the normal physiological range over the term of the study. The high-energy diets and the current rapid growth intensive husbandry conditions have not led to any malfunctions concerning anatomical and physiological responsiveness of the liver of LD chickens.

6 Summary

The poultry industry has specialized in meat and eggs as the two major fields of production. Due to economic reasons, the fattening of male layer breeds is considered to be inefficient in modern chicken industry. Therefore, about 50 million male chicks of laying hens are killed in Germany every year on their first day of life. As an alternative to this, dual-purpose chickens have received much attention in the last years.

The new genetic chicken line Lohmann Dual (LD) combines a good laying performance with an acceptable increase of body weight. However, there is still a demand for reliable data on the basic anatomical and physiological responsiveness of the gastrointestinal tract and the liver of LD chickens when fed high-energy diets. The aim of this doctoral thesis was to carry out comparative examinations on the gastrointestinal tracts and the livers of the new LD chicken line and the conventional broiler chicken line Ross 308.

The body weight (BW), the weight of the stomach, intestine and liver as well as the intestinal length were measured. Villus and epithelial height, crypts´ depth, the enlargement factor of the intestinal mucosal surface and the thickness of the tunica muscularis were determined. Ultrastructural characteristics of the jejunum were analyzed via transmission electron microscopy. In addition, liver fat content and lymphatic aggregations in the liver were studied histologically; the ultrastructure of the hepatocytes was examined by electron microscopy.

Results showed that the weight of the gastrointestinal tract and the liver of LD chickens increased in proportion to the body weight gain, with no abnormalities or deformations observed. Anatomical differences between the LD chickens and the faster-growing Ross 308 birds were found that may have contributed to a slower growth rate of LD chickens. LD chickens may have lower nutrient absorption capacity due to their shorter intestinal tract and smaller intestinal mucosal surface area, resulting in slower body growth rates than Ross chickens.

Liver lipid content of the two chicken lines examined in this study, were within the normal physiological range. Liver lipid content correlated negatively with age and body weight in both lines.

In summary this study has not lead to indications that under the described experimental conditions the development of the gastrointestinal tract and the liver of LD chickens are impaired by breeding and selection methods.

7 Zusammenfassung

Makroskopische, mikroskopische und morphometrische Vergleichsstudie des Magens, des Darmes und der Leber einer Zweinutzungs- und einer Broiler-Hühnerlinie

Die Geflügelindustrie ist auf die zwei Hauptproduktionsbereiche Fleisch und Eier spezialisiert. Da die Mast der männlichen Küken von Legehennen für die Fleischindustrie als ineffizient erachtet wird, werden in Deutschland jedes Jahr am ersten Lebenstag etwa 50 Millionen männliche Legehennenküken getötet. Als Alternative dazu werden Mehrzweckhühner („Dual-purpose"-Hühner) mehr in den letzten Jahren diskutiert. So kombiniert die neue Hühnerlinie Lohmann Dual (LD) eine gute Legeleistung mit einem akzeptablen Mastresultat.

Das Ziel der vorliegenden Arbeit waren Untersuchungen am Magen-Darm-Trakt und an der Leber der neuen Hühnerlinie LD im Vergleich mit einer herkömmlichen Broiler-Hühnerlinie (Ross 308), um zuverlässige Daten zur anatomischen Robustheit dieser Organe von LD-Hühnern bei Mast mit energiereicher Nahrung zu erhalten.

Das Körpergewicht, das Gewicht des Magen-Darm-Trakts und der Leber sowie die Darmlänge wurden erfasst. Ebenso wurden die Zotten- und Epithelhöhe, die Tiefe der Darmkrypten, der Vergrößerungsfaktor der Darmschleimhautoberfläche sowie die Dicke der intestinalen Tunica muscularis gemessen. Jejunumproben wurden mittels Transmissionselektronenmikroskopie untersucht. Zusätzlich wurden der Leberfettgehalt und Lymphaggregationen in der Leber histologisch und die Ultrastruktur der Hepatozyten transmissionselektronenmikroskopisch analysiert.

Die Ergebnisse zeigten, dass sich das Gewicht des Magen-Darm-Trakts und der Leber von LD-Hühnern proportional zur Körpergewichtszunahme entwickelte, ohne dass Anomalien oder Deformationen beobachtet wurden.

Einige anatomische Unterschiede zwischen den LD-Hühnern und den schneller wachsenden Ross 308-Vögeln wurden beobachtet, die möglicherweise im Zusammenhang mit der langsameren Wachstumsrate von LD-Hühnern zu sehen sind.

Die Ergebnisse geben Hinweise darauf, dass LD-Hühner aufgrund ihrer kürzeren Darmlänge- und kleineren Darmschleimhautoberfläche eine geringere Nährstoffaufnahmefähigkeit haben, was zu langsameren Körperwachstumsraten im Vergleich zu dem der Ross-Hühner führen könnte.

Die Veränderungen des Leberlipidgehalts der beiden Hühnerlinien lagen im Untersuchungszeitraum im normalen physiologischen Bereich. Der Leberlipidgehalt korrelierte in beiden Linien negativ mit Alter und Körpergewicht.

Insgesamt ergaben diese Untersuchungen keine Hinweise auf Beeinträchtigungen der Entwicklung des Magen-Darm-Trakts und der Leber von LD-Hühnern unter den beschrieben Versuchsbedingungen.

8 References

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9 List of own publications

Full papers

Harash, G., Richardson, K., **Alshamy, Z**., Hünigen, H., Hafez, H. M., Plendl, J., Al Masri, S. (2020). Basic morphometry, microcomputed tomography and mechanical evaluation of the tibiotarsal bone of a dual-purpose and a broiler chicken line. PLOS ONE 15(3): e0230070.

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Alshamy, Z.; Röhe, I.; Hünigen, H.; Hafez, M. H.; Plendl, J.; Al-Masri, S. (2019):
Validation of a histological procedure to determine liver fat content in chicken.
12. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences".
Berlin: 27.09.2019.
Berlin: Mensch und Buch Verlag. ISBN: 978-3-96729-006-6. Poster.

Harash, G., **Alshamy, Z**., Hünigen, H., Hafez, M. H., Plendl, J., Al Masri, S. (2019):
Microstructural properties of the tibiotarsal bone: comparison between a dual-purpose and a broiler chicken line.
12. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences“.
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Alshamy, Z.; Hünigen, H.; Hafez, M. H.; Plendl, J.; Al-Masri, S. (2019):
Morphometric Evaluation of the Intestine and its Interpretation: Comparison between a dual- purpose and a conventional chicken line.
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Anatomia Histologia Embryologia; Volume 47, Issue S1, S. 64–67.
Poster. Schlütersche Verlagsgesellschaft
Young Scientists Award for Anatomists in the session: Digestive system.

Al Masri, S.; **Alshamy, Z.**; Hünigen, H.; Röhe, I.; Zentek, J.; Plendl, J. (2018):
Development and validation of a histological method to measure liver fat content in chicken.
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11 Declaration of academic honesty

I hereby confirm that the text at hand is solely my own work. I assure that I only used the cited sources and the thesis has not been submitted in any form for another degree at any university or other institute.

Berlin, 18.09.2020

Zaher Alshamy

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