Differences in intestinal size, structure, and function contributing to feed efficiency in broiler chickens reared at geographically distant locations

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ABSTRACT The contribution of the intestinal tract to differences in residual feed intake (RFI) has been inconclusively studied in chickens so far. It is also not clear if RFI-related differences in intestinal function are similar in chickens raised in different environments. The objective was to investigate differences in nutrient retention, visceral organ size, intestinal morphology, jejunal permeability and expression of genes related to barrier function, and innate immune response in chickens of diverging RFI raised at 2 locations (L1: Austria; L2: UK). The experimental protocol was similar, and the same dietary formulation was fed at the 2 locations. Individual BW and feed intake (FI) of chickens (Cobb 500FF) were recorded from d 7 of life. At 5 wk of life, chickens (L1, n = 157; L2 = 192) were ranked according to their RFI, and low, medium, and high RFI chickens were selected (n = 9/RFI group, sex, and location). RFI values were similar between locations within the same RFI group and increased by 446 and 464 g from low to high RFI in females and males, respectively. Location, but not RFI rank, affected growth, nutrient retention, size of the intestine, and jejunal disaccharidase activity. Chickens from L2 had lower total body weight gain and mucosal enzyme activity but higher nutrient retention and longer intestines than chickens at L1. Parameters determined only at L1 showed increased crypt depth in the duodenum and jejunum and enhanced paracellular permeability in low vs. high RFI females. Jejunal expression of *IL1B* was lower in low vs. high RFI females at L2, whereas that of *TLR4* at L1 and *MCT1* at both locations was higher in low vs. high RFI males. Correlation analysis between intestinal parameters and feed efficiency metrics indicated that feed conversion ratio was more correlated to intestinal size and function than was RFI. In conclusion, the rearing environment greatly affected intestinal size and function, thereby contributing to the variation in chicken RFI observed across locations.

Key words: chickens, intestinal permeability, gene expression, visceral organs, residual feed intake

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INTRODUCTION

In chicken production, traits related to production efficiency have been under selection for generations, resulting in a correlated improvement of feed efficiency (**FE**) (Zuidhof et al., 2014). Nowadays, residual feed intake (**RFI**), calculated as the difference between predicted and observed feed intake (**FI**), is often used as the metric for FE in livestock and reflects inherent inter-animal variation in biological processes associated with FE (Bottje and Carstens, 2009). By using disproportionate amounts of energy relative to their weight, the gastrointestinal tract and liver are important energy sinks, accounting for about 20% of the whole body energy expenditure (Choct, 2009). The extent of intestinal nutrient uptake is modulated by the interplay between digestive secretions and the condition of the intestinal absorptive surface (Caspary, 1992; Nain et al., 2012). Moreover, the integrity of the intestinal epithelium modifies nutrient uptake, translocation of intestinal antigens, and thus growth efficiency (Choct, 2009).

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Therefore, it can be assumed that, in order to maximize the utilization of dietary energy and nutrients, more feed efficient animals should have greater digestive and absorptive capacity than less efficient animals. This hypothesis has been more intensively studied in beef cattle and pigs than in meat-type chickens (Fitzsimons et al., 2014; Montagne et al., 2014; Vigors et al., 2016; Metzler-Zebeli et al., 2017a). One characteristic of low RFI animals is a drastically lower FI compared to less feed efficient (high RFI) animals (Metzler-Zebeli et al., 2016; Vigors et al., 2016). In general, FI substantially influences the size and energy requirement of the intestine and can therefore elevate the basal nutrient demands of the animal (Johnson et al., 1990; Herd and Arthur, 2009; Fitzsimons et al., 2014). Increasing the daily protein intake has been shown to decrease the efficiency of protein digestion and amino acid absorption in chickens (Hernández et al., 2012). Available data for chickens are inconclusive regarding whether the increased FI associated with high RFI chickens results in enlarged visceral organs and reduced nutrient digestion. Some authors reported no relation between RFI and nutrient digestibility (Luiting et al., 1994), whereas others found positive relationships between RFI and fecal nitrogen excretion (Aggrey et al., 2014). Likewise, low RFI also has been associated with lighter liver weight in pullets (van Eerden et al., 2004), whereas adult hens of low RFI had larger liver size and duodenal absorptive villi surface than high RFI hens (Nain et al., 2012). In addition, evidence for RFI-related variation in the function of the duodenum in meat-type chickens has been found using global gene expression profiling (e.g., Aggrey et al., 2014; Lee et al., 2015), whereas similar information for other intestinal segments is scarce.

Most evidence regarding the underlying mechanisms for variation in RFI in chickens has been derived from one contemporary population of chickens of similar biotype and management conditions at one experimental setting (Bottje and Carstens, 2009). Therefore, it is not known whether the underlying biological differences for diverging RFI concur when chickens are raised in different rearing environments. Substantial batch-to-batch variation was reported for the intestinal microbiota of chickens raised in one environment (Stanley et al., 2013, 2016; Ludvigsen et al., 2016). The intestinal microbiota differed in chickens of diverging FE, but FE effects on the intestinal microbiota varied between batches (Stanley et al., 2016). As the intestinal microbiota influence development and function of the gastro-intestinal tract of chicks early in life (Schokker et al., 2015), environment-dependent differences in the intestinal microbiota may affect the RFI-related intestinal function as well. This led to the hypothesis that the environment may modify RFI-related differences in intestinal size, structure, and function in chickens, which will have implications for intervention approaches to manipulate underlying physiological mechanisms in attempts to improve FE in chickens.

The objective was therefore to examine the differences in nutrient digestion, visceral organ size, intestinal morphology, intestinal permeability, expression of genes in relation to barrier function, and innate immune response of the jejunal mucosa in chickens of diverging RFI raised at 2 different experimental sites under very similar experimental conditions.

MATERIALS AND METHODS

Chicken Trials

Two chicken experiments using similar protocols comprising the experimental setup, diets, data, and sample collection were conducted at the Institute of Animal Nutrition and Functional Plant Compounds [University of Veterinary Medicine Vienna, Austria; location 1 (**L1**)] and at the Agriculture Branch of Agri-Food and Biosciences Institute [Hillsborough, Northern Ireland, United Kingdom; location 2 (**L2**)] using a completely randomized study design. At both locations, 3 replicate batches were performed using day-old mixed-sex Cobb 500FF chicks, resulting in a total population of 78 females and 79 males at L1 and in a total population of 96 females and 96 males at L2. Within each replicate batch, equal numbers of females and males, except for batch 2 with one more male at L1, were used. Due to the geographic distance, chickens at L1 and L2 came from different commercial hatcheries. The chicken batches were run simultaneously at both locations. All animal experimentation procedures were approved by the institutional ethics committee at the University of Veterinary Medicine Vienna and the Austrian national authority according to paragraph 26 of Law for Animal Experiments, Tierversuchsgesetz 2012 – **TVG** 2012 (GZ 68.205/0131—II/3b/2013). At Agri-Food and Biosciences Institute, the animal procedures were conducted under Project License PPL 2781 obtained from the Department of Health, Social Services and Public Safety (**DHSSPS**) and which adhere to the Animals (Scientific Procedures) Act, 1986.

At hatch, chicks were sexed at the hatcheries and transported to L1 and L2 within the first d of life. Upon arrival, chickens were weighed and group-housed for the first d of life. From d 7 of life, chickens were individually housed until the end of the experimental period. The cage floors were made of wire mesh (10 mm × 10 mm) and padded with rubber tubing. The temperature was maintained at 33 °C for the first 5 d, after which it was gradually decreased until reaching a temperature of 21 °C on d 21 of life. The chickens received 18 h of light and 6 h of dark. Each cage was equipped with one manual feeder and drinker with feed and demineralized water freely available.

Chickens were fed starter, grower, and finisher diets based on corn and soybean meal from d 1 to 10, d 11 to 21, and d 22 to 42 of life, respectively (Supplementary Table S1). Diets did not contain anti-microbial growth promoters or coccidiostats. Starter, grower, and finisher diets were mixed according to the same diet formulation

at each location. At each location, starter, grower, and finisher diets for the replicate batches came from the same batch of commercially prepared crumbles (starter diet) and pellets (3 mm; grower and finisher diets) and were stored in cool (<15°C) and dry conditions for a duration of no longer than 6 months.

Data Collection and Feed Efficiency Measurement

The FI was determined weekly. Feed leftovers and spills were collected before recording FI on d 14, 21, 28, 35, 36, and 38 of life. Once a wk (upon arrival, d 7, 14, 21, 28, and 35) and on the d when the RFI was determined (d 36 and d 38 of life at L1 and L2, respectively), body weight (**BW**) of all chickens was recorded. Determination of RFI and selection of low, medium, and high RFI chickens were planned to occur for the experimental period between d 7 and d 38 of life. However, as chickens at L1 grew faster than chickens at L2, selection of low, medium, and high RFI chickens at L1 took place 2 d earlier on d 36 of life in order to achieve approximately similar BW at euthanization and hence to minimize the effect of BW and body composition on parameters of interest. Chickens at L2 were weighed again and ranked according to their RFI values on d 38 of life. Data for net total FI (**TFI**), metabolic mid-weight (**MMW**), and total BW gain (**TBWG**) were used to estimate RFI values as the residuals over the test interval with a nonlinear mixed model in SAS (SAS Stat Inc., version 9.2; Cary; NC) as described in Metzler-Zebeli et al. (2016):

The MMW was calculated as:

$$\text{MMW} = [(\text{BW at d 7 of life (g)} + \text{BW at d 36 or d 38 of life (g)})/2]^{0.75}.$$

The RFI and residual body weight gain (**RBG**) were calculated as:

$$\text{RFI (g)} = \text{TFI} - (a_1 + b_1 \times \text{MMW} + b_2 \times \text{TBWG}),$$

Where: a_1 is the intercept, and b_1 and b_2 are partial regression coefficients of MMW and TBWG on TFI, respectively. In addition, RBG, residual intake over gain (**RIG**), and feed conversion ratio (**FCR**) for the test interval were calculated for the selected chickens:

$$\text{RBG (g)} = \text{TBWG} - (a_2 + b_3 \times \text{MMW} + b_4 \times \text{TFI}),$$

Where: a_2 is the intercept, and b_3 and b_4 are partial regression coefficients of MMW and TFI on TBWG, respectively.

The RIG was calculated as:

$$\text{RIG (g)} = \text{RBG (g)} - \text{RFI (g)}.$$

The FCR was calculated as:

$$\text{FCR (g/g)} = \text{TFI (g)}/\text{TBWG (g)}.$$

Experimental Design

In each replicate batch and location, separately for females and males, the 3 chickens with the lowest RFI (high FE), the 3 chickens with the highest RFI (poor FE), and the 3 chickens with medium RFI, which was close to zero, were selected. For all 3 replicate batches, each RFI group was represented by 9 females and 9 males at L1. At L2, in turn,6 low RFI, 11 medium RFI, and 6 high RFI female chickens and 10 low RFI, 9 medium RFI, and 9 high RFI male chickens were selected. Only FE data and excreta samples from the selected chickens at both locations were used for analysis. At the end of the experimental period, the selected chickens were euthanized to collect intestinal samples. The remaining chickens were removed from the experiment. TFI and TBWG were adjusted for the test interval from d 7 to 36 of life across locations.

Sampling Procedures

Total excreta were collected from the cage floor and the tray below the cage from 08:00 h on d 34 to 08:00 h on d 36 of life and stored at −20°C. Chickens were weighed before being sacrificed between d 37 and 42 of life. At L1, selected chickens were euthanized with an overdose of sodium pentobarbital (450 mg/kg, Release, WTD-Wirtschaftsgenossenschaft Deutscher Tierärzte, Garbsen, Germany) by i.v. injection into the caudal tibial vein from d 37 to 42 of life, whereas at L2, selected chickens were sacrificed on d 41 and 42 of life. After opening the abdominal cavity, the liver and the gastrointestinal tract were removed. The weight of the liver and pancreas was recorded. Following collection of intestinal samples for morphometric analysis, the intestinal segments were opened at the mesenterium, intestinal digesta removed, and intestinal segments were washed in neutral-buffered saline and blotted dry on paper towels. The weight of the dried empty intestinal segments was then determined. Thereby, the empty weight of the crop, gizzard, and proventriculus were measured only at L1, whereas the empty weight of the duodenum, jejunum, ileum, ceca, and colon, and the lengths of the total intestinal tract and of the individual segments were determined at both locations. The duodenum was defined as the segment from the pylorus to the end of the pancreatic loop. The ileum was defined as the segment between the ileo-cecal junction and the beginning of the caudal mesenteric blood supply. To adjust for differences in BW among chickens, the weight of liver and pancreas, and the empty weight of

intestinal segments, as well as total and segmental gut length, were expressed per kg of BW.

Mucosa Sampling and Enzyme Activity Measurement

The mucosa was scraped off using a glass slide from the jejunum between the Meckel's diverticulum and 35 cm towards the duodenum. Mucosa samples were immediately snap-frozen in liquid nitrogen, and subsamples were stored at −80°C for subsequent brush border enzyme activity measurements and RNA isolation. Enzyme activity measurements in jejunal samples from both locations were performed at L1. Preparation of jejunal homogenates (20%, w/v) and mucosal enzyme activity measurements were performed essentially as previously described (Martin et al., 2013; Metzler-Zebeli et al., 2017a). All enzyme activities were expressed as micromoles of substrate hydrolyzed per min per g protein (U/g protein).

Candidate Gene Expression

Candidate gene expression analysis for jejunal samples from both locations was performed at L1. Total RNA was isolated from jejunal mucosal scrapings of low and high RFI chickens as described (Metzler-Zebeli et al., 2015) using mechanical homogenization and the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA isolates were treated with DNase I (RNA Clean & Concentrator-5 Kit, Zymo Research, Irvine, CA). The quality of RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the subsequent determination of RNA integrity numbers (**RIN**). The majority of samples had a RIN between 9 and 10; 5 samples had RIN values between 4.5 and 6.7. Single stranded cDNA was synthesized from 1 μg of total RNA using the High Capacity Reverse Transcription Kit (Life Technologies, Foster City, CA).

Primers were designed using the Primer Express Software version 3.0 (Life Technologies; Supplementary Table S2). If possible, primer pairs were located on different exons. Candidate genes were monocarboxylate transporter 1 (*MCT1*), intestinal alkaline phosphatase (*ALPI*), tight-junction proteins [claudin 1, (*CLDN1*), claudin 5 (*CLDN5*), zona occludens 1 (*ZO1*), and occludin (*OCLN*)], interleukin-1β (*IL1B*), tumor-necrosis-factor-α (*TNFA*), and toll-like receptors 2 (*TLR2*) and 4 (*TLR4*) (Supplementary Table S2). In total, 6 potential housekeeping genes (**HKG**) were included. Beta-actin (*ACTB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-2 microglobulin (*B2M*), and ornithine decarboxylase antizyme1 (*OAZ1*) were chosen based on previous gene expression data of jejunal tissue from pigs (Metzler-Zebeli et al., 2015). The small nuclear ribonucleoprotein D3 polypeptide (*SNRPD3*) was added as an additional HKG, as this gene is uniformly expressed among a variety of human tissue types and has been shown to be suitable for normalization of RT-qPCR data from other mammalian species (Eisenberg and Levanon, 2013; Scarlet et al., 2015). The expression stability of all 6 HKG was assessed using the geNorm software tool (Vandesompele et al., 2002). The geometric mean of the 2 most stably expressed genes (*GAPDH*, *SNRPD3*) was used for normalization of the target gene expression levels.

Amplifications of target and HKG were performed on a ViiA 7 Real-time PCR system (Life Technologies, Carlsbad, CA). RT-qPCR was carried out in 20 μl reactions, including 25 ng cDNA template, 200 nM of each primer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 1 × buffer B2 (Solis BioDyne, Tartu, Estonia), 50 nM ROX reference dye (Invitrogen, Carlsbad, USA), 0.4 × EvaGreen fluorescent dye (Biotium, Fremont, CA) and 1 unit of HOT FIREPol DNA polymerase (Solis BioDyne; Metzler-Zebeli et al., 2015). All reactions were run in duplicate using the following temperatures: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by the generation of dissociation curves. Reverse transcription controls (RT minus) were included in order to control for residual DNA contamination. The fold change in the target gene, normalized to the mean of the 2 most stably expressed HKG, was calculated relative to the expression of one high RFI female from L1 using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Amplification efficiencies ($E = 10^{(-1/slope)-1}$) of all primer sets are provided in Supplementary Table S2 and prepared by using a 5-fold serial dilution of samples.

Morphometric Measurements (Performed at L1 only)

Pieces of the intestinal tube (1 cm) for morphometric measurements were collected from the *Flexura duodeni*, Meckel's diverticulum, the first centimeter of the proximal ileum, and proximal to the blind end of the ceca. Tube pieces were thoroughly washed in phosphate-buffered saline and fixed in neutral-buffered (pH 7.0) formalin (4% vol/vol). After fixation, intestinal tube samples were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Three discontinuous 3 to 4 μm-thick sections per intestinal site and chicken were processed for evaluation. These sections were stained with hematoxylin and eosin. Slides were examined on a Leica DM2000 light microscope (Leica Microsystems, Wetzlar, Germany), and digital images were captured for morphometric analysis. The villus height from the tip to the villus-crypt junction, villus width at one-third and two-thirds of the length of the villus, and the crypt depth from the base of the villus to the mucosa were measured using the image analysis software ImageJ (version 1.47; https://imagej.nih.gov/ij/index.html). For each trait, 15 measurements were taken from intact well-oriented,

crypt-villus units. The criterion for villus selection was based on the presence of intact lamina propria. Villus height and width were measured at 4-times objective magnification and crypt depth at 10-times objective magnification. The villus surface area was estimated (Nain et al., 2012; Sohail et al., 2012):

$$\text{Villus surface area} = 2\pi \times (\text{average villus width}/2) \times \text{villus height}.$$

In addition, the thickness of the circular and longitudinal muscular layers was measured. Goblet cells were counted per 250 μm of villus or crypt epithelium profile length for a total of 15 replicates per gut section at a 10-times objective magnification. Intraepithelial lymphocytes were counted per 400 μm villus epithelium profile length for 12 replicates per intestinal section at a 20-times objective magnification.

As more RFI-related histo-morphological differences were observed in females, the relative absorptive and secretory surface area of the jejunum in low and high RFI females was additionally assessed using vertical uniform random sampling according to Howard and Reed (2005). Of the paraffin-embedded samples, 100 4 μm-thick serial sections per tissue block were cut. Every 10th section was stained with periodic acid-Schiff reagent. The distance between the examined sections was 40 μm, allowing evaluation of a total length of 400 μm per jejunum sample. Pictures of the sections were taken at 4-times objective magnification and analyzed using the digital software package ELLIPSE (version 2.0.8.1, Kosice, Slovakia). Mucosa and serosa surfaces within the samples were estimated stereologically according to Howard and Reed (2005). Results are presented as mucosal surface to serosal surface ratio.

Ussing Chamber Experiment (Performed at L1 Only)

Differences in intestinal electrophysiological parameters and permeability marker flux were evaluated for 4 replicate samples per chicken and 3 chickens per sampling d (one low, medium, and high RFI chicken of the same sex) as described in Metzler-Zebeli et al. (2017b). This resulted in 6 observations per RFI group and sex. A 20-cm tissue tube piece for the Ussing chamber experiment was collected distal to the Meckel's diverticulum in the direction of the ileum, immediately transferred into ice-cold transport buffer (for buffer composition, see Supplementary Material), which was pre-gassed with carbogen gas (95% O_2–5% CO_2), and transported to the laboratory within 10 min of the death of the animal. For each chicken, jejunal tube pieces were opened at the mesenterium and rinsed with transport buffer to remove digesta particles. Clean tissue pieces were stripped of the outer serosal layers (*Tunica serosa* and the *Tunica muscularis*). The first centimeter of the tissue sample was discarded, after which 4 jejunal mucosal pieces were consecutively cut from the proximal 10 cm of the jejunal tube, which were each mounted in an Ussing chamber. The apical and basolateral sides of the tissue piece had an exposed area of 0.91 cm^2 and were each incubated in a total volume of 10 mL serosal and mucosal buffer solution (pH 7.4, 38°C; for buffer composition, see Supplementary Material). Continuous gassing with carbogen was provided on both the mucosal and the serosal sides to ensure oxygenation and circulation of the buffer by gas lift. The temperature was maintained at 38°C using a circulating thermostatic water jacket.

Each Ussing chamber apparatus was connected to 2 pairs of dual channel current and voltage Ag–AgCl electrodes, which were connected via 3% agar bridges filled with 3 M potassium chloride to allow for electrophysiological measurement by a microprocessor-based voltage-clamp device and software (version 9.10; Mussler, Microclamp, Aachen, Germany). The tissue was alternatively pulsed with a positive or negative pulse of 20 μA and 100 ms duration. After an equilibration period of 20 min under open-circuit conditions, the tissue was short-circuited by clamping the voltage to zero. The potential difference (mV), short-circuit current (I_{sc}, $\mu A/cm^2$) and transepithelial resistance ($\Omega \times cm^2$) were continuously recorded using a microprocessor-based voltage-clamp device and software (version 9.10; Mussler, Microclamp, Aachen, Germany). The tissue conductance (G_T, mS/cm^2) was calculated as the reciprocal of the R_T.

After recording electrophysiological measurements for 5 min, fluorescein 5(6)-isothiocyanate (**FITC**; 389.38 g/mol; Sigma-Aldrich, Schnelldorf, Austria) and horse-radish peroxidase (**HRP**; 44,000 g/mol; Carl Roth GmbH+Co.KG, Karlsruhe, Germany) were added to final concentrations of 0.1 mM and 1.8 μM, respectively, to assess the mucosal-to-serosal flux and, as a result, the paracellular permeability of the distal jejunum. Samples from the basolateral buffer solution were taken at 60, 120, and 180 min, whereas samples from the mucosal side were collected at 70 and 170 min after marker addition to measure marker flux rates. The buffer samples were stored at −20°C and were later analyzed for concentrations of FITC and HRP. At the end of the experiment (185 min after initiating voltage clamp), the tissue survival was monitored by adding theophylline (inhibitor of the phosphodiesterase; final concentration, 8 mmol/L) to both chamber halves. Concentrations of FITC and HRP in mucosal and serosal buffers were analyzed, and mucosal-to-serosal flux rates of FITC and HRP were calculated as described in Metzler-Zebeli et al. (2017b).

Chemical Analysis of Feed and Feces

The DM content of feed and freshly dropped excreta samples was determined by oven-drying at 105°C overnight (method 3.1; Naumann and Basler, 2012). Total excreta samples were pooled for each chicken and

freeze-dried prior to proximate nutrient analysis. Dried fecal samples were ground (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) to pass through a 0.5-mm screen. Proximate nutrients [DM, crude ash, CP (nitrogen × 6.25) by the Kjeldahl method] in diets and feces at both locations, and only at L1 real protein by the Barnstein method, ether extract, crude fiber, total starch, sugar, calcium in diets and phosphorus (**P**) were analyzed in diets and feces according to Naumann and Basler (2012). Acid-insoluble ash (**AIA**; used as indigestible marker) contents of finisher diet and fecal samples were analyzed at both locations after ashing the samples and boiling the ash with 4 M hydrochloric acid (Naumann and Basler, 2012).

The apparent total tract digestibility (**ATTD**, %) of DM and real protein as well as the apparent retention of ash, CP, and P (% of intake) were calculated as:

$$\begin{aligned}&\text{ATTD or retention (\%)}\\&= 100 - [100 \times (\%\ \text{AIA in feed}/\%\ \text{AIA in feces})\\&\quad \times (\%\ \text{nutrient in feces}/\%\ \text{nutrient in feed})] \quad (5)\end{aligned}$$

Daily nutrient excretion was calculated as [6]:

$$\begin{aligned}&\text{Nutrient excretion(g/day)}\\&= \text{Nutrient content in feces(g/kg)}\\&\quad \times \text{DM intake(g/day)}\\&\quad \times (\%\ \text{AIA in feed}/\%\ \text{AIA in feces}) \quad (6)\end{aligned}$$

Dry matter intake was calculated for the collection d of total excreta on d 34 and 35 of life.

Statistical Analysis

Data for BW, RFI, size of intestine, liver, and pancreas, jejunal permeability, brush border enzyme activity, histo-morphology, and candidate gene expression were first analyzed for normality using the Shapiro–Wilk test with the PROC UNIVARIATE in SAS (version 9.4; SAS Inst. Inc., Cary, NC). The Cook's distance (Cook's D) test in SAS was used to determine any influential observation on the model. All variables were normally distributed and analyzed by ANOVA using the PROC MIXED procedure in SAS. Overall, 2 different models were run. The first accounted for the fixed effects of sex, batch, location, and RFI group. Because chickens were sacrificed at different d of life and to consider that chickens were consecutively sampled, the first model included the random effects of chicken nested within d of life × chicken order at sacrifice. Sex as fixed effect was significant for most parameters; therefore, variables were analyzed separately for female and male chickens using the second model. This model was fitted to take into account the fixed effects of the RFI group, experimental location, and their 2-way-interaction. The random effect considered the chicken nested within batch, d of life × chicken order at slaughter. For parameters determined only at L1 (i.e., jejunal permeability, morphology, and weight of the crop, gizzard, and proventriculus), location and the RFI × location interaction were omitted as fixed effects. Where applicable, orthogonal contrasts were used to evaluate linear effects of the RFI group. Degrees of freedom were approximated by the method of Kenward–Roger. Least squares means were computed using the pdiff statement and significance declared at $P \leq 0.05$. A trend was considered at $0.05 < P \leq 0.10$.

For variables that were available from both locations, Pearson's correlation analysis (PROC CORR in SAS) was used to establish and quantify the relationship among individual RFI, RBG, RIG, FCR, TFI, and TBWG values and the intestinal variables visceral organ size, nutrient retention and excretion, and mucosal enzyme activity and gene expression in the jejunum.

RESULTS

Chicken Performance and Feed Efficiency

The RFI ranged on average from −231 to 215 g for low to high RFI in females and from −197 to 267 g for low to high RFI in males ($P < 0.001$; Supplementary Table S3) representing a difference of 330 and 498 g in TFI between the most and least efficient female and male chickens ($P < 0.001$), respectively. Body weight development and TBWG were similar among chickens of diverging RFI. The FCR linearly increased on average by 13% from low to high RFI in both sexes ($P < 0.001$). Total FI was similar across locations, whereas female and male chickens gained 354 and 418 g less, respectively, at L2 compared to L1 ($P < 0.001$). This led to a 12% lower FCR in chickens at L1 compared to L2 ($P < 0.001$). At sacrifice, male chickens at both locations had similar BW across locations and RFI group, whereas female chickens at sacrifice weighed 270 g more at L1 than at L2 ($P = 0.001$), but their BW was similar among RFI groups.

Nutrient Retention and Excretion

Irrespective of sex, selected chickens at L2 had higher nutrient retention and lower nutrient excretion than at L1 ($P < 0.01$; Table 1). Female chickens at L2, but not at L1, had increased ATTD of DM from low to high RFI, whereas male chickens at L1 had a 20% higher daily DM excretion from low to high RFI ($P < 0.05$). In males, retention of CP linearly decreased from low to high RFI, while daily CP excretion increased from low to high RFI in male and female chickens at L1 ($P < 0.05$). Both males and females at L1 had a linearly increasing ATTD and decreasing daily excretion of real protein from low to high RFI ($P < 0.05$), whereas uric acid-nitrogen excretion was similar for the RFI groups.

Table 1. Least squares means of nutrient digestibility, retention, and excretion in female and male broiler chickens of diverging residual feed intake (RFI) raised at 2 different locations.

	Location 1				Location 2						
Item[1]	Low RFI	Medium RFI	High RFI	SEM	Low RFI	Medium RFI	High RFI	SEM	FE, $P^{2,3}$	location, P^{2}	FE × location, P^{2}
Females											
Dry matter											
ATTD (%)	73.2	73.1	74.2	1.32	78.2	80.9	82.8	1.39	0.15	<0.001	0.43
Excretion (g/day)	44.4	47.5	46.8	2.21	34.4	31.8	30.1	2.32	0.85	<0.001	0.30
Crude protein											
Retention (%)	57.7	52.2	52.7	2.50	70.6	76.4	77.8	2.63	0.89	<0.001	0.051
Excretion (g/day)	13.6^{b}	16.4^{a,b}	16.7^{a}	0.92	7.0	7.2	6.9	0.95	0.22	<0.001	0.23
ATTD of real protein (%)[4]	68.2^{a}	64.1^{a,b}	63.9^{b}	1.45	–	–	–	–	0.086*	–	–
Real protein excretion (g/d)[4]	11.4^{b}	13.7^{a,b}	14.2^{a}	0.86	–	–	–	–	0.065*	–	–
Uric acid-nitrogen excretion (g/d)	0.35	0.43	0.40	0.05	–	–	–	–	0.56	–	–
Crude ash											
Retention (%)	20.2	20.5	21.4	5.07	40.0	35.2	46.0	5.32	0.51	<0.001	0.62
Excretion (g/day)	6.9	7.4	8.1	0.55	9.3	8.3	7.4	0.58	0.86	0.007	0.27
Phosphorus											
Retention (%)	57.9	55.3	55.3	2.06	–	–	–	–	0.59	–	–
Excretion (g/day)	0.40	0.47	0.48	0.04	–	–	–	–	0.21	–	–
Males											
Dry matter											
ATTD (%)	74.4	73.2	71.1	1.73	77.5	80.8	81.8	1.73	0.83	<0.001	0.093
Excretion (g/day)	48.4	51.6	58.8	3.42	36.9	36.8	41.3	3.42	0.081*	<0.001	0.68
Crude protein											
Retention (%)	61.7	57.4	52.7	1.71	72.2	76.3	76.4	2.72	0.63	<0.001	0.053
Excretion (g/day)	14.0	15.9	18.6	1.16	8.3	8.4	9.2	1.17	0.062	<0.001	0.28
ATTD of real protein (% of intake)[4]	70.7^{a}	67.6^{a,b}	63.4^{b}	2.25	–	–	–	–	0.090*	–	–
Real protein excretion (g/d)[4]	12.0^{b}	13.5^{a,b}	16.2^{a}	1.2	–	–	–	–	0.061*	–	–
Uric acid-nitrogen excretion (g/d)	0.38	0.40	0.32	0.05	–	–	–	–	0.54	–	–
Crude ash											
Retention (%)	26.5	23.1	21.9	5.85	31.6	37.1	41.9	5.86	0.88	0.009	0.43
Excretion (g/day)	7.3	11.1	10.0	0.68	9.4	7.8	8.4	0.69	0.12	<0.001	0.76
Phosphorus											
Retention (%)	61.5	56.5	54.0	2.93	–	–	–	–	0.21	–	–
Excretion (g/day)	0.43	0.49	0.55	0.04	–	–	–	–	0.16	–	–

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; ATTD, apparent total tract digestibility; FE, feed efficiency; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2]P: probability level of fixed effects feed efficiency, location, and their 2-way interaction.

[3]Linear polynominal contrast: $^{*}P \leq 0.05$.

[4]Nitrogen × 6.25.

[a,b]Least squares means within a row without a common lowercase superscript differ among RFI groups ($P < 0.05$).

Visceral Organ Weights and Gut Length

The size and weight of the intestinal tract of chickens was largely affected by location ($P < 0.05$; Table 2), and hence RFI-related differences in visceral organ size were different between locations. In particular, the length and weight of the ileum were 3.5- and 5-times greater at L2 compared to L1 ($P < 0.001$), irrespective of sex. Overall, FE × location interactions were observed for liver and ileum weight in females, as well as for ceca length and colon weight in males, which indicated FE-related differences at L2, but not at L1 ($P < 0.05$). Accordingly, at L2, low RFI female chickens had a heavier liver and ileum compared to medium RFI females ($P < 0.05$). Also at L2, male chickens of low RFI had shorter ceca compared to high RFI males ($P < 0.05$), whereas medium RFI males had a heavier colon than low and high RFI males ($P < 0.05$). Moreover, in males, the weight of the liver increased by 21% from low to high RFI at L1 ($P < 0.05$) but not at L2.

Intestinal Morphology

Intestinal histo-mophological measures were determined only in chickens at L1. Differences in intestinal morphology due to diverging RFI were mostly found in female chickens, whereas in males, differences could not be distinguished (Table 3; Supplemental Table S4). As such, crypt depth linearly increased from low to high RFI in the duodenum of females ($P < 0.05$; Table 3). Likewise, jejunal villus height and crypt depth linearly increased from low to high RFI in the mid-jejunum of females ($P < 0.05$), leading to a 29%-increase in jejunal apparent villus surface area from low to high RFI ($P < 0.05$). Because of these differences for female chickens, we also determined the relative absorptive and secretory surface area in the jejunum of chickens; however, this was similar between low RFI and high RFI females. Goblet cell and intraepithelial lymphocyte numbers were not different between the RFI groups at any intestinal segment.

Table 2. Least squares means of visceral organ weight and intestinal length in female and male broiler chickens of diverging residual feed intake raised at 2 different locations.

	Location 1				Location 2						
Item[1]	Low RFI	Medium RFI	High RFI	SEM	Low RFI	Medium RFI	High RFI	SEM	FE, P^2	location, P^2	FE × location, P^2
Females											
Organ weight (g/kg BW)											
Liver	20.16	22.90	23.36	1.122	24.17[a]	20.12[b]	22.30[a,b]	1.193	0.50	0.96	0.019
Pancreas	1.60	1.82	1.72	0.087	1.77	1.63	1.76	0.093	0.81	0.96	0.13
Crop	2.42	2.33	2.35	0.211	–	–	–	–	0.76	–	–
Gizzard	3.04	3.56	3.72	0.355	–	–	–	–	0.38	–	–
Proventriculus	7.62	7.85	7.87	0.368	–	–	–	–	0.87	–	–
Duodenum	4.21	4.73	4.60	0.277	5.47	5.15	4.92	0.294	0.79	0.007	0.23
Jejunum	12.26	12.36	13.27	0.879	10.58	8.87	9.45	0.934	0.60	<0.001	0.48
Ileum	1.48	1.44	1.60	0.234	8.08[a]	6.30[b]	7.36[a,b]	0.249	0.001	<0.001	0.003
Average cecum[3]	0.72	0.66	0.70	0.118	2.73	2.45	2.29	0.126	0.18	<0.001	0.26
Colon	0.76	0.89	0.84	0.091	1.15	1.01	1.14	0.096	0.91	0.001	0.34
Length (cm/kg BW)											
Duodenum	9.99	10.53	10.23	0.534	11.35	11.19	11.56	0.555	0.91	0.015	0.75
Jejunum	27.98	31.58	30.22	1.271	28.55	25.99	28.75	1.352	0.67	0.050	0.063
Ileum	8.31	7.84	8.86	1.015	28.33	27.15	29.88	1.079	0.19	<0.001	0.70
Average cecum[3]	5.02	5.00	5.18	0.273	7.05	7.05	6.71	0.290	0.94	<0.001	0.58
Colon	2.46	2.59	2.59	0.170	3.05	2.80	3.10	0.199	0.72	0.008	0.57
Males											
Organ weight (g/kg BW)											
Liver	19.47	19.50	23.43	1.003	21.08	20.42	22.16	0.986	0.012	0.61	0.33
Pancreas	1.64	1.61	1.38	0.099	1.44	1.59	1.54	0.097	0.37	0.80	0.17
Crop	2.74	3.46	3.34	0.328	–	–	–	–	0.28	–	–
Gizzard	3.40	3.15	3.39	0.304	–	–	–	–	0.80	–	–
Proventriculus	8.10	7.66	7.85	0.502	–	–	–	–	0.83	–	–
Duodenum	3.94	4.39	4.84	0.324	4.95	5.32	5.36	0.319	0.13	0.003	0.71
Jejunum	11.77	14.02	13.53	0.997	9.52	9.77	9.76	0.980	0.41	<0.001	0.57
Ileum	1.46	1.54	1.6	0.346	7.33	7.86	7.66	0.340	0.64	<0.001	0.82
Average cecum[3]	0.68	0.66	0.67	0.120	2.67	2.71	2.46	0.118	0.53	<0.001	0.51
Colon	0.66	0.66	0.74	0.063	0.90[b]	1.21[a]	1.00[b]	0.062	0.049	<0.001	0.025
Length (cm/kg BW)											
Duodenum	9.40	9.34	9.89	0.488	9.94	11.66	9.93	0.480	0.22	0.018	0.060
Jejunum	28.07	28.76	28.71	1.571	23.12	25.00	24.17	1.545	0.70	0.001	0.93
Ileum	7.52	6.78	7.98	0.706	25.59	27.16	25.28	0.670	0.82	<0.001	0.086
Average cecum[3]	4.92	4.69	4.73	0.228	5.77[b]	6.73[a]	6.06[b]	0.224	0.23	<0.001	0.036
Colon	2.08	2.09	2.24	0.124	2.53	2.83	2.69	0.122	0.33	<0.001	0.39

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; BW, body weight; FE, feed efficiency; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2]*P*: probability level of fixed effects feed efficiency, location, and their 2-way interaction.

[3]Average cecum represents the average (weight or length) of both ceca.

[a,b]Least squares means within a row without a common lowercase superscript differ among RFI groups ($P < 0.05$).

Mucosal Enzyme Activity

Location affected the mucosal activity of maltase and sucrase in the mid-jejunum of males and females by being ∼10 to 50% higher in chickens at L1 than at L2 ($P < 0.01$; Table 4), whereas the detectable lactase activity was similar at both locations. Variation in jejunal maltase activity between the RFI groups was observed only in males at L1, where lower activity was found in low RFI compared to medium RFI animals ($P < 0.05$).

Candidate Gene Expression in Jejunal Mucosa

Location-related differences in the relative expression of genes in the mid-jejunal mucosa were observed for expression of *OCLN* and *IL1B* in both sexes and for the expression of *ZO1*, *TNFA*, *TLR2*, and *IAP* in males, which were higher at L2 than at L1 ($P < 0.05$; Table 5). Only the relative *MCT1* expression of the jejunal mucosa was about 22% higher at L1 compared to L2 ($P < 0.05$). A RFI-related variation in relative gene expression was distinguishable for the expression of *IL1B* in females, and of *TLR4* and *MCT1* in males ($P < 0.05$). Low RFI females had a lower relative *IL1B* expression than high RFI chickens at L2, but not at L1 ($P < 0.05$). In contrast, relative expression of *TLR4* in the jejunum was 50% higher in low RFI males compared to high RFI males at L2 ($P < 0.05$), but not at L1. Likewise, relative expression of the short-chain fatty acid transporter *MCT1* was higher in low RFI males compared to high RFI males, and this effect was observed at both locations ($P < 0.001$).

Jejunal Permeability

Short-circuit current, G_T, and mucosal-to-serosal flux rates of FITC and HRP were used to determine

Table 3. Least squares means of morphological characteristics of different intestinal segments in female broiler chickens of diverging residual feed intake raised at location 1.

Item[1]	Low RFI	Medium RFI	High RFI	SEM	FE, $P^{2,3}$
Duodenum					
Villus height (μm)	1375	1579	1547	177.1	0.12
Villus width (μm)	178	180	164	6.3	0.15
Crypt depth (μm)	131	141	159	6.1	0.010*
Muscle layer (μm)	183	218	211	13.4	0.18
Villus height: crypt depth ratio	10.6	11.3	9.8	0.49	0.13
Apparent villus surface area (mm^2)	0.68	0.83	0.83	0.072	0.29
Goblet cells per 250 μm villus surface	8.7	8.5	8.6	0.61	0.98
Intraepithelial lymphocytes per 400 μm villus surface	10.4	10.1	10.0	0.65	0.89
Jejunum					
Villus height (μm)	893	920	1048	50.7	0.080*
Villus width (μm)	129	153	149	7.5	0.080
Crypt depth (μm)	95	102	113	5.0	0.036*
Muscle layer (μm)	179	189	195	15.8	0.79
Villus height: crypt depth ratio	9.4	9.1	9.4	0.41	0.83
Apparent villus surface area (mm^2)	0.38	0.43	0.49	0.036	0.117*
RASS (mucosal surface, μm/serosal surface, μm)[4]	17.6	–	16.6	1.11	0.54
Goblet cells per 250 μm villus surface	11.0	11.8	11.1	0.52	0.79
Intraepithelial lymphocytes per 400 μm villus surface	13.2	12.4	11.6	0.74	0.34
Ileum					
Villus height (μm)	624	647	741	42.1	0.14
Villus width (μm)	138	152	150	9.5	0.55
Crypt depth (μm)	98	98	108	4.7	0.22
Muscle layer (μm)	229	224	236	17.2	0.88
Villus height: crypt depth ratio	6.3	6.7	6.9	0.37	0.53
Apparent villus surface area (mm^2)	0.28	0.31	0.35	0.069	0.38
Goblet cells per 250 μm villus surface	16.5	16.5	15.2	1.11	0.64
Intraepithelial lymphocytes per 400 μm villus surface	12.3	12.1	12.2	0.93	0.99
Ceca					
Crypt depth (μm)	320	317	331	24.6	0.91
Goblet cells per 250 μm villus surface	6.5	6.6	6.3	0.61	0.92

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); FE, feed efficiency; RFI, residual feed intake.
[1]Values are least squares means ± standard error of the mean (SEM).
[2]P: probability level of the fixed effect feed efficiency.
[3]Linear polynominal contrast: $^*P \leq 0.05$.
[4]RASS, relative absorptive and secretory surface area (mucosal surface to serosal surface ratio). The RASS was determined only for the jejunum of low and high RFI female chickens.

Table 4. Least squares means of mucosal disaccharidase activities in mid jejunum of female and male broiler chickens of diverging residual feed intake raised at 2 different locations.

	Location 1				Location 2						
Enzyme activity (U/g protein)[1]	Low RFI	Medium RFI	High RFI	SEM	Low RFI	Medium RFI	High RFI	SEM	FE, P^2	Location, P^2	FE × location, P^2
Females											
Maltase	2240	2033	2448	163.8	1227	1527	1248	174.1	<0.001	0.096	0.51
Sucrase	123	188	231	19.4	213	168	135	20.6	<0.001	0.074	0.76
Lactase	9.6	7.7	7.4	1.84	9.1	8.7	8.2	1.96	0.81	0.91	0.44
Males											
Maltase	1989[b]	3208[a]	2755[a,b]	290.5	1687	1397	1573	285.5	0.26	<0.001	0.038
Sucrase	209	252	254	31.7	172	161	169	31.1	0.77	0.008	0.64
Lactase	8.0	5.2	7.8	1.57	9.8	7.7	7.2	1.55	0.29	0.35	0.58

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; FE, feed efficiency.
[1]Values are least squares means ± standard error of the mean (SEM).
[2]P: probability level of fixed effects feed efficiency, location, and their 2-way interaction.
[a,b]Least squares means within a row without a common lowercase superscript differ among RFI groups ($P < 0.05$).

electrogenic ion transport and tissue permeability in the distal jejunum (Table 6). Gut electrophysiological parameters (I_{sc} and G_T) and marker fluxes were not different in the distal jejunum of male chickens of diverging RFI, whereas RFI-related differences were detectable in females. Females had a linearly decreasing I_{sc} ($P < 0.05$) and G_T ($P < 0.001$) from low to high RFI in the distal jejunum. Similarly, the mucosal-to-serosal flux of FITC linearly decreased ($P < 0.05$) from low to high RFI in the distal jejunum of females.

Table 5. Least squares means of mucosal expression of target genes in mid jejunum of female and male broiler chickens of diverging residual feed intake raised at 2 different locations.

	Location 1			Location 2					
Relative expression[1]	Low RFI	High RFI	SEM	Low RFI	High RFI	SEM	FE, P^2	Location, P^2	FE × location, P^2
Females									
CLDN1	1.076	1.157	0.1254	1.051	1.073	0.1343	0.70	0.68	0.82
CLDN5	1.504	2.180	0.7672	1.794	1.725	0.7990	0.70	0.92	0.64
ZO1	1.223	0.768	0.3595	1.765	1.562	0.3851	0.39	0.085	0.74
OCLN	1.007	0.694	0.2304	1.624	1.168	0.2468	0.12	0.031	0.77
IL1B	0.486	0.527	0.2068	0.562	1.399	0.2134	0.048	0.034	0.071
TNFA	6.723	1.442	3.1561	1.797	4.384	3.9548	0.71	0.78	0.27
TLR2	0.679	0.933	0.2890	0.766	1.359	0.3009	0.17	0.39	0.57
TLR4	0.537	0.625	0.1071	0.731	0.544	0.1154	0.66	0.62	0.23
IAP	1.018	0.819	0.2800	0.732	1.770	0.3539	0.21	0.32	0.070
MCT1	1.515	1.374	0.1975	1.493	1.272	0.2128	0.39	0.76	0.85
Males									
CLDN1	1.804	1.354	0.3673	1.678	1.085	0.3474	0.16	0.59	0.84
CLDN5	1.986	1.483	0.4553	2.012	1.387	0.4553	0.23	0.94	0.90
ZO1	0.806	0.700	0.1969	1.499	1.156	0.1979	0.26	0.007	0.55
OCLN	0.730	0.608	0.1677	1.196	1.000	0.1685	0.35	0.016	0.83
IL1B	0.707	0.361	0.2288	0.858	1.389	0.2436	0.70	0.019	0.074
TNFA	1.364	1.707	0.4333	3.080	2.301	0.5397	0.66	0.027	0.26
TLR2	0.617	0.464	0.2266	1.808	1.508	0.2266	0.33	<0.001	0.75
TLR4	0.734	0.670	0.0941	0.834	0.417	0.0969	0.017	0.43	0.074
IAP	0.730	0.759	0.2500	1.575	1.474	0.3062	0.90	0.010	0.82
MCT1	2.032	1.443	0.1828	1.729	0.989	0.1774	<0.001	0.044	0.68

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); location 2, Agri-Food and Biosciences Institute, Hillsborough, UK; FE, feed efficiency; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2]*P*: probability level of fixed effects feed efficiency, location, and their 2-way interaction.

Table 6. Least squares means of electrophysiological data and tissue permeability in distal jejunum of female and male broiler chickens of diverging residual feed intake raised at location 1.

Item[1]	Low RFI	Medium RFI	High RFI	SEM	FE, $P^{2,3}$
Females					
I_{sc} (μA/cm^2)	23.0^a	18.8^{a,b}	10.1^b	3.762	0.087*
G_T (ms/cm^2)	10.3^a	6.5^b	6.5^b	0.644	0.001**
J_{ms} of FITC (nmol/cm^2 × h)	0.119^a	0.035^b	0.049^b	0.021	0.030*
J_{ms} of HRP (pmol/cm^2 × h)	0.22	0.16	0.15	0.046	0.60
Males					
I_{sc} (μA/cm^2)	19.2	12.6	20.9	3.959	0.33
G_T (ms/cm^2)	8.23	6.41	8.03	0.975	0.38
J_{ms} of FITC (nmol/cm^2 × h)	0.126	0.065	0.082	0.023	0.19
J_{ms} of HRP (pmol/cm^2 × h)	0.41	0.27	0.27	0.093	0.47

Location 1, University of Veterinary Medicine Vienna (Vienna, Austria); FE, feed efficiency; G_T, tissue conductance; I_{sc}, short-circuit current; J_{ms}, mucosal-to-serosal flux; RFI, residual feed intake.

[1]Values are least squares means ± standard error of the mean (SEM).

[2]*P*: probability level of the fixed effect feed efficiency.

[3]Linear polynominal contrast: $^*P \leq 0.05$, $^{**}P < 0.001$.

[a,b]Least squares means within a row without a common lowercase superscript differ among RFI groups ($P < 0.05$).

Pearson's Correlations Among RFI and Intestinal Size and Function

Pearson's correlation analysis was used to correlate FE metrics, TFI, and TBWG data with variables that were measured at both locations (Supplemental Tables S5 and S6). Only a few correlations between chickens' TFI and intestinal variables, such as jejunal relative *TLR4* and *MCT1* expression in males, could be established. Irrespective of sex, chickens' TBWG correlated ($r \geq 0.30$; $P < 0.05$) with small and large intestinal length and weight as well as with retention and excretion of DM and CP. It also was correlated with jejunal expression of *IL1B, TLR2* (negatively), and *TNFA* (positively) in females ($P < 0.05$). Positive correlations between RFI and excretion of DM and CP could be established in males, but not in females. In females, jejunal expression of *IL1B* positively correlated to chickens' RFI ($r = 0.40$; $P < 0.05$). Other variables, such as visceral organ size and mucosal enzyme activity in the jejunum, were not correlated to chickens' RFI values. Correlations between RIG and intestinal variables were similar to those of RFI, whereas chickens' RBG correlated with duodenal weight in females as well as

excretion of DM and CP in males ($r \geq 0.33$; $P < 0.05$; Supplementary Tables S5 and S6). Many variables, including length and weight of almost all segments of the small and large intestines in both sexes, retention and excretion of DM and CP in both sexes, jejunal maltase and sucrase activity in females, and the relative expression of *IL1B* and *ZO1* (females) as well as of *OCLN*, *CLDN1*, and *MCT1* (males), correlated to chickens' FCR ($r \geq 0.33$; $P < 0.05$; Supplementary Tables S5 and S6).

Moreover, nutrient retention and intestinal length and weight were correlated and showed weak ($r = 0.30$ to 0.49; $P < 0.05$), medium ($r = 0.50$ to 0.69; $P < 0.001$), or strong ($r = 0.70$ to 0.87; $P < 0.001$) relationships for almost all combinations in both sexes (Supplemental Table S7).

DISCUSSION

In the current study, all birds represented Cobb 500FF genetics (Supplementary Figures S1), and the trials across the 2 geographical locations were designed to mirror each other as closely as possible. However, the present results demonstrate that the environment has a great impact on RFI-related differences in intestinal structure and function in chickens. Strong location-associated effects existed for all parameter categories, including growth performance, nutrient retention and excretion, intestinal size, mucosal disaccharidase activity, and expression of some target genes in the jejunum. Only a few intestinal variables, such as the excretion of DM and CP in males as well as jejunal expression of *IL1B* in females, were clearly associated with chickens' RFI at both locations and as such might be targeted to improve RFI in chickens, irrespective of the environment in which the chickens were raised. Present results from L1 further emphasized that biological profiles of RFI-related variation in intestinal size, structure, and function should be separately generated for males and females, as differences in small intestinal structure and jejunal permeability was associated with variation in RFI in females, but not in males. Regardless of the poor correlation between RFI and intestinal variables, FCR in chickens appeared to be more useful for the identification of common intestinal features that explain FE variation across different environments.

Due to the similar experimental procedures, the largely diverging TBWG from d 7 to 36 of life across locations was not expected but was likely associated with the longer and heavier small and large intestines found in chickens at L2 compared to L1. The intestinal tract has a high metabolic activity; therefore, changes in its size can have a profound impact on the energy efficiency and growth of the whole animal (Johnson et al., 1990; Choct et al., 1999; Herd and Arthur, 2009). The higher nutrient retention in chickens at L2 compared to chickens at L1 also was not expected and was evidently not sufficient to compensate for the higher basal needs of the intestine to maintain growth. Due to the higher retention of nitrogen, chickens from L2 had a reduced daily excretion of environmental pollutants compared to chickens from L1. However, at production scale, lower growth performance adds d to reach market weight, which may counterbalance a reduced daily excretion of nitrogen (Willems et al., 2013). The reduced mucosal maltase and sucrase activity in the mid-jejunum of chickens from L2 compared to chickens at L1 also may be seen as an adaptation to the longer small intestine. Mucosal lactase activity was equal across locations and might represent similar microbial disaccharidase activity.

To explain the diverging intestinal development and function in chickens from the 2 locations, a genetic influence is probable, although chicks were not related to each other (Supplementary Figure S2 and Supplementary Table S8). A certain variation probably originated from the diets. As the diets were produced locally, natural variation in nutrient concentrations of the raw materials used to prepare the diets at the 2 locations, i.e., corn and soybean meal (e.g., dietary fiber composition; Rodehutscord et al., 2016), may have altered digestive, absorptive, and fermentative processes, thereby leading to the diverging intestinal size and performance. Notably, the protein content of the starter diet at L1 was 2% higher than at L2, which may have had consequences for intestinal development, muscle protein metabolism, and growth (Everaert et al., 2010). With regards to intestinal development, nutrient digestion, and growth, the microbial colonization is another important factor to consider, which is also influenced by the diet (Oakley et al., 2014; Apajalahti and Vienola, 2016). The early microbial colonization largely influences the gene expression profiles in the jejunal mucosa of chickens within the first wk of life (Schokker et al., 2015). Microbes encountered in the environment (e.g., personnel, housing, water, and diet) probably differed between locations, modifying the early microbial colonization and successional changes during the test period. Location-related differences in the relative expression of genes related to the innate immune response in both sexes and expression of the short-chain fatty acid transporter *MCT1* at the jejunal mucosa in males also point to diverging microbial profiles and activity between locations.

In accordance with earlier observations in laying hens and broilers (Luiting et al., 1994), as well as in other livestock species such as beef cattle and pigs (Fitzsimons et al., 2014; Montagne et al., 2014), the present findings supported that the digestion of nutrients played a small role in the variation of RFI in our chicken populations across both locations. Only at L1, selection of chickens for RFI reduced the excretion of nitrogenous substances, which was mainly caused by a reduction in nitrogen losses from the digestive tract as indicated by the excretion of real protein and uric acid.

Increasing the FI level may enlarge the intestinal size to metabolize the supplied nutrients, which

subsequently increases the intestinal energy demand (Johnson et al., 1990). Previous studies in laying hens and chickens selected either for RFI or AME_N, respectively, supported heavier intestinal tracts in less efficient animals (van Eerden et al., 2004; de Verdal et al., 2010). In spite of the current differences in TFI, visceral organ size was similar among RFI groups across locations. Only by analyzing the data individually for location and sex, RFI-related linear patterns in visceral organ size became apparent. As such, the weight of the ceca may have contributed to the RFI-variation in females at L2. At L1, liver weight may explain some of the variation in RFI of male chickens, thereby supporting the theory of greater basal energy needs in less efficient animals (van Eerden et al., 2004; Fitzsimons et al., 2014).

Besides visceral organ size, we observed strong sex-related differences in RFI-effects on intestinal structure and functioning at L1. Notably, in females, but not in males, differences in small intestinal structure (e.g., villus height in the jejunum and crypt depth in the duodenum and jejunum) and jejunal permeability contributed to the variation in RFI. In general, longer villi may be expected in more efficient chickens, allowing for greater absorption of nutrients and hence compensating lower FI (Caspary, 1992; de Verdal et al., 2010). In contrast to this proposition and to findings in laying hens (Nain et al., 2012), but corresponding to observations in chickens (de Verdal et al., 2010), our results for smaller jejunal villus height and apparent villus surface area in low RFI females supports an energy-saving mechanism by reducing the maintenance needs for the renewal of the epithelial surface in the jejunum. To confirm this and similar to de Verdal et al. (2010), smaller crypts were found in the duodenum and jejunum of more FE females, as longer villi have deeper crypts to ensure their renewal (Samanya and Yamauchi, 2002; de Verdal et al., 2010). Since the maltase and sucrase activities per gram of mucosal protein at the jejunal brush border were similar among females of diverging RFI, it can be speculated that the greater villus surface in high RFI females augmented glucose release at the jejunal brush border.

Unlike pigs selectively bred for low and high RFI (Mani et al., 2013), the present jejunal electrophysiological data and mucosal-to-serosal flux of FITC in females indicated that part of the compensation of the reduced FI to maintain similar growth may be explained by greater paracellular nutrient uptake in the distal jejunum. In spite of this, gene expression levels of *ZO1*, *OCLN*, *CLDN1*, and *CLDN5* in mid-jejunal mucosa scrapings were not different among female chickens of diverging RFI, neither at L1 nor at L2. All 4 genes code for proteins that are important components of the tight junction protein complex, which establishes the paracellular barrier (Turner, 2009). Due to translational regulation of gene expression, levels of tight junction proteins likely did not correspond to the functional protein level. However, correlation analysis indicated that a certain association between chickens' FE and up-regulated expression of tight junction protein genes existed when FCR was used as a FE metric. It should also be considered when comparing the results of the gene expression and Ussing chamber experiments that tissue samples did not originate from the same jejunal spot, and the regulation of tight junction proteins and paracellular permeability may have differed between the mid and distal jejunum.

Greater energy fueling of the basal immune response may have a negative impact on feed efficiency (Mani et al., 2013). Previously, differences in the contribution of the immune system to variation in RFI were more often reported in chickens under challenge conditions (Cotter and Van Eerden, 2006). As part of the innate defense mechanism, similar goblet cell numbers between chickens of diverging RFI may imply similar mucin production. Likewise, intraepithelial lymphocytes along the small intestine were equal among RFI groups at L1, and RFI-related differences in the expression of genes related to the innate immune response were small. Lower *IL1B* expression in the jejunum of low RFI females was in line with the lower cytokine expression in the colon of low RFI pigs, which may indicate a potential energy-saving mechanism compared to high RFI animals (Vigors et al., 2016). In contrast, low RFI male birds in the present study had a greater *TLR4* expression in the jejunal mucosa. Toll-like receptor 4 interacts with lipopolysaccharide on the cell wall of Gram-negative bacteria (Tremaroli and Bäckhed, 2012) and may therefore indicate differences in the bacterial composition of the jejunum towards a greater proportion of Gram-negative bacteria in low RFI birds. Along with this, by being substrate induced (Cuff et al., 2002), the increased jejunal *MCT1* expression in low RFI males may be associated with a greater generation of short-chain fatty acids.

In the correlation analysis, data from both locations were used, and results support a weak relationship between RFI in chickens and intestinal size, structure, and functioning. Evidence for the contribution to the variation of RFI of chickens could be established only for excretion of DM and CP in males. Controversially, many intestinal parameters correlated to the TBWG and FCR of the selected chickens. Chickens' FCR values corresponded to chickens' RFI values, but they were higher at L2 than at L1. Hence, by taking the differences in TBWG into account, FCR may better reflect location-associated differences in intestinal variables. Based on FCR, some variation in the FE of female chickens can be explained by increased nutrient retention, lower expression of genes related to the innate immune response (*IL1B* and *ZO1*), higher disaccharidase activity at the jejunal mucosa, and smaller intestinal size in low FCR animals. In males, underlying mechanisms for improved FCR at the intestinal level could be nutrient retention, smaller intestinal size, up-regulated expression of *OCLN*, and lower expression of *CLDN1* and *MCT1*.

In conclusion, aside from sex-related variation, the present results showed that the environment in which the chickens were raised largely affected differences in intestinal structure and function, which in turn contributed to the variation in RFI of the selected chickens. Location-related variation in intestinal size, nutrient digestion, and jejunal gene expression may have been associated with the different growth rates of chickens between L1 and L2. At L1, energy-saving mechanisms, such as shorter villi and crypts, increased paracellular permeability and improved protein retention in females, and reduced liver size in males contributed to improved RFI. At L2, low RFI was mainly associated with lower ATTD of DM and heavier ilea in females and higher jejunal *TLR4* expression in male chickens. By contrast, according to the correlation analysis, lower excretion of DM and CP in males and jejunal *IL1B* expression in females were common factors reflecting a lower RFI at both locations. Due to the greater RFI-associated variation in gut structure and function in females than in males, this may imply that the RFI may be more applicable for female chickens than for males in selection programs. Controversially, present correlations also suggested that the FCR may better reflect intestinal function profiles linked to FE in chickens raised in different environments.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Figure S1. Principal component analysis plot of single nucleotide 94 polymorphisms from all Cobb500 chickens used at location 1 (Austria) and location 2 (UK). 95 1, 2, 3 indicates the batch number at each location.

Figure S2. G-relationships among chickens from both locations.

Table S1. Ingredients and chemical composition of diets.

Table S2. Oligonucleotide primers for target and housekeeping genes.

Table S3. Least squares means of feed efficiency metrics, total feed intake, total body weight gain, and body weight of female and male broiler chickens of diverging residual feed intake raised at 2 different locations[1].

Table S4. Least squares means of morphological characteristics of different intestinal segments in male chickens of diverging residual feed intake raised at location 1.

Table S5. Selected Pearson's correlation coefficients for RFI, RBG, RIG, FCR, TFI, and TBWG with intestinal variables of female chickens of diverging RFI raised at 2 locations.

Table S6. Selected Pearson's correlation coefficients for RFI, RBG, RIG, FCR, TFI, and TBWG with intestinal variables of male chickens of diverging RFI raised at 2 locations.

Table S7. Pearson's correlation coefficients for retention and excretion of nutrients and intestinal length and weight of male and female chickens of diverging residual feed intake raised at 2 locations.

Table S8. Genomic relationships among chickens.

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