Institute of Animal Science - University of Hohenheim

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EFFECTS OF STRAIN, LIFESPAN AND DIETARY MYO-INOSITOL SOURCES ON POULTRY METABOLISM

DISSERTATION

Submitted in fulfillment of the requirements for the degree

“Doktor der Agrarwissenschaften”

(Dr. sc. agr. / PhD in Agricultural Science)

to the

Faculty of Agricultural Science

presented by

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Born in Bogotá D.C., Colombia

2020

This thesis was accepted as a doctoral thesis (Dissertation) in fulfillment of the regulations to acquire the doctoral degree "Doktor der Agrarwissenschaften by the Faculty of Agricultural Sciences at University of Hohenheim on October the 8th, 2020.

Date of the oral examination: November the 13th, 2020.

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This project was financially supported by the German Research Foundation (DFG HU838/16-1) to which I gratefully acknowledge.

Project FOR 2601:

Inositol phosphates and myo-inositol in the domestic fowl: Exploring the interface of genetics, physiology, micro-biome, and nutrition

Subproject 5:

Potential differences in physiological processes between brown and white laying hens

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List of abbreviations

Ca	Calcium
Day-old	Post-hatching day
DM	Dry matter
FDR	False Discovery Ratio
FTU	Phytase unit
HMIT	H+/myo-inositol transporter
IMPase 1	Inositol monophosphatase 1
InsPs	Inositol phosphates
KEEG	Kyoto Encyclopedia of Genes and Genomes
LB	Lohmann Brown-Classic hens
LSL	Lohmann LSL-Classic hens
LysoPC	Lysophosphatidylcholines
MI	*Myo*-inositol
MIOX	*Myo*-inositol oxygenase
P	Phosphorus
PC	Phosphatidylcholine
Phytase	Inositol hexakisphosphate phosphohydrolase
PI	Phosphatidylinositol
SMIT1/2	Sodium-dependent *myo*-inositol transporter 1 and 2
SM	Sphingomyelin
Week-old	Post-hatching week

1. General introduction

Poultry production represented one of the most important industries for the European Union. Statistics indicated the European Union produced 15,200,000 tons of poultry meat in 2018, of which Germany produced 10.4%. During 2019 poultry meat production was expected to increase by 2.5% while expected per capita consumption was 25.3 kg (EC, 2019). Likewise, European Union expected to increase egg production from 7,210,570 tons in 2018 to 7,349,640 tons in 2019 with Germany representing the second producer from the European Union with around 926,000 tons of eggs produced in 2019 (EC, 2020).

The need for poultry industry rapid growth has triggered more and fast meat and egg production, accelerating metabolic rate, and general performance of birds (Kuenzel and Kuenzel, 1977). Maintaining an adequate balance between poultry health and performance has depended mainly on an optimal feeding strategy (Collins and Sumpter, 2007). An example is a reduction of the dietary anti-nutritional factors in feedstuffs (Makkar, 1993), which has been associated with increases in nutrient availability and thus improvements on nutrient absorption and assimilation.

During the last years, attention has been placed on nutrient releasing from anti-nutritional factors found on poultry feedstuffs. One of the most investigated anti-nutritional factors is phytate (Inositol hexakisphosphate or $InsP_6$). $InsP_6$ is widely known due to its property of binding 60-80% of P and other nutrients from the plant-based diets (Abdel-Megeed and Tahir, 2015). It has been demonstrated broilers and laying hens could hydrolyze $InsP_6$ through their endogenous mucosa (Maenz and Classen, 1998; Sommerfeld et al., 2019) and microbial enzymatic activity (Dersjant-Li et al., 2015). However, it is a matter of the fact that the complete fraction of dietary $InsP_6$ is not degraded completely by the bird endogenous enzymatic system so that a significant portion of $InsP_6$-bound nutrients are not harnessed,

causing low nutrient absorption by the poultry intestine, but also increases on production costs and eutrophication of water sources (Maenz, 2001).

The need for additional strategies to degrade $InsP_6$ has led to the optimization of dietary enzyme utilization. Phytase is an enzyme able to degrade $InsP_6$ into lower inositol pentakisphosphate ($InsP_5$), this first step is crucial to further formation of lower inositol phosphates (InsPs), which are hydrolyzed gradually until reaching their complete dephosphorylation to *myo*-inositol (MI) with a concomitant nutrient releasing **(Figure 1)**. This process was identified to be carried out at the small intestine of broilers after super-dosing phytase supplementation (Sommerfeld et al., 2018).

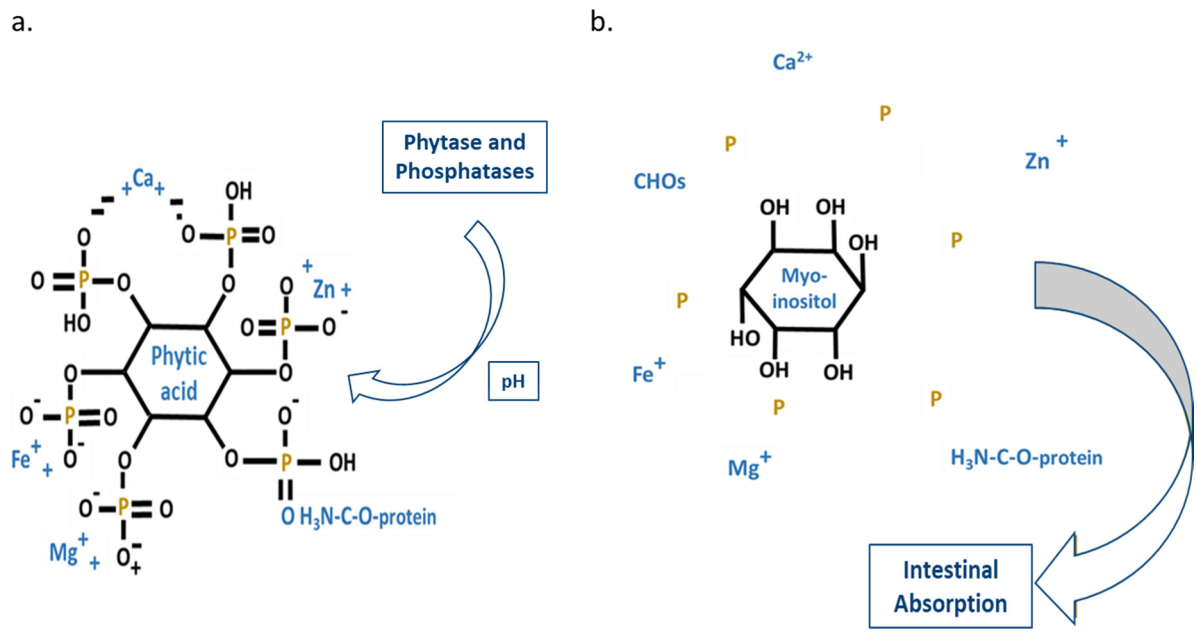

Figure 1. a) Structure of the phytate molecule and its interactions with nutrients residues (Coulibaly et al., 2011). b) Nutrient residues released by the action of phytase and phosphatases.

The increases of MI in the small intestine of poultry was associated with higher blood MI concentrations; however, it is not clear how intestinal MI is absorbed across the intestinal epithelium and if blood MI responds on a phytase dose-dependent manner (Cowieson et al., 2015; Sommerfeld et al., 2018). Furthermore, the effects of dietary sources of MI (either by MI released from phytate but also pure MI supplementation) on general poultry metabolism

are not researched extensively. It has been hypothesized that MI plays several physiological processes in animals (**Figure 2**), as is discussed along the manuscripts included in this thesis (chapter 3).

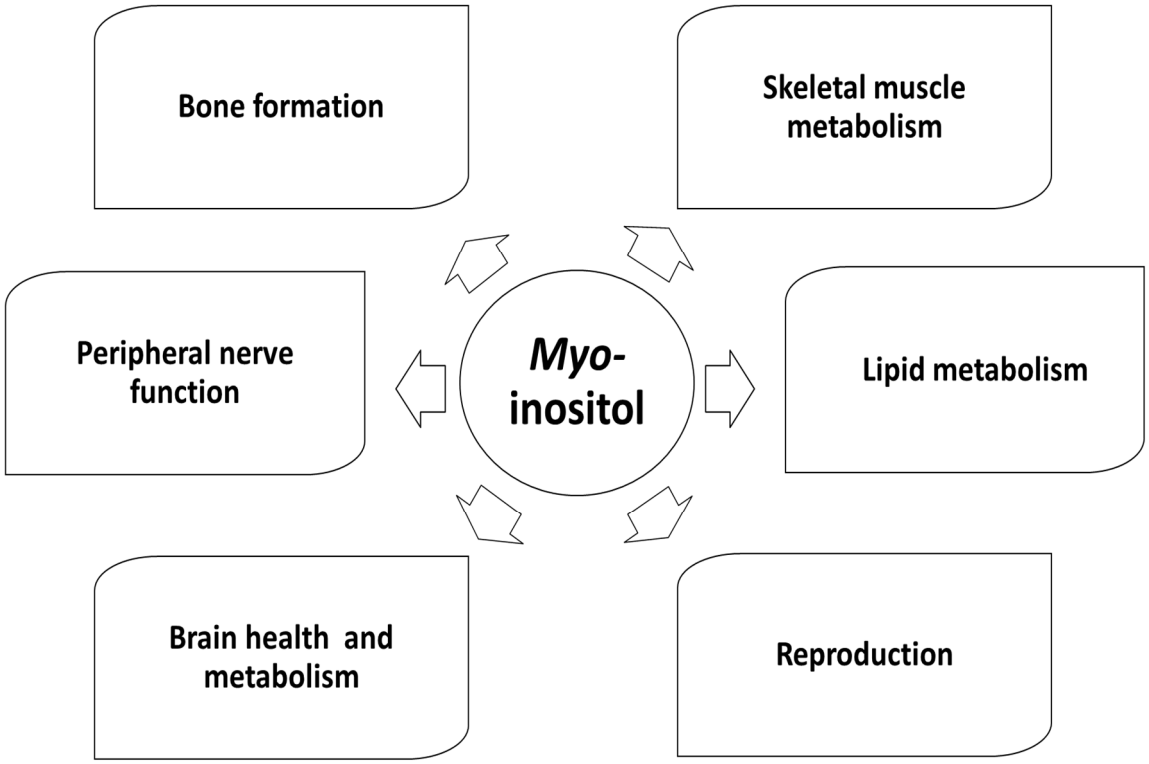

Figure 2. Physiological roles attributed to dietary MI in poultry.

As aforementioned, several questions about MI metabolism in poultry have emerged. Until now, it has remained unknown necessary information such as the basal concentrations of MI in organs of poultry and beyond it, which are the effects of breed and age on poultry, MI metabolism, and, in general, on metabolite profiles.

Broilers are selected for rapid growth, accelerated metabolic rate, and high feed intake so that the number of days required to raise broilers to slaughter weight has decreased significantly during the last years. In modern breeds, breast muscle constituted 14 % of the body mass, and this tended to rise to 18 % by day 35 (Tallentire et al., 2016). In laying hens, age appeared to

be determinant for metabolic changes. They undergo a variety of physiological changes that consist of three molts. The first molt is determinant for immune and digestive systems. The second molt (7^{th} to 11^{th} week of life) has shown to be crucial for body growth through cortical bone, skeletal muscle, and frame development. Around the 17^{th} week (3^{rd} molt), the ovary reproductive tract and the onset of sexual maturity are developed, reaching maturity at around 24 weeks of age. The heaviest body weight coincides with the highest egg production, reaching the laying peak around 30 weeks of age (Phillips, 2014). Moreover, layer strains also appeared to differ physiological and behaviorally (Cockrem, 2007; de Hass et al., 2013; Pusch et al., 2018). The expression of albumen ribitol and N-butyrylglycine has been influenced by strain as well (Goto et al., 2019).

Interactions between MI metabolism and poultry development have not been studied extensively. This thesis intended to give initial insights about MI concentrations and metabolism in broiler and laying hens. Understanding the roles of diet, strain, and productive period on metabolic-related factors in poultry not only would indicate the grade of the importance of MI for poultry development but also would provide further information about the full effects of poultry raising interventions, allowing the optimization of poultry performance, behavior, and health.

2. Overview and aims of the included studies

This thesis had as its primary objective to gain a comprehensive understanding of the potential roles of strain, productive period, and dietary sources of MI on poultry metabolism. To study it, a detailed revision of literature, as well as experimental sets with different dietary interventions, strains and productive periods, were carried out on broiler chickens and laying hens.

Aiming to compile information from literature, a comprehensive review of dietary MI was carried out (manuscript 1, published). This review aimed to provide information about the meaning of MI in animal metabolism as well as to discuss potential implications of dietary MI in poultry health and performance to identify open questions in poultry research.

Considering the use of dietary phytase as a source to increase intestinal MI availability, a second study was performed to compare plasma metabolite profiles of broiler chickens fed diets supplemented with different levels of exogenous phytase vs. pure MI (manuscript 2, published).

Subsequently, since no information about systemic MI is available in the literature, a third study was performed to get insights on the effects caused by dietary phytase on systemic MI. The study aimed to determine critical factors involved in MI metabolism in the gastrointestinal tract, blood, and organs of broiler chickens non-supplemented or supplemented with phytase (manuscript 3, submitted).

Finally, the study detailed in the "draft of the manuscript" intended to give a general description about the influence of the productive period on the MI and MI key enzymes concentrations as well as on the general metabolite profile of Lohmann Classic Brown and Lohmann LSL Classic laying hens.

3. Included manuscripts

3.1. Manuscript 1

Myo-inositol: Its metabolism and potential implications for poultry nutrition- a review

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Published in Poultry Science (2020)

Volume 99, Issue 2, pp. 893-905

The original publication is available at

https://www.sciencedirect.com/science/article/pii/S0032579119441692

DOI: https://doi.org/10.1016/j.psj.2019.10.014

ABSTRACT

Research on physiological roles of *myo*-inositol (MI) have increased during the last years. MI as an abundant molecule in nature has been considered crucial for diverse metabolic and regulatory processes such as lipid signaling, osmolarity, glucose, and insulin metabolism. In mammals, dietary MI has appeared to be a valuable alternative for treatment of several diseases as well as for metabolic performance improvements. In poultry few studies about MI on metabolism have been performed, indicating its supplementation might be associated to changes on metabolism, performance and health. This review intended to provide new information about the meaning of dietary MI in poultry metabolism as well as to discuss potential implications of dietary MI in poultry health and performance.

Keywords: *myo*-inositol, phytate, metabolism, physiology, poultry nutrition

3.2. Manuscript 2

Dietary phytase and myo-inositol supplementation are associated with distinct plasma metabolome profile in broiler chickens

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Published in Animal (2019)

Volume 15, pp. 1-11

The original publication is available at

https://www.cambridge.org/core/journals/animal/article/dietary-phytase-and-myoinositol-supplementation-are-associated-with-distinct-plasma-metabolome-profile-in-broiler-chickens/5F22A2F6288FAD7DC5EC2434092E1A11

DOI: https://doi.org/10.1017/S1751731119002337

ABSTRACT

Phytase is commonly used in broiler nutrition to improve phosphorous bioavailability through the releasing of phosphate groups from the phytic acid molecule. *Myo*-inositol is a product of the complete dephosphorylation of phytic acid. As a bioactive compound, *myo*-inositol presents beneficial modulatory effects on metabolism in humans; nonetheless, it is not well understood if and how complete phytic acid degradation products, especially *myo*-inositol, could modulate metabolism in broiler chickens. This study aimed to investigate the effects of dietary *myo*-inositol and phytase on the plasma metabolome profile of broiler chickens. Broilers were fed on a nutrient-adequate control diet or the same diet supplemented with either 3.5 g myo-inositol or 500, 1500 or 3000 units of phytase per kilogram of feed (grower diet). Broilers were group-housed in floor pens (eight pens per diet) and provided one of the treatment diets for 22 days. Then, blood was collected from one bird per pen (eight replicated measurements per diet). A targeted metabolomics approach was applied to the heparin plasma. Body weight of the birds was not significantly affected by the treatments. Plasma *myo*-inositol concentrations were significantly increased by myo-inositol supplementation and phytase supplementation at 500 and 1500 units/kg. Dietary phytase affected concentrations of metabolites belonging to acyl-carnitines, phosphatidylcholines, sphingomyelins, lysophosphatidylcholine, biogenic amines and amino acids, being particularly interesting the increases observed on kynurenine and creatinine, as well as the lower concentrations of histamine and cis-4-hydroxyproline. On the other side, *myo*-inositol supplementation significantly increased plasma concentrations of dopamine and serotonin, demonstrating a clear difference between of both dietary molecules on chicken metabolite profile. We conclude that *myo*-inositol, either as a directly added supplement or indirectly released from phytate upon phytase supplementation, can affect specific metabolic pathways whereas effects found on phytase supplementation may be related to intermediary phytate degradation products. Results are indicative for innovative hypothesis to be tested in future experiments with regard to relationships between phytase or *myo*-inositol supplements and bird immunity or behavior.

Keywords: dopamine, metabolomics, phosphate, phytic acid, serotonin

3.3. Manuscript 3

Research Note: Jejunum phosphatases and systemic myo-inositol in broiler chickens fed without or with supplemented phytase

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Published in Poultry Science (2020)

Volume 99, Issue 11, pp. 5972-5976

The original publication is available at

https://www.sciencedirect.com/science/article/pii/S0032579120305848

DOI: https://doi.org/10.1016/j.psj.2020.08.045

ABSTRACT

Myo-inositol (MI) has been hypothesized to be crucial in several metabolic and regulatory pathways of poultry. Dietary phytase releases MI from phytate in the poultry digestive tract, increasing systemic MI concentrations. This study aimed to study the activity of total phosphatases in the jejunum as well as the systemic plasma MI concentration in broilers not supplemented or supplemented with phytase, this by using analyses based on modifications from commercial enzyme activity kits. Three hundred sixty male Ross 308 broilers were randomly allocated to 24 pens (15 birds per pen) in 4 dietary groups. The positive control (PC) group was fed with adequate basal diet. The negative control group (NC) was fed with a reduced level of P and Ca. Groups Phy1500 and Phy3000 were fed with the NC diet plus 1,500 or 3,000 FTU of phytase per kilogram of feed, respectively. One bird per pen was chosen for quantification of jejunal phosphatase activity, MI concentration in plasma, the liver, and the kidney; and key MI enzyme expression (liver inositol monophosphatase 1 [IMPase 1] and kidney myo-inositol oxygenase [MIOX]). Endogenous phytase and alkaline phosphatase activity as well as IMPase 1 and MIOX expression were not statistically different among the dietary groups. Supplementation of 1500 FTU resulted in increase of plasma ($P < 0.001$) and kidney ($P < 0.05$) but not liver MI concentrations. It is possible that systemic MI reflected increases on MI released from dietary sources, which did not appear to change expression of enzymes related to endogenous MI synthesis in the liver and catabolism in the kidney. New and larger studies are necessary to reach stronger evidence on the effects of dietary phytase on endogenous phosphatases activity as well as intestinal and systemic MI concentrations in broilers.

Keywords: broiler, kidney, liver, *myo*-inositol, phytase activity

3.4. Draft of the manuscript

Influence of egg-laying on basal myo-inositol concentrations, expression of myo-inositol key enzymes and metabolite profiles in Lohmann Brown-Classic and Lohmann LSL-Classic hens

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ABSTRACT

Laying hen metabolism changes during productive period, mainly because of the morphological and physiological adaptations in response to egg-laying. Previous studies demonstrated the importance of *myo*-inositol (MI) metabolism on poultry; however, in laying hens, MI concentrations and metabolite profiling are unknown so far. This study aimed to observe the influence of the hen strain and the stage of production on basal concentrations of MI, MI key enzymes (namely, inositol monophosphatase 1 (IMPase 1) and *myo*-inositol oxygenase (MIOX)) as well as on the metabolite profiles as likely markers for specific physiological processes. **Methods:** 50 Lohmann Brown-Classic (LB) and 50 Lohmann LSL-Classic (LSL) were housed in groups during the productive period and sampled at five stages of production (10, 16, 24, 30 and 60 weeks of age). All hens were provided the same nutrient-adequate diet specifically for each age. The targeted AbsoluteIDQ p180 Kit was used for metabolite quantification in plasma, whereas a MI enzymatic kit and ELISAs were used to quantify MI concentrations and MI key enzymes (IMPase 1 and MIOX), respectively. **Results:** Liver and muscle MI and IMPase 1 protein concentrations increased significantly at week 60 in LB and LSL hens ($p<0.05$); however, no differences were observed between strains at any time point. Kidney MIOX but not MI expressed different time patterns after week 16 ($p<0.05$) and between LB and LSL hens at weeks 16, 24, 30, and 60 ($p<0.01$). Principal component analysis (PCA) demonstrated marked differences in metabolite profiles at week 24 among individuals in both strains in comparison to week 16, but no apparent difference between strains. False discovery ratio (FDR) adjusted p-value <0.05 identified up- and down-regulated metabolites at the onset of egg-laying in both strains (16 vs. 24 weeks of age). Phosphatidylcholine concentrations increased in LB and LSL hens, whereas 18 and 31 metabolites, consisting of amino acids, biogenic amines, lysophosphatidylcholines and sphingomyelins decreased at week 24 on LB and LSL hens, respectively. Interestingly, sarcosine concentrations decreased significantly from 16 to 24 weeks but only in LSL hens. Regarding strain differences at 24 weeks of age, partial least square discriminant analysis (PLS-DA) and variable of important projection (VIP) showed L-methionine, sarcosine and creatinine, were significantly higher in LB while L-citrulline and carnosine were higher in LSL hens ($p<0.05$). **Conclusion:** Laying hens could increase endogenous MI production in liver and muscle to supply hen systemic levels during egg production. Besides, amino acid, polyamines, and phospholipid metabolism might occur differently by LB and LSL during the onset of egg-laying. Present findings may stimulate further research related to metabolic interventions and to handling of hen's health and nutrition. However, more research is necessary to elucidate metabolic interactions during the onset of laying.

Keywords: egg-laying onset, *myo*-inositol, laying hens, metabolite profiling

INTRODUCTION

The Commission Implementing Regulation (EC) 2017/1185 from the European Union has estimated that by 2019, a total of 413 million laying hens were produced by the EU member states, being Germany the largest producer with 54, 900,379 laying hens (13.3% of total). The importance of egg market for the EU was represented by the fact that exports exceeded by around 300,000 tons the imports during 2019 (EC, 2020), nonetheless concerns have emerged due to the coronavirus crisis. It has been estimated global egg market will decline by 1.2% between 2019 and 2020 (Wood, 2020). In fact, European Poultry egg sectors issued a warning to the European Commission to manage egg-production chain in order to prevent an egg market crisis (Fortune, 2020).

Reasons such as those above have triggered the need for research to optimize egg production and the general layer´s productivity. The productive periods of laying hens have been associated with many metabolic changes. The first 10 weeks of age are crucial for immune and digestive system development as well as for bone and muscle growth (Phillips, 2014). Around the week 15 reproductive tract started maturation with a concomitant increase of fat cells in abdominal fat depots (Leclercq 1984; Phillips, 2014). During this period hens continued growing and developing while egg-laying started (Gilbert et al., 1978). Circa at week 24, hens become sexually mature, and egg production increased until reaching its peak around week 30 (Thiele, 2012). After week 30, hens growth has been terminated and egg production decreased overtime, although egg production was still carried out beyond 60 weeks of age (Huneau-Salaun et al., 2011).

Several physiological indicators are worth to consider in terms of layer development. During the last decade, attention has been placed on the physiological differences between laying strains. Tona et al. (2010) demonstrated that embryos developed faster in Lohmann Brown than Lohmann White, suggesting the incubation conditions should be determined individually according to the strain. In this way, it was demonstrated that Lohmann Brown showed larger and heavier bones (Silversides et al., 2012). Moreover, chronic stress has shown to induce lasting and trans-generational modifications in hen physiology when experienced at different phases of life (Ericsson et al., 2016). In this way, additional evidence indicated Brown Hy-line hens presented lower corticosterone

concentrations and higher food intake and egg production while handling stress in comparison to white layers (Fraisse and Cockrem, 2006; Pusch and Navara, 2018), categorizing brown and white layers as proactive and reactive, respectively (Cockrem, 2007). Plasma serotonin concentration, a metabolite associated to energy homeostasis and bird behavior, also was lower in White Leghorn hens compared to Rhode Island Red hens (Uitdehaag et al., 2011).

It has been established that hens required specific nutrition in accordance with productive period to match their physiology needs (Flock et al., 1999). Nonetheless, research about metabolic pathways behind layer metabolic phenotype is rare. The use of metabolomics has shown to be a powerful instrument to describe pathways associated with the metabolic phenotype of laying hen strains at different stages of production. Targeted metabolomics showed a snapshot-like quantification of a certain number of metabolites, which were associated with several metabolic pathways, indicating a wide range of physiological processes (Roberts et al., 2012). Identification of metabolite expression could generate a new hypothesis allowing the identification of potential biomarkers of metabolic balance as indicators of hen´s health.

The metabolite *myo*-inositol (MI), a cyclic sugar polyalcohol, has demonstrated to be synthesized endogenously from D-glucose and inositol phosphates phosphorylation by the action of inositol monophosphatase 1 (IMPase 1); its catabolism is carried out mainly by the action of non-heme di-iron oxygenase, the myo-inositol oxygenase (MIOX). MI has gained relevance in physiology research during the last decade as a relevant metabolite for health in poultry (Cowieson et al., 2015; Pirgozliev et al., 2019; reviewed by Gonzalez-Uarquin et al., 2019). The KEGG library (internet link is missing indicated the MI pathway is related to several metabolic pathways in *Gallus gallus.* MI acted as a precursor of phosphatidylinositol (PI), which in turn was part of the glycerophospholipid metabolism. It had also been shown as precursor, by mean its interconversions, of the glycolysis/gluconeogenesis processes, the Citric acid cycle and the pentose phosphate pathway (Kanehisa, 2019). Additionally, MI appeared to be potentially involved in physiological processes such as energy and lipid metabolism, bone and muscle formation, reproduction (reviewed by Gonzalez-Uarquin et al., 2020), and behavior, although no studies have been performed on laying hens so far.

Considering that hen metabolic development appeared to be characterized by strain and stage of production, it was hypothesized that metabolite profiles and MI metabolism vary according to hen genetic background and along their productive period. Therefore, this study aimed to observe the influence of hen stage of production on MI metabolism and metabolite expression of brown and white hen strains, as likely markers of physiological changes.

MATERIALS AND METHODS

Animals and diets

Experimental design and management procedures were approved by Regierungspräsidium Tübingen following the German animal welfare regulations (Project no., HOH50/17TE). See the complete description of the project in Sommerfeld et al., 2020.

In this project, 50 Lohmann Brown-Classic (LB) and 50 Lohmann LSL-Classic (LSL) hens with the fixation index (F_{st}) of 0.25 were used in this trial. All hens were from the same hatchery (Lohmann Tierzucht GmbH, Cuxhaven) and raised with the same conditions for eggs and birds. A 2x5-factorial arrangement of treatments was used by using hen strain and 5 stages of production as factors (10, 16, 24, 29, and 60 weeks of age). All hens were housed together on deep litter bedding during the experimental phase. 10 days before each sampling, 10 animals per strain were randomly chosen and kept individually in metabolism cages (1 m^3) in a randomized block design. The temperature was set to 18-22 °C during sampling periods. All nutrients were calculated according to the recommended levels (Lohmann Tierzucht GmbH) for each production stage. Diets were based on corn and soybean meal to ensure minimum plant intrinsic phytase activity. Feed and tap water were given ad libitum. On the day of sampling, the 20 hens were anesthetized by a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and euthanized by CO_2 asphyxiation.

Plasma and tissue sampling

Following euthanasia, hens were immediately decapitated, and trunk blood was collected in EDTA tubes and then centrifuged at 2490×g for 10 min at 6°C (Megafuge 2.0 R, Thermo Fisher Scientific, Waltham, MA). Subsequently, circa 500 µl of plasma was collected in 2 ml tubes, chilled on liquid nitrogen, and stored on dry ice. Hens carcasses were eviscerated. Jejunal mucosa from cleaned

proximal jejunum was stripped using microscopic slides. Breast muscle and medial sections of the liver and kidney were also sampled carefully to preserve the tissues. All organ samples were washed in PBS 1x (0.02 mol/l, pH 7.0-7.2), cut, shock frozen into liquid nitrogen, and collected in pre-chilled 5 ml cryotubes. Immediately, plasma and organ samples were transported on dry ice and stored at -80°C for further analysis.

Dry matter calculation in tissues

Weight from empty, cleaned glass containers was recorded and placed into a desiccator during 4 h. After that, organ samples were placed in the containers, weighed and recorded. The weight of containers was subtracted from the total weight to determine the weight of the tissues before drying (an initial record). Containers were placed on an oven at 103°C for 8 h and then cooled at room temperature into a desiccator for 2 h. Once containers were cooled, they were weighted and the records were subtracted from the initial records to determine the weight of the tissues after drying. Subsequently, records from dry weight were divided by wet weight and multiplied by 100 to obtain the percentage.

Tissue grinding and homogenization

Liver, kidney, and breast muscle samples were ground by using a mortar and a pestle chilled with liquid nitrogen. 420 mg of ground tissue was mixed with 500 µl PBS 1x buffer plus protease inhibitor (Complete mini, Hofmann-La Roche, Mannheim, Germany) in lysing matrix tubes (5076-400, MP Biomedicals, France) containing silica beads. Subsequently, tissues were homogenized by using Fast-prep homogenizer (FastPrep®-24 5G, MP Biomedicals, China) at 6 m/sec for 30 s 3x. Upon homogenization, tissues were centrifuged for 15 minutes at 1500 g (Centrifuge 5424R Eppendorf, Germany). Supernatants were stored at -80°C until further analysis.

Protein quantification

Tissue homogenates were diluted at 1:400 in distilled water for liver, kidney, and breast muscle. Protein concentrations were determined by the method, according to Bradford (Bradford Reagent, 5 ×, SERVA, Heidelberg, Germany) in triplicate.

Myo-inositol determination in liver, kidney and breast muscle

Myo-inositol concentrations were measured by commercially available kit (K-INOSL 02/14, Megazyme International, Ireland,) in tissue homogenates previously diluted at 1:120 for liver and kidney and at 1:40 for muscle in distilled water. The K-INOSL assay was down-scaled to 96 microtiter plates (655101, Greiner bio-one, Germany), and eight samples were run per assay. All samples were assessed in duplicate, and concentrations were calculated according to the standards provided by the kit. Final values were normalized over tissue dry matter (mg MI /g DM).

Inositol monophosphatase 1 and myo-inositol oxygenase expression

Concentrations of kidney MIOX as well as liver and muscle IMPase 1 were measured by using commercial enzyme-linked immunoassay kits (Chicken MIOX ELISA Kit, MBS7215577 and Chicken IMPA1 ELISA Kit, MBS7235623, Mybiosource). Intra- and interassay coefficients of variation were reported as 5.5 and 7.3%, respectively. Protocols were performed according to manufacturer´s guidelines. In brief, aliquots of 100 µl from liver, kidney and muscle homogenates (containing means ± SEMs of 60.6 ± 7.8, 56.7 ± 7.5 and 120.6 ± 4.5 mg/ml protein, respectively) were buffered with balance solution (provided by the kit) and incubated for 1 h at 37 °C together with IMPase and MIOX conjugated with horseradish peroxidase (HRP). The plates were washed 5 times and incubated with the substrate for HRP. After 15 min, a blue colored complex was formed, and a stop solution was added to end the reaction, creating a yellow color. The colorimetric intensity was measured at 450 nm using a microplate reader (Infinite® Infinite M Nano, TECAN, Austria). MIOX and IMPase 1 concentrations were extrapolated from the standard values by a 5 parameter logistic (5-PL) curve-fit (Magellan software, Tecan GmbH 2016, Austria). All values were normalized against tissue homogenate protein concentration (pg enzyme /mg total protein).

Targeted metabolomics approach

Plasma metabolite profile analysis was performed by using the AbsoluteIDQ p180 Kit (Biocrates Life Science AG, Innsbruck, Austria) by of Biocrates Life Science AG. Briefly, AbsoluteIDQ p180 Kit was created for identifying 188 metabolites belonging to amino acids, biogenic amines, acylcarnitines, phosphatidylcholines, lysophosphatidylcholines, sphingolipids, and hexoses. The assay was based on phenylisothiocyanate (PITC) derivatization in the presence of internal standards followed by FIA-MS/MS (acylcarnitines, (lyso-) phosphatidylcholines, sphingomyelins, hexoses)

and LC-MS/MS (amino acids, biogenic amines) using a SCIEX 4000 QTRAP® (SCIEX, Darmstadt, Germany) or a Xevo TQ-S Micro (Waters, Vienna, Austria) instrument with electrospray ionisation (ESI). The experimental metabolomics measurement technique is described in detail by patent US 2007/0004044 (Ramsay et al., 2007). All pre-analytical and analytical procedures were performed, documented, and reviewed according to the ISO 9001:2008 certified in-house quality management rules and guidelines of Biocrates Life Sciences AG.

Statistical analyses

Parametric statistics

Variance components estimation from MI and key MI enzymes from all the production stages were performed by using restricted maximum likelihood (REML) using Kenward and Roger as the method to determine degrees of freedom ($_{df}$). Least square (LS) means comparison between hen lines and productive periods were analyzed to calculate means and SEM by using mixed model procedures (SAS version 9.4, SAS Institute Inc., Cary, N C). For this experiment the following model was used: $Y_{ijklm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\gamma\beta)_{kj} + \delta_l + \phi_m + \varepsilon_{ijklm}$, where Y_{ijklm} = response variable, μ = overall mean, α_i = effect of strain (fixed), β_j = effect of period (fixed), the interaction between strain and period (fixed), γ_k = block (random), the interaction between block and period (random), δ_l = cage (random), ϕ_m = father/rooster (random), and ε_{ijklm} = residual error. Data was tested and confirmed to be normally distributed by the use of the D'Agostino & Pearson omnibus normality test. Values for the table were given as LSmeans ± SEM. Different letters indicated significant differences over productive periods ($p<0.05$), whereas * or ** indicates indicated difference ($p<0.05$) and high difference ($p<0.01$), respectively, between LB and LSL hens at a specific production period.

Metabolomics data analyses and visualization

Plasma metabolite concentrations from each hen were provided in µmol/L by Biocrates as original data. Metabolomics data were analyzed and visualized by using MetaboAnalyst 4.0 (Chong et al., 2019). Briefly, metabolite concentrations from all the hens were normalized by generalized logarithm transformation, mean-centered, and divided by the square root of the standard deviation

of each variable (Pareto scaling). Phosphatidylcholines (PCs), lysophosphatidylcholines (LysoPCs) and sphingomyelins (SMs) were summed and analyzed as the sum of PCs, sum of lysoPCs, and sum of SMs, respectively. Principal component analysis (PCA) as an unsupervised method was used to display differences in metabolic profiles between stage of productions and between hen strains. Once a change in metabolite profiles was identified and selected, False Discovery Rates (FDR) adjusted p-values from t-student´s test were performed to identify which metabolites caused the variation in metabolite profile within each strain. Comparisons and visualization between LB and LSL at the were performed by using unpaired t-student´s test (GraphPad Prism version 6.07, La Jolla, CA, USA). Values for tables and figures were given as student´s t-test means ± SEM. Different letters indicated significant differences over productive periods (FDR-adjusted p or p <0.05). Finally, a computational network was performed by using the metabolomics platform Cytoscape 3.7.2 plug-in Metscape 3 (https://cytoscape.org/) to depict an overview of differential and high impact metabolites at 24 weeks of age as well as their potential linkages.

RESULTS AND DISCUSSION

Liver and muscle MI and IMPase 1 concentrations increased at week 60 in LB and LSL laying hens

MI concentrations in the liver of LB and LSL hens increased at week 60. The same pattern was observed in liver IMPase 1 concentrations of both strains, which increased at week 60 in LB and from week 24 in LSL hens (**Table 1**). Higher concentrations of IMPase 1 and MI during egg-laying might reflect enhanced production of phosphatidylinositol (PI) for yolk development (Lordan et al., 2017). Besides that, supplemented MI to a diet high in sucrose has proven to be able to lower hepatic triglyceride and total lipid concentrations in rats, this presumably, by modifying the the abundance of Lactobacillus spp. In the gut, which has demonstrated to protect against fattly liver disease (Okazaki et al., 2018). This indicated one additional role of higher liver MI and IMPase 1 concentrations after the peak of egg-laying might be the up-regulation of MI synthesis in response to the elevation on total lipid production during ovogenesis, which is known as a metabolically costly period (Vézina et al., 2003). Reduction on liver lipids production could be explained by the fact MI decreased hepatic lipogenesis (Katayama, 1997; Okazaki et al., 2018).

MI concentrations in muscle of LB and LSL hens decreased significantly at week 30, showing a significant recovery at week 60. IMPase 1 concentrations in muscle of both strains increased significantly at week 60 (**Table 1**). Lower concentrations of muscle MI and IMPase 1 coincided with the stage hens reached physical maturity and egg-laying peak, indicating a potential negative nutritional balance. Increasings observer at week 60 indicated at this age MI could be used mostly for egg development, causing adaptive increases in endogenous MI synthesis to maintain muscle MI concentrations. The effects of dietary sources of MI on skeletal muscle have been reported previously (Schmeisser et al., 2017; Gonzalez-Uarquin et al., 2020).

Kidney MI concentrations did not vary, but MIOX decreased after laying peak in LB and LSL laying hens

Kidney MI concentrations did not vary along time within strains and between strains (**Table 1**). MI has been characterized as a crucial cellular osmolyte in mammal kidney cells (Sizeland et al., 1993); under basal conditions concentrations of MI might be strongly regulated to maintain kidney osmoregulation.

Kidney MIOX concentrations decreased with the progression of egg production, reaching its lowest point at week 60 (**Table 1**). MIOX concentrations were significantly higher in LSL hens at week 10; however, from week 16 until week 60, LB hens presented a higher MIOX expression. Explanations behind MIOX expression could depend on several metabolic factors. In mammals, MI appeared to be catabolized through MIOX to D-glucuronic acid (Arner et al., 2001). If reduced amount of enzyme reflected diminished activity, less MI degradation occurred in LSL hens. Furthermore, it is known, Differences in MIOX activity could depend on additional metabolic mechanisms (Nayak et al., 2011: Tominaga et al., 2016).

The Principal Component Analysis showed different metabolite profiles during the onset of egg-laying in LB and LSL hens

An overview of the changes of metabolite profiles from LB and LSL laying hens showed that hens presented different metabolite profiling along time (**Figure 1**). A clear differentiation was observed in both strains between 16 and 24 weeks of age, indicating a metabolism-related change that

corresponded to the onset of egg-laying. According to this analysis, 56.8% of the accumulated variance is explained by the first two principal components.

These results indicated that the first weeks of hen development were driven by growth and development as aforementioned. This could explain the differentiation seen between week 10 and the other stages of production (**Figure 1**), however differences between week 10 and the other stages of production are not discussed in this manuscript. Moreover, week 16 could be interpreted as an adaptation state to egg production so that a particular metabolite profile was also observed. Later stages of production showed no clear differentiation on metabolite profiling, indicating completed adaptation to processes associated with egg-laying.

In this experiment, a clear division was depicted between sexually immature (weeks 10 and 16) mature laying hens (weeks 24, 30 and 60), indicating the onset of egg-laying (the transition between weeks 16 and 24) might be a critical metabolic point for LB and LSL hens.

Statistical analyses indicated significant differences in 15 metabolites comparing LB and LSL hens between weeks 16 and 24.

Comparing metabolite concentrations of LB and LSL strains during the onset of egg-laying (16 vs. 24 weeks) revealed strain similarities and differences in metabolite profiles. A total of 17 metabolites changed in both strains over time; 16 of them decreased significantly (8 amino acids, 6 biogenic amines, the sum of lysoPCs and the sum of SMs), being the only exception the sum of PCs which increased statistically at the week 24 (**Tables 2 and 3**).

Furthermore, a total of 15 metabolites were differently expressed over time exclusively in one of the two strains. One metabolite belonged to LB hens: L-lysine (**Table 2**) whereas 14 belonged to LSL hens: 7 amino acids (glycine, L-aspartate, L-serine, L-valine, L-methionine, L-leucine and L-isoleucine), 6 biogenic amines (ADMA, Met-SO, kynurenine, taurine, putrescine and creatinine) and the sum of hexoses (**Table 3**). All the metabolites, regardless of strain, showed lower concentrations at week 24, except for the polyamine putrescine, which appeared to be up-regulated in LSL hens at week 16. Differential metabolite concentrations between strains during the onset of egg-laying might depend on factors such as different requirements and utilization of nutrients and own strain physiology processes. Indeed, a recent study indicated the metabolite profiles of egg

yolk and albumen (mainly sugar alcohol metabolites) from two strains of hens were influenced by feed or strain (Goto et al., 2019).

Providing a detailed discussion about metabolite profile behavior is difficult since metabolomics results are based on mere descriptive recognition of phenotypic patterns. This means metabolomics is a useful tool to provide the basis for new hypotheses generation rather than offering mechanistic conclusions of multifactorial events.

Studies performed by colleagues of the same project indicated an increase of plasma corticosterone from week 24 in LB and LSL (J. Schmucker, 2020, University of Hohenheim, Germany, personal communication,), indicating the onset of egg-laying would be a stage of metabolically stress regardless of the strain. In humans with hypercortisolism (chronic high levels of circulating cortisol) decreases on plasma aromatic and branched chain amino acid concentrations have been reported (Di Dalmazi et al., 2017) whereas supplementation of L-lysine and L-arginine has shown to reduce anxiety and to normalize stress hormones (Smriga et al., 2007). Moreover, high corticosterone concentrations also have shown to decrease endogenous neuronal anti-oxidants (Spiers et al., 2015) so that it could trigger the decreases observed on basal plasma trans-4-OH-Pro, and methionine sulfoxide concentrations which have been associated to protection against oxidative stress in horses (Kenez et al., 2018).

Decreases on some metabolites could also be consequences from precursor availability. For instance, LSL hens showed decreases on L-threonine, L-serine, L-proline, trans-4-hydroxy-L-proline, and sarcosine concentrations at week 24. This could explain the down-regulation observed in the amino acid glycine, which is an amino acid considered semi-essential for chickens (Wu et al., 2011). Worthy, L-proline, trans-4-hydroxy-L-proline, and glycine together comprise 57% of total amino acids in collagen (Li and Wu, 2017).

Interestingly, although LB and LSL hens presented lower concentrations of L-tryptophan, only LSL hens presented decreases on its product kynurenine, suggesting probably lower action of cytokines and, therefore, lower inflammatory processes (Birkl et al., 2019). It could be related to the up-regulation seen in putrescine at the 16 weeks, whose supplementation has shown to improve intestinal development and immune function in chickens (Hashemi et al., 2014).

Finally, variations in metabolite concentrations also could be attributed to egg formation. Phospholipids have been reported to be essential components of egg yolk (Lordan et al., 2017). Elevations in the sum of plasma PCs at week 24 could be explained by the fact that hen PCs biosynthesis is the primary source of egg yolk phospholipids. PCs are essential components of egg yolk, comprising 45-80% of total phospholipids (Lordan et al., 2017; Antova et al., 2019). Moreover, increases on PCs might be carried out, presumably, at the expense of LysoPCs and SMs concentrations in egg yolk (Antova et al., 2019), which could explain the significant decreases on the sum of lysoPCs and sum of SMs observed at the week 24.

Partial least square discriminant analysis showed differences in metabolite profiles between LB and LSL hens at the week 24

A direct comparison between LB and LSL laying hens at week 24 indicated a difference in five metabolites between strains. The rate of the supervision model (R^2Y) was 0.95, which indicated that PLS-DA was a reliable model for showing differences between LS and LSL hens. The value Q^2 was 0.73, indicating a high predictive ability of the model.

Together, these results showed that LB and LSL hens had significant differences in their metabolite profiles (**Figure 2a**). The variable importance projection value (VIP) of the PLS-DA model showed L-citrulline, sarcosine, L-methionine, carnosine, and creatinine were the metabolites responsible for group separation (**Figure 2b**).

A deeper comparison between LB and LSL metabolite expression performed by Volcano Plot analysis corroborated that L-methionine (t_{18}=2.59, p=0.01), sarcosine (t_{18} = 2.38, P = 0.02) and creatinine (t_{18} = 2.13, P =0.04) increased significantly in LB hens (**Figure 2c**) whereas L-citrulline (t_{18} = 3.97, P < 0.01) and carnosine (t_{18} = 2.99, P < 0.01) increased significantly in LSL hens (**Figure 2d**).

In humans, plasma sarcosine and creatinine are both sub-products from homocysteine and L-methionine inter-conversions so that it is plausible that more rate on endogenous L-methionine synthesis increased sarcosine and creatinine (Pajares, 2016). Moreover, in chickens, plasma L-citrulline concentrations appeared to participate in thermoregulation by lowering the temperature

364 to cope with heat stress (Chowdhury et al., 2017), while in rats, plasma carnosine has been reported
365 as a protecting factor against metabolic stress (Stegen et al., 2015).
366 The metabolic map depicting possible pathway interrelationships from metabolite concentrations is
367 shown in **Figure 3**. The amino acids, polyamines, urea, collagen, and glycerophospholipid
368 pathways were altered in by onset of egg-laying in LB and LSL hens; however, each strain
369 presented different expression on several metabolites, indicating own strain was associated with
370 different metabolite profile. Moreover, alteration on MIOX expression would be associated not only
371 to MI degradation but also to glucose metabolism, indicating MI and PI pathways might be affected
372 by egg production and its concomitant physiological processes.
373 Metabolomics tools open the way to a characterization of laying hen metabolite profile and its
374 implications for poultry health and welfare. Results showed here suggested LB and LSL hens
375 present differences on general metabolite profile, at least during the onset of egg-laying, what would
376 indicate both hen strains were associated with different adaptation processes during the onset of
377 egg-laying. Additional mechanistic research is necessary to confirm the findings.
378

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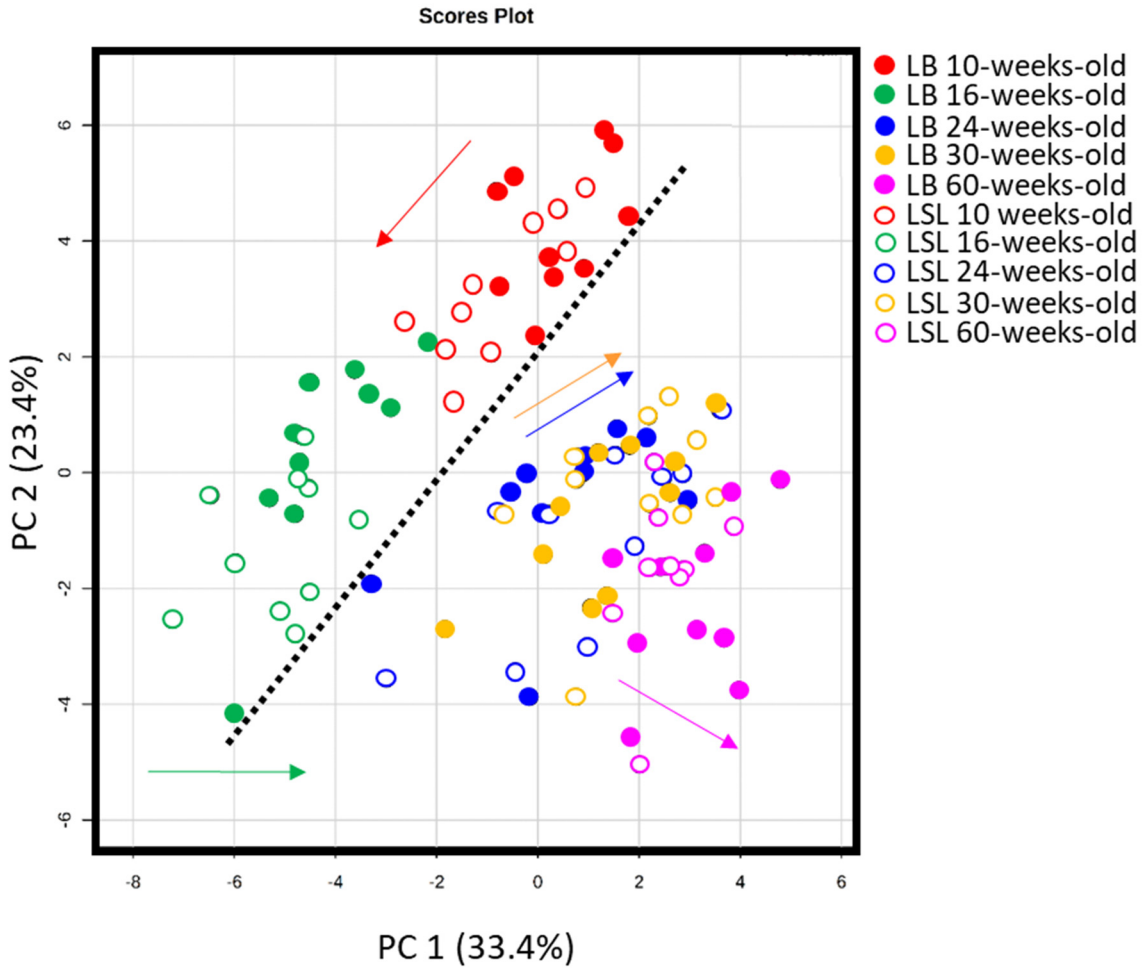

520

521 **Figure 1.** Principal component analysis (PCA) of plasma metabolite profiles data at five ages of LB and LSL hens. In
522 this longitudinal study through layer hen production 7 groups of metabolites classes were measured (amino acids, biogenic
523 amines, acylcarnitines, phosphatidylcholines, lysophosphatidylcholines and sphingomyelins). Each point indicates one
524 hen and each color indicates one specific stage of production (see conventions). Filled points indicate LB hens whereas
525 unfilled points indicate LSL hens. Dashed line indicated a clear separation between hens before and after laying-egg
526 period. Color arrows showed the metabolic pattern according to each stage of production. As the main finding, PCA
527 scores indicate a clear differentiation of metabolite profiles during the onset of egg laying (the transition from 10-16 to
528 24 weeks of age) for both strains.

529

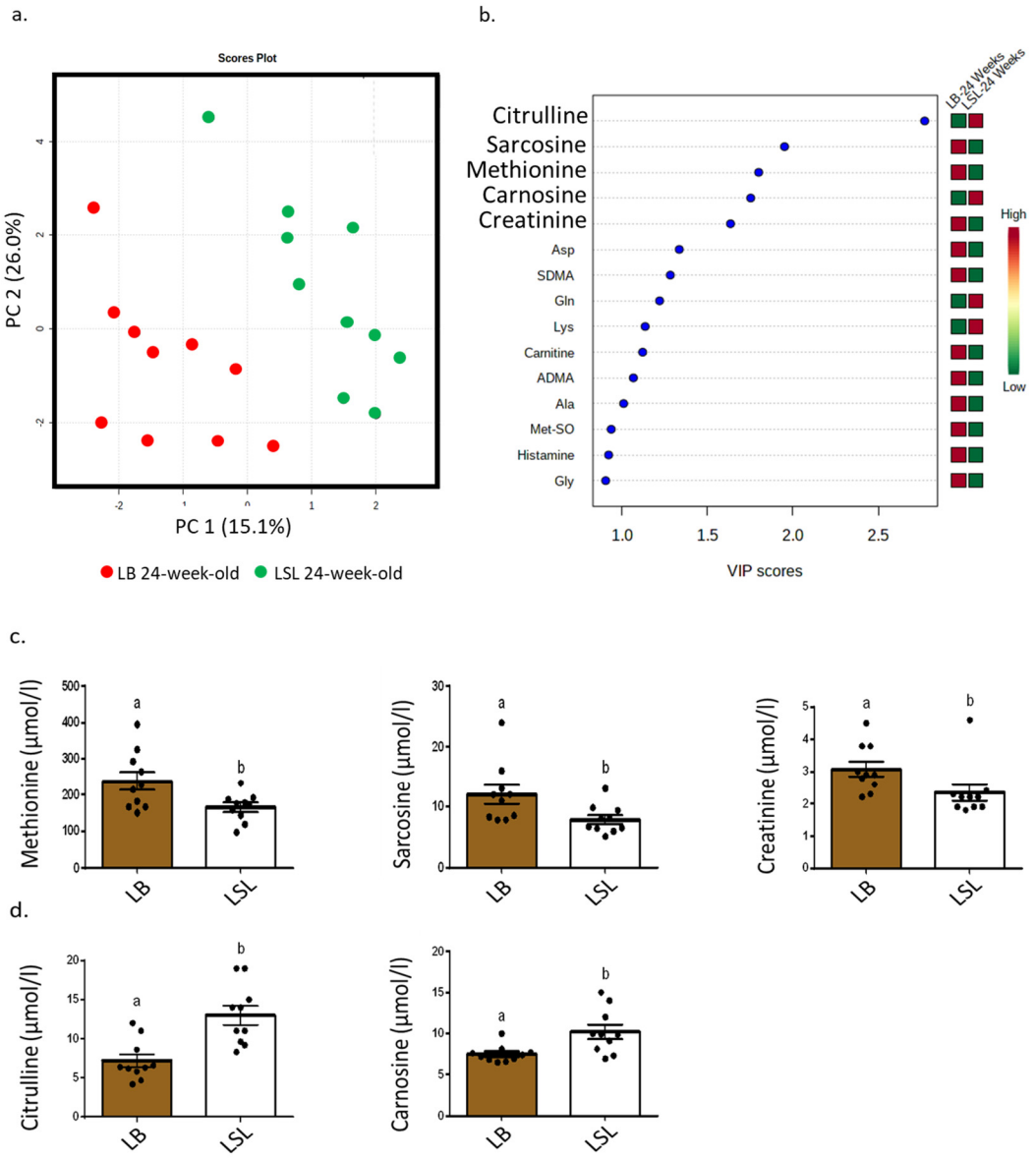

Figure 2. (a) Partial least square discriminant analysis (PLS-DA) of plasma metabolite profiling data at the week 24 of LB and LSL hens. In this longitudinal study through layer hen production 7 groups of metabolites classes were measured (amino acids, biogenic amines, acylcarnitines, phosphatidylcholines, lysophosphatidylcholines and sphingomyelins). Each point indicates one hen and each color indicates one strain at one specific stage of production. Dashed line was performed to indicate clear differentiation between groups. (b) Differentially expressed metabolites between LB and LSL hens at 24 weeks of age identified by PLS-DA and variable importance projection value (VIP). The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each strain. Dark green showed low relative concentration while dark red showed high relative concentration. The x-axis represents the VIP value, and the blue dots represent the different metabolites. (c) Concentrations of metabolites significantly up-regulated on LB hens at week 24 (d) Concentrations of metabolites significantly up-regulated on LB hens at week 24. Dashed line indicated a clear separation between LB and LSL hens at week 24. Bars show mean ± SEM (n=10 hens per treatment). Student´s t-test was used were used as the statistical test to analyze the data. Different lower case letter indicates significant ($P < 0.05$) differences. Only metabolites presenting statistical difference were selected for the analysis.

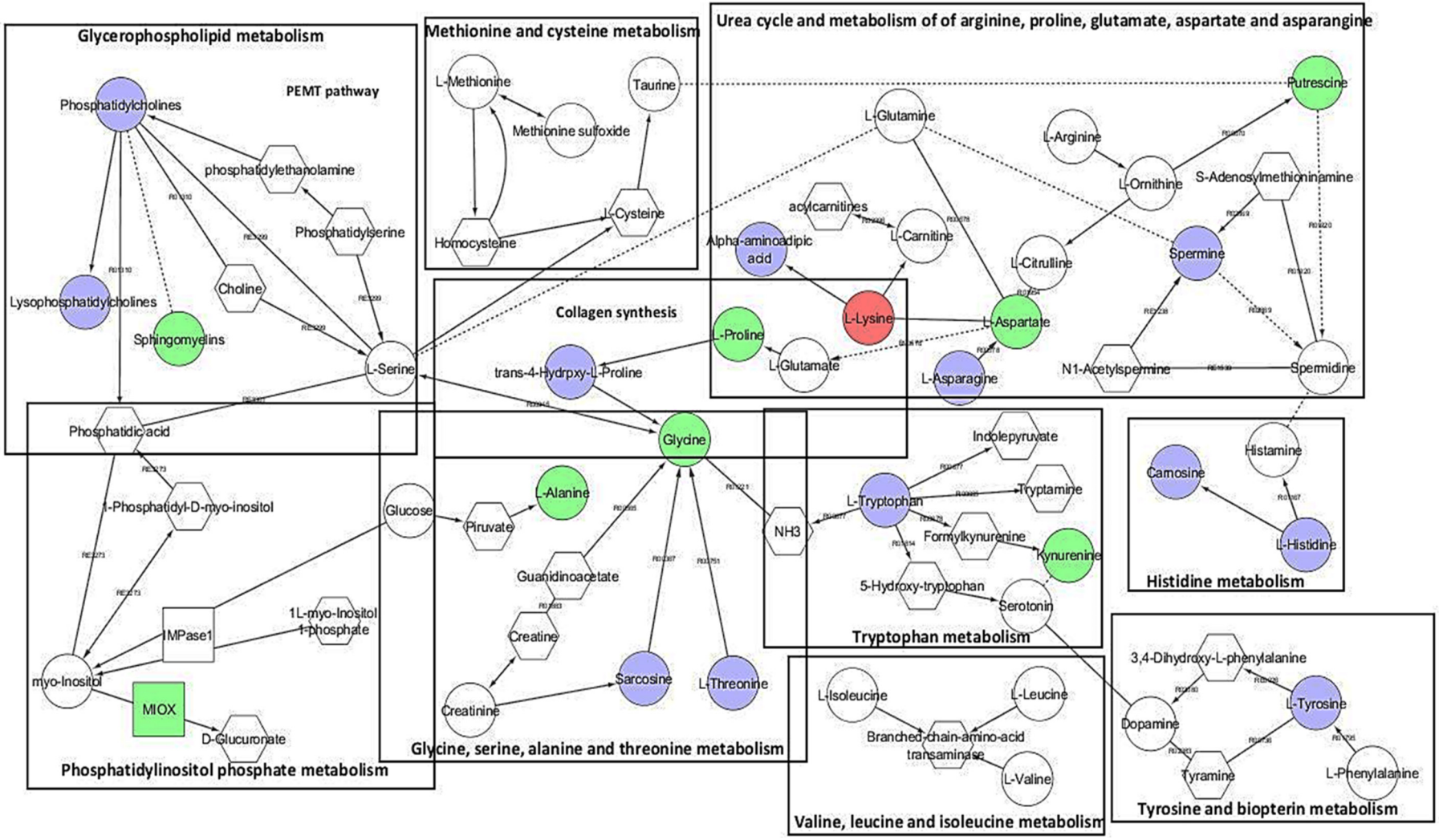

Figure 3. Metabolic map showing the measured metabolites (in circles) on LB and LSL hens during the onset of egg laying. The map depicts the metabolites that changed significantly according to the volcano plot analyses in LB (red), LSL (green) and both (purple) strains at the 24 in comparison to the 16 week of age. Metabolite profiling indicated onset on egg laying affected metabolic routes such as amino acid, polyamine glycerophospholipid urea and collagen pathways. Interestingly LSL hens showed significant variation in MIOX expression and 7 metabolites more than the LB hens, which only presented significant (lower) variations for the amino acid L-Lysine. Metabolites in squares changed between LB and LSL at week 24. Networks were generated using Cytoscape 3.7.2 with the MetScape 3 plug-in. Metabolites depicted in hexagons where not measured in this study.

Table 1. MI[1] and MI key enzymes concentration in plasma during five productive periods of LB and LSL hens

		10 Weeks	16 Weeks	24 Weeks	30 Weeks	60 Weeks	P-value
MI (mg/g DM)							
Liver	LB	6.9 ±0.68 ^{ab}	6.6 ±0.87 ^{a}	5.47 ±0.67 ^{a}	6.2 ±1.02 ^{a}	8.6 ±0.47 ^{b}	Strain: 0.09 Period: <0.01 Inter.: 0.51
	LSL	7.2 ±0.56 ^{xz}	5.3 ±0.72 ^{xy}	3.9 ±0.64 ^{y}	5.3 ±0.65 ^{xy}	8.7 ±0.60 ^{z}	
Muscle							
	LB	1.1 ±0.12 ^{a}	1.5 ±0.26 ^{a}	1.3 ±0.22 ^{a}	0.5 ±0.12 ^{b}	1.9 ±0.15 ^{c}	Strain: 0.30 Period: <0.01 Inter.: 0.44
	LSL	0.7 ±0.14 ^{x}	1.5 ±0.16 ^{y}	1.2 ±0.22 ^{y}	0.5 ±0.09 ^{x}	1.6 ±0.17 ^{y}	
Kidney							
	LB	8.5 ±0.61	7.5 ±0.54	7.1 ±0.46	8.3 ±0.88	9.5 ±0.97	Strain: 0.73 Period: 0.08 Inter.: 0.62
	LSL	8.2 ±0.62	6.9 ±0.67	8.2 ±0.51	7.8 ±0.69	9.1 ±0.79	
IMPase 1[2] (pg/mg protein)							
Liver							
	LB	0.08 ±0.02 ^{a}	0.20 ±0.03 ^{ab}	0.17 ±0.07 ^{ab}	0.10 ±0.02 ^{a}	0.32 ±0.09 ^{b}	Strain: 0.51 Period: <0.01 Inter.: 0.57
	LSL	0.05 ±0.01 ^{x}	0.15 ±0.03 ^{xy}	0.28 ±0.08 ^{yz}	0.18 ±0.07 ^{xyz}	0.35 ±0.08 ^{z}	
Muscle							
	LB	0.1 ±0.01 ^{a}	0.07 ±0.008 ^{a}	0.08 ±0.006 ^{a}	0.06 ±0.01 ^{a}	1.07 ±0.07 ^{b}	Strain: 0.47 Period: <0.01 Inter.: 0.34
	LSL	0.12 ±0.01 ^{y}	0.08 ±0.007 ^{y}	0.11 ±0.02 ^{y}	0.04 ±0.01 ^{y}	0.96 ±0.08 ^{z}	
MIOX[3] (pg/mg protein)							
Kidney							
	LB	1.4 ±0.04 ^{ac}	1.35 ±0.05 ^{ac}	1.47 ±0.08 ^{a}	1.28 ±0.09 ^{c}	1.0 ±0.07 ^{b}	Strain: <0.01 Period: <0.01 Inter.: <0.01
	LSL	1.47 ±0.06 ^{x}	1.56 ±0.07 ^{x}	0.92 ±0.09 ^{y}	1.03 ±0.05 ^{y}	0.63 ±0.04 ^{z}	

Basal concentrations of myo-inositol and MI key enzymes in liver, muscle and kidney of LB and LSL hens at 10, 16, 24, 30 and 60 weeks of age. Values for each variable are given as LSmeans ± SEM. Different letter indicates statistical differences within each strain along productive period and ** indicated highly significant differences ($p<0.01$) between strains at each specific week, respectively. Value of n was 10 hens/line/productive period, with the exception of LSL hens at 60 weeks old, where n was 9 hens. Interactions between strain and period are indicated by "Inter." $P < 0.05$. [1] MI: *myo*-inositol. [2] IMPase 1: inositol monophosphatase 1, [4] MIOX: *myo*-inositol oxygenase.

557
558 **Table 2.** LB hens - Differential metabolite concentrations between weeks 16 and 24 in plasma
559 according to FDR adjusted P-value from t- student´s test.

Metabolite (µmol/l)	**Week 16**	**Week 24**	**FDR adjusted P-value**	**t-value** $_{df}$
Amino acids				
Tyrosine	337±9.9	172±7.3	<0.001	13.09 $_{18}$
Threonine	557±21.2	313±20.1	<0.001	7.70 $_{18}$
Tryptophan	144±6.4	91.8± 4.74	<0.001	6.77 $_{18}$
Histidine	262±10.2	162±16	<0.001	5.41 $_{18}$
Asparagine	319±14.1	210±15	<0.001	5.04 $_{18}$
Arginine	503±10.7	389±22.1	<0.001	4.56 $_{18}$
Glutamine	1297±35.7	883±95.8	<0.01	3.98 $_{18}$
Alanine	1156±46.6	813±63.7	<0.01	3.96 $_{18}$
Lysine	474±31.7	310±38.5	<0.01	3.96 $_{18}$
Proline	763± 29.2	592±35.6	<0.01	3.58 $_{18}$
Biogenic Amines				
Carnosine	20.3±1.4	7.5± 0.32	<0.001	12.76_{18}
trans-4-OH-Pro	134.4±7.9	30.8±3.6	<0.001	11.44_{18}
Alpha-AAA	2.1±0.18	1.04±0.1	<0.001	5.35_{18}
Sarcosine	21.6±0.8	12±1.57	<0.001	5.32_{18}
Spermine	0.58±0.06	0.30±0.01	<0.001	4.99_{18}

Phospholipids

Sum of PCs	1862±95.8	4728±455	<0.001	-8.56_{18}
Sum of lysoPCs	54.4±3.38	19.3±1.11	<0.001	10.91_{18}
Sum of SMs	237.4±11.3	160.5±14.2	<0.01	4.15_{18}

Comparison between LB hens at 16 (n=10) and 24 (n=10) weeks of age. Values are showed as means ± SEM. FDR-adjusted $p<0.05$ as significance level was used. t-value indicates the ratio between the difference between and within both groups. Degrees of freedom (df) are shown in the figure as subscript of t-values. Abbreviations: trans-4-OH-Pro (trans-4-Hydroxy-L-proline), Alpha-AAA (Alpha-Aminoadipic acid) Sum of PCs (Sum of phosphatidylcholines) Sum of lysoPCs (Sum of lysophosphatidylcholines) Sum of SMs (Sum of sphingomyelins).

598
599 **Table 3.** LSL hens - Differential metabolite concentrations between weeks 16 and 24 in plasma
600 according to FDR adjusted P-value from t- student´s test

Metabolite (µmol/l)	**Week 16**	**Week 24**	**FDR adjusted P-value**	**T-value** $_{df}$
Amino acids				
Threonine	548±21.6	275±24.4	<0.001	7.71 $_{18}$
Tryptophan	168±6.3	89.3±7.5	<0.001	7.58 $_{18}$
Asparagine	347±18.4	200±12	<0.001	7.11 $_{18}$
Histidine	260±11.4	153±10.5	<0.001	6.58 $_{18}$
Glycine	1050±32.3	689±44.6	<0.001	6.54 $_{18}$
Alanine	1090±47.2	680±44.7	<0.001	6.29 $_{18}$
Tyrosine	289±13.4	183±13.1	<0.001	5.73 $_{18}$
Proline	898±50.4	536±48.4	<0.001	5.34 $_{18}$
Arginine	546±18.1	408±20.1	<0.001	5.16 $_{18}$
Aspartate	72±5.7	45± 3.5	<0.001	4.44 $_{18}$
Serine	1071±29.1	868±42.4	<0.01	3.96 $_{18}$
Valine	516±21.7	348±37.4	<0.01	3.96 $_{18}$
Methionine	230±12	165±12.3	<0.01	3.59 $_{18}$
Leucine	664±29.3	516±37.2	<0.01	3.40 $_{18}$
Glutamine	1294±42.7	1057±65.8	<0.01	3.26 $_{18}$
Isoleucine	291±11.7	228±20.0	<0.05	3.03 $_{18}$

Biogenic amines				
trans-4-OH-Pro	134±6.4	25±1.79	<0.001	20.48 $_{18}$
Sarcosine	24± 1.3	7.9± 0.74	<0.001	10.92 $_{18}$
Carnosine	26.7±1.2	10.2± 0.85	<0.001	10.53 $_{18}$
ADMA	1.14±0.05	0.75±0.03	<0.001	6.34 $_{18}$
Alpha-AAA	2.4± 0.2	1.2± 0.1	<0.001	5.33 $_{18}$
Spermine	0.62± 0.05	0.36± 0.04	<0.001	4.54 $_{18}$
Met-SO	19.9± 1	13.47±1.32	<0.01	4.07 $_{18}$
Kynurenine	0.44±0.07	0.18±0.02	<0.01	3.73 $_{18}$
Taurine	774±116	380±49	<0.01	3.17 $_{18}$
Putrescine	0.96±0.1	1.69±0.2	<0.01	-3.15 $_{18}$
Creatinine	2.95±0.17	2.34±0.2	<0.05	2.41 $_{18}$
Phospholipids				
Sum of PCs	1951±65.7	4726±393	<0.001	-9.44 $_{18}$
Sum of lysoPCs	60.5±4	19.9±1	<0.001	13.72 $_{18}$
Sum of SMs	246±12.2	161±12	<0.001	4.88 $_{18}$
Hexoses				
Sum of hexoses	21253±998	17995±857	<0.05	2.53 $_{18}$

Comparison between LSL hens at 16 (n=10) and 24 (n=10) weeks of age. Values are showed as means ± SEM. FDR-adjusted $P < 0.05$ as significance level was used. t-value indicates the ratio between the difference between and within both groups. Degrees of freedom (df) are shown in the figure as subscript of t-values. Abbreviations: trans-4-OH-Pro (trans-4-Hydroxy-L-proline), ADMA (Asymmetric dimethylarginine), Alpha-AAA (Alpha-Aminoadipic acid), Met-SO (methionine sulfoxide), Sum of PCs (Sum of phosphatidylcholines) Sum of lysoPCs (Sum of lysophosphatidylcholines) Sum of SMs (Sum of sphingomyelins).

3.5. Contribution of the author to each manuscript and draft

Manuscript 1 status: *published*

"Myo-inositol: its metabolism and potential implications for poultry nutrition - a review"

- Compilation of literature, paper drafting and final corrections.

Manuscript 2 status: *published*

"Dietary phytase and myo-inositol supplementation are associated with distinct plasma metabolome profile in broiler chickens"

- Data analysis, paper drafting and final corrections.

Manuscript 3 status: *published*

"Research Note: Jejunum phosphatases and systemic *myo*-inositol in broiler chickens fed without or with supplemented phytase"

- Experimental design, Lab and data analyses, manuscript drafting.

Draft of the manuscript status: *Drafted*

"Influence of egg-laying on basal myo-inositol concentrations, expression of myo-inositol key enzymes and metabolite profiles in Lohmann Brown-Classic and Lohmann LSL-Classic hens"

- Experimental design, Lab and data analyses, manuscript drafting.

4. General discussion

This study aimed to gain a comprehensive understanding of the potential relationships between *myo*-inositol and poultry metabolism. Up to now, there is little scientific literature about basal MI metabolism as well as the effects of MI supplementation on the metabolism of poultry. In this section, methodological considerations and the following additional topics emerged from main results from the manuscripts, and the draft of the manuscript (chapter 3) are presented: Did basal MI and MI key enzyme concentrations differ between broilers and laying hens? Could dietary MI have a role in dopamine-serotonin synthesis up-regulation? Which might be the hypothetical consequences for feather pecking behavior? Discussion about the first point was limited to compare MI and MI key enzyme concentrations between broilers and laying hens. The third point was shortly discussed based on the main result from manuscript 2 (chapter 3).

4.1. Sampling and methodological limitations

Experimental variability was inherent in each measurement, although every effort was made in order to avoid it in these experiments. The main sources of variation corresponded to animal sampling, analytical measurements, and data handling. Given that it is reasonable to mention them to improve the analyses of the present findings.

Animal sampling coexisted with experimental variability. The main reason is that is not possible to slaughter all animals at the same time, then investigators could not assure metabolic variations between animals were associated only to biological reasons such as age. This was observed in the experiment with hens, which were slaughtered at different production periods and at different seasons. It could be argued animals were housed under the same conditions over time, however, they were transported outdoor to the slaughterhouse minutes before euthanasia, which could affect biological parameters (e.g., hormones, metabolite

concentrations) but comparable in all animals. Analytical measurements might be considered another source of variations. Nevertheless, all the implemented kits were validated previously. It is right samples were not run at the same time; however, samples were frozen at - 80°C, and each experiment was performed equally and within its every standard curve, intending to minimize experimental variations at this stage.

Finally, data handling itself might be not considered as a source of variation; however, in some analyses, data were transformed and scaled, which could cause loss of biological information. This problem is commonly related to big data analyses and should be considered in the interpretation of data.

4.2. Did basal MI and MI key enzyme concentrations differ between broilers and laying hens?

In this section, a mere description of MI and MI key enzymes concentrations on broilers and hens was performed. Providing discussion about differences between breeds was difficult, initially because broilers and hens experiments were performed individually and responding to different research questions. To the present, it is unknown to what extent MI metabolism is similar between broilers and hens. In the experiments of this thesis, broilers were sampled at 22 days of age, whereas hens were sampled at different productive periods at 10, 16, 24, 30, and 60 weeks of age.

Here, 22-days-old broilers were compared to 16-weeks-old laying hens (**Figure 3)**. These ages were selected because both were productive periods considered metabolically and nutritionally critical for body growth and performance. Findings indicated that MI and MI key enzyme concentrations were within the same range of units for broilers and laying hens. Basal concentrations of MI in plasma were 0.25 ± 0.02 (mean ± SEM) and 0.15 ± 0.01 mmol/l for broilers and hens, respectively (**Figure 3a**). Liver and kidney MI concentrations were significantly different between broilers (7.93 ± 0.68 and 9.38 ± 1.31 mg/g DM, respectively)

and hens (5.96 ± 0.37 and 7.21 ± 0.43 mg/g DM, respectively) (**Figures 3b and 3c**). In relation to enzyme expression, liver IMPase 1 concentrations (**Figure 3b**) were highly significant in broilers (1.25 ± 0.11 pg/mg protein) compared to hens (0.18 ± 0.02 pg/mg protein) whereas kidney MIOX concentrations (**Figure 3c**) did not change significantly (1.38 ± 0.23 and 1.45 ± 0.06 pg/mg protein, for chickens and hens, respectively).

As mentioned before, analyses based on direct comparisons are not possible from a scientific point of view. To make an effective comparison, specific studies looking for performance trait differences must be designed. From this simple comparison, it was striking that broilers presented higher systemic MI concentrations (as well as its marker of endogenous synthesis, IMPase1) than hens. It has been argued that breeds for meat production are selected based on their high metabolic rate and fast growth (Scheele, 1997), which means faster muscle development (Scheuermann et al., 2003) but also higher abdominal fat production (Leclercq 1984). MI has been related to skeletal muscle development (Dang et al., 2010) but also with promotion of adipose tissue lipid storage capacity for prevention of ectopic fat deposition (Plows et al., 2017), then it is plausible, but must be proven, that due to their shorted lifespan, broilers presented more levels of endogenous MI for PI and inositol phosphates synthesis than young hens.

Furthermore, MI is an important cellular osmolyte in mammal kidney (Sizeland et al., 1993). It might be possible that basal MI concentrations were strongly regulated in broilers and hens to maintain kidney osmoregulation, although it is interesting the fact that broilers concentrations of kidney MI and MIOX, in opposite to hens, presented a high variability.

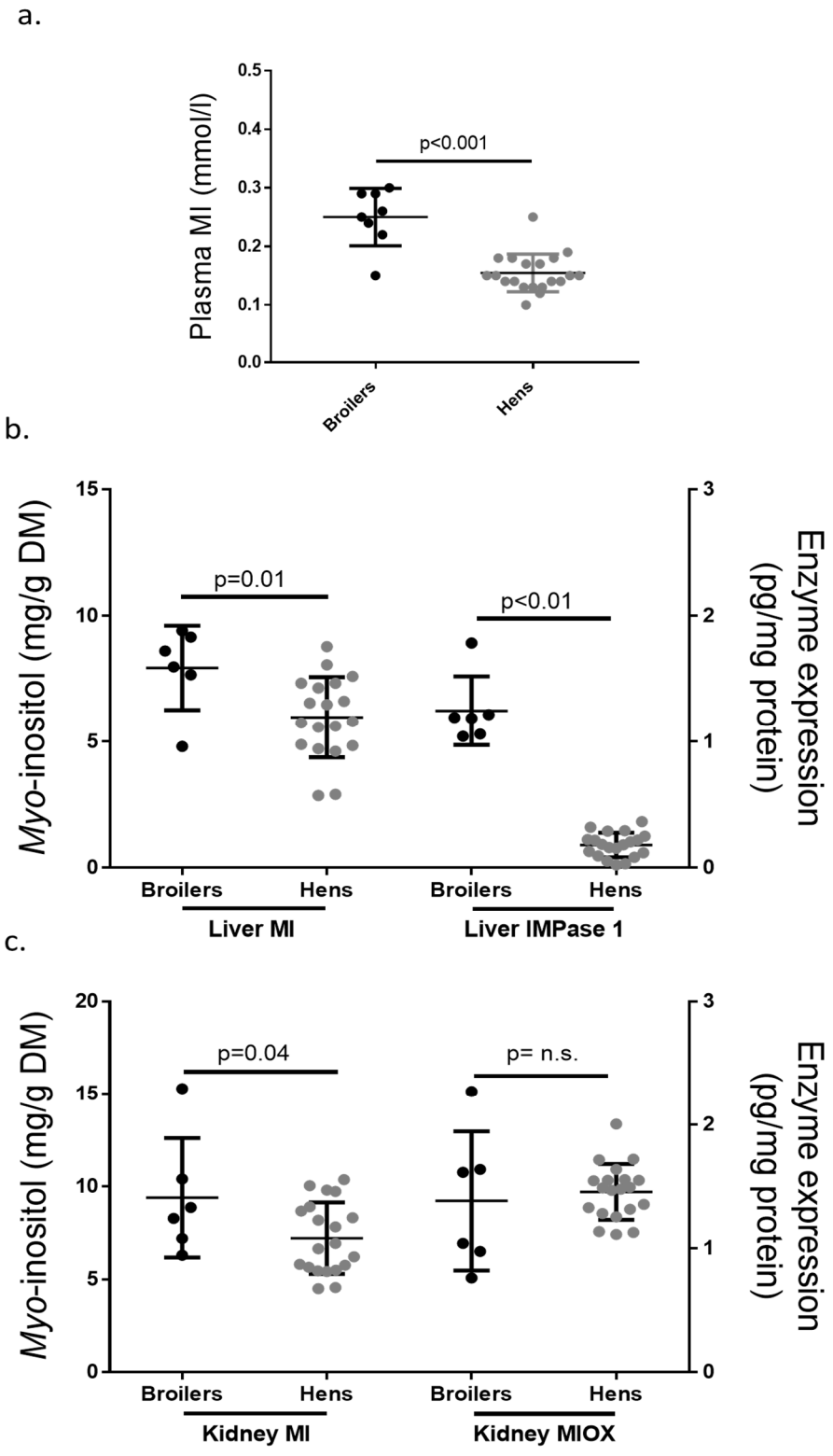

Figure 3. Comparison between basal MI and MI-key enzymes concentrations on plasma, liver and kidneys from broilers and hens. (a) Plasma MI concentrations. (b) Liver MI and IMPase 1 concentrations (c) kidney MI and MIOX concentrations. Left-y-axes indicate *myo*-inositol concentrations, whereas right-y-axes indicate *myo*-inositol key enzymes expression. 22-days-old broilers and 16-weeks-old hens were measured for this comparison. Each point represents one individual. Hen results represented pools from LB and LSL at week 16. Student´s t-tests were used as the statistical test to analyze the data. Bars show mean ± SD. Significance was set at $P < 0.05$. Plasma concentrations from hens (a) were obtained by Vera Sommerfeld, 2020 (University of Hohenheim, Germany, personal communication), who performed a parallel study with the same hens. MI and MI-key enzymes analyses were performed as described by the materials and methods section from the manuscript 3 and the draft of manuscript (chapter 3).

4.3. Could dietary MI have a role in dopamine-serotonin synthesis?

Dietary MI is significantly plasma serotonin and dopamine concentrations in broilers (manuscript 2, chapter 3). This section intended to offer novel information about concentrations of metabolites which acted as precursors of dopamine and serotonin (**figure 4**). Briefly, MI supplementation increased significantly plasma MI concentrations (0.55 ± 0.06 mmol/l, mean ± SEM) in comparison to controls (0.25 ± 0.018 mmol/l). No differences were observed in the amino acids L-tyrosine (control= 220.4 ± 33.47; MI= 220.5 ± 23.69) and L-tryptophan (control= 77.26 ± 6.08 μmol/l; MI= 66.71 ± 5.55 μmol/l), neither in the L-tyrosine product L-DOPA (control= 0.15 ± 0.02 μmol/l; MI= 0.14 ± 0.01 μmol/l). 5-hydroxy-L-tryptophan, the precursor of serotonin, was not measured. Moreover, tyramine, a minor precursor of dopamine synthesis, was also not measured.

Dopamine and serotonin are both synthesized by the enzyme *aromatic L-amino-acid decarboxylase* (not measured). The experiments performed in this thesis showed that MI supplementation did not affect concentrations of serotonin and dopamine precursors, indicating MI could be regulating additional pathways for serotonin and dopamine synthesis (**figure 4**). Even though there is no previous evidence about interactions between MI and *L-amino-acid decarboxylase* activity*,* it could be hypothesized MI supplementation might participate in its up-regulation. The fact dopamine and serotonin concentrations were significantly different but correlated significantly in controls (R^2=0.81; p=0.002), and MI-supplemented (R^2=0.86; p=0.001) broilers chickens could be an indication of enzyme functionality; however, it must be proven by mechanistically studies.

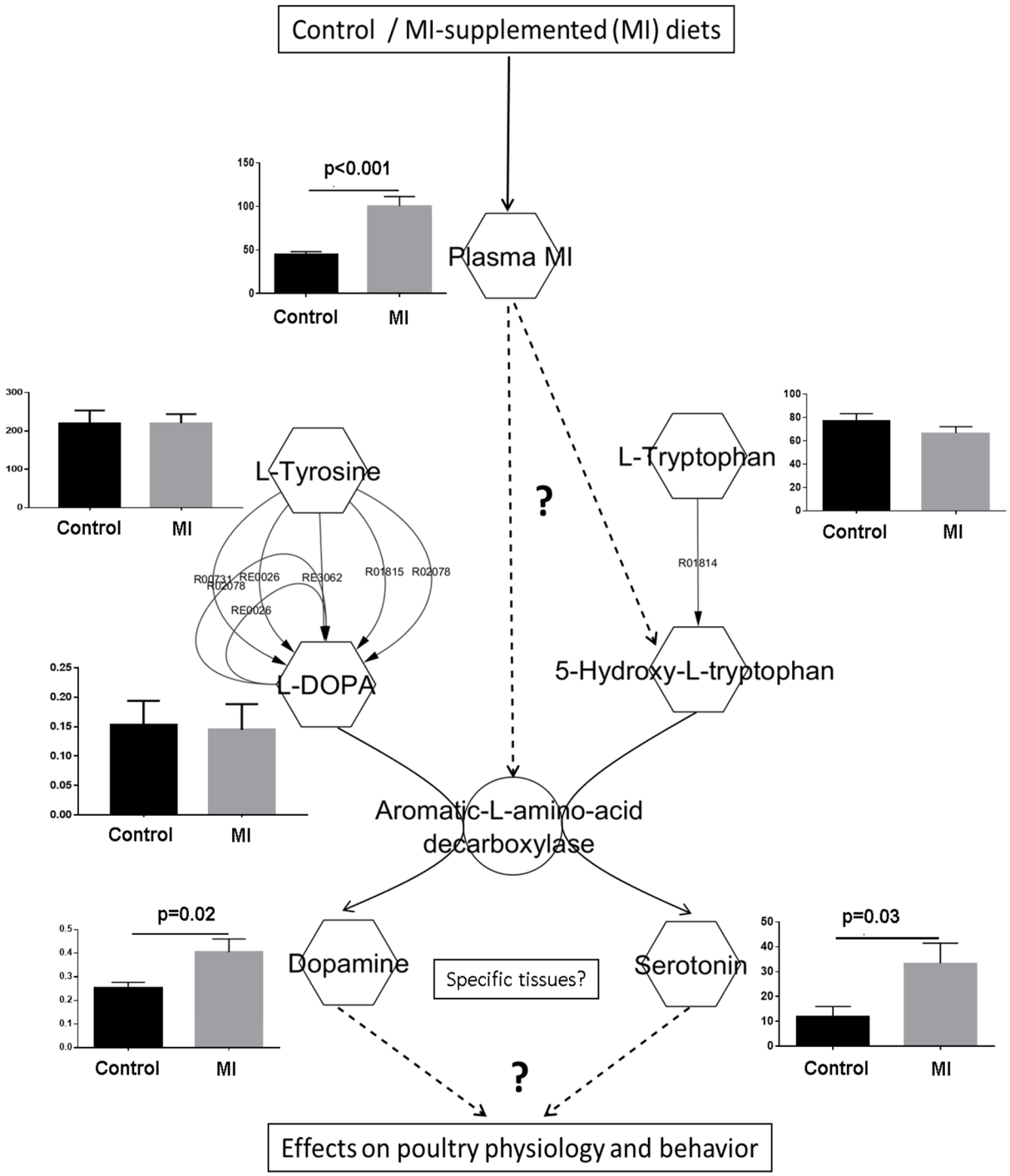

Figure 4. Possible effects of dietary *myo*-inositol (MI) on plasma dopamine and serotonin synthesis based on targeted metabolomics results. Rhomboid shapes indicate metabolites, whereas the circular shape indicates the main enzyme involved in dopamine and serotonin synthesis. ROs and REs represent the KEGG nomenclature of each enzyme involved in metabolite conversions. Black arrows indicate precursors and products of different reactions, whereas dashed arrows indicate potential metabolite associations and consequences on poultry physiology and behavior. Question marks indicated the unanswered questions for further research. Student´s t-tests were used as the statistical test to analyze the data. Bars show mean ± SD. n=8 per group. Metabolites differing significantly between control and MI-supplemented diets showed reported p-value on the top of the bars.

Which might be the consequences for feather pecking behavior?

Brain serotonin and dopamine are monoamines associated with aggression and the reward system, respectively (Narvaes et al., 2014). Feather pecking behavior has been associated with central dopamine and serotonin down-regulations (Uitdehaag et al., 2011; de Haas and van der Eijk. 2018). In humans and rats, plasma serotonin showed correlations with central concentrations (Audhya et al., 2012). Indeed, it has been demonstrated that high feather pecking birds presented lower plasma serotonin concentrations (Buitenhuis et al., 2006).

It is a matter of fact that dietary interventions could contribute to decreasing feather pecking behavior (van Krimpen et al., 2005). Results showed in this thesis indicated that MI supplementation increased plasma serotonin and dopamine (see manuscript 2, chapter 3). In a previous study, rats that were intraperitoneally injected with MI (1.25 g/kg) presented increases in the time spent in the open arms of an elevated plus-maze (Einat et al., 1998). Interestingly, a new study showed that dietary MI was related to the reduction of fear response and vent feathers but also with the increase of mortality in laying hens (Herwig et al., 2019). Increased mortality could be attributed to the fact that lower fear and avoidance behavior could increase cannibalism and less feather cover the vent area (Savory and Mann, 1997). This evidence indicated MI supplementation modulates anxious and aggressive behaviors so that more studies are necessary to establish e.g., the optimal MI doses to be used to prompt reductions in stress and anxiety, avoiding, in turn, aggressive behaviors and cannibalism-associated behaviors.

4.4. Conclusion and perspectives for future research

Increases on MI concentrations in plasma and kidneys of broiler chickens associated with phytase supplementation suggested that intestinal MI availability would be reflected MI in blood and organs; nevertheless, MI transport across the intestine has not been confirmed yet. Findings indicated that metabolites were affected differently upon phytase or pure MI

supplementation. In this case, while phytase supplementation was associated with decreases of plasma metabolites belonging to acylcarnitines, phosphatidylcholines, sphingomyelins, lysophosphatidylcholines and biogenic amines, pure MI supplementation increased plasma concentrations of dopamine and serotonin. These findings are documented by the first time in the present thesis and might indicate phytase supplementation modulates metabolite profile beyond MI releasing, indicating different dietary MI sources could participate in diverse physiological functions that are not understood yet.

Moreover, MI endogenous concentrations in liver and muscle would be up-regulated by stage of production in LB and LSL laying hens. Besides that, amino acid, polyamine, and phospholipid profile might be characterized differently by LB and LSL during the onset of egg-laying. The corresponding metabolic phenotype indicated the involvement of amino acid synthesis adaptation to egg-laying period, reflected by their decreases in plasma amino acid profile.

Observations presented in this thesis offer novel information and a better understanding of the role of basal and supplemented MI in laying hens and broilers, respectively. Furthermore, a significant effect of strain, productive period, and diet on metabolite profile indicates that these variables could influence metabolic flexibility; nonetheless, future mechanistically research needs to be carried out to explain the effects of those variables in poultry metabolism.

Findings showed so far prompt to the importance of interdisciplinary research. It is known that metabolic interpretations depend on the crosstalk between areas such as physiology, behavior, nutrition, genetics, and microbiome so that potential interactions around MI metabolism should be studied (some questions showed in **figure 5**) for the sake of improving parameters of health, performance, and welfare.

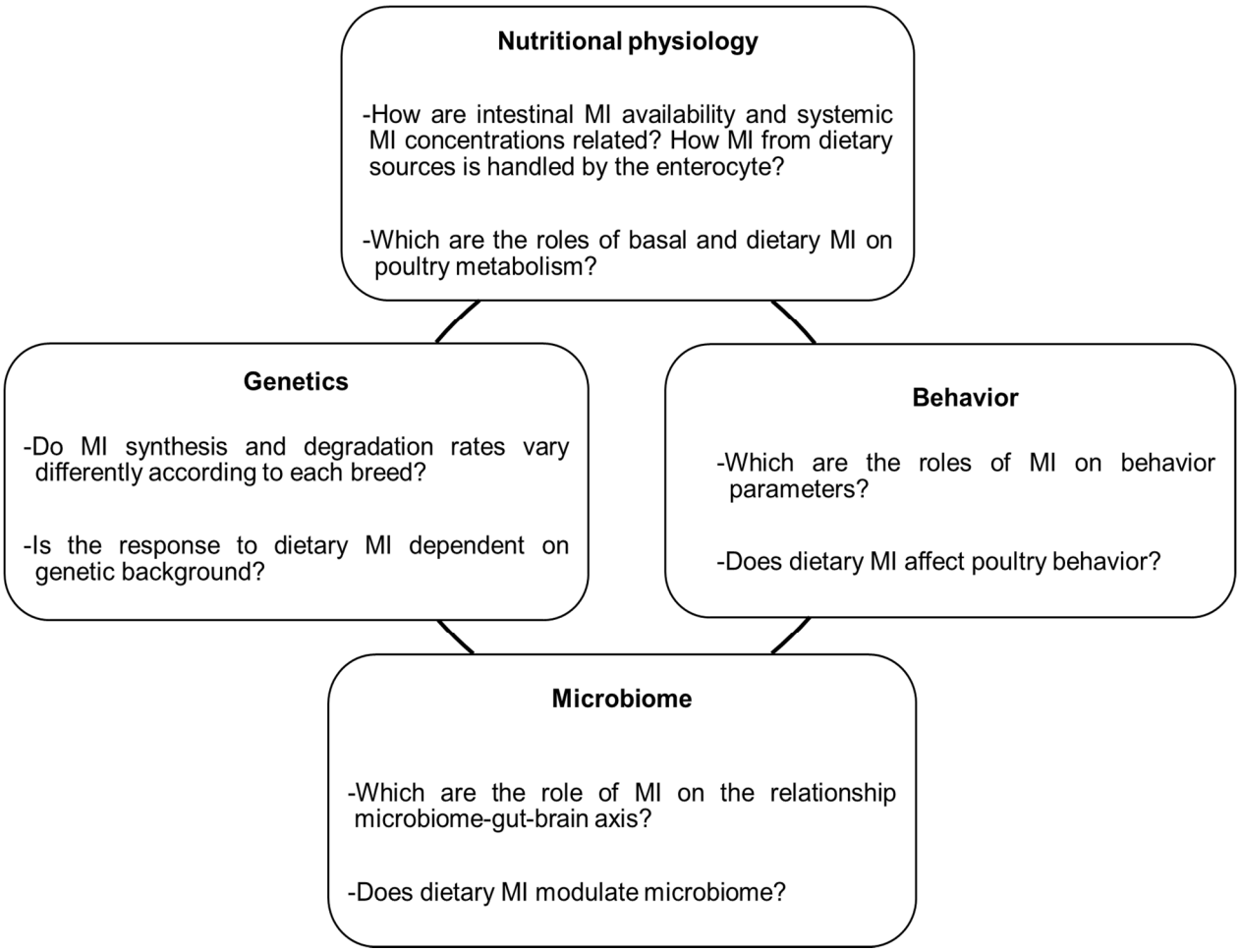

Figure 5. Relevant questions for future research about MI metabolism in poultry.

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6. Summary

Poultry production has shown a significant increase during the last decade. Meat and egg industry rapid growth implicates accelerating metabolic rate and general performance of birds. To maintain a high level of production, several strategies to achieve optimal raising and feeding have been implemented. Previous studies demonstrated the importance of MI metabolism on animal physiology; however, at present there is a substantial lack of information about the roles of MI and its metabolism in poultry. For instance, no information is available about MI concentration in organs of poultry. Moreover, it remains no elucidated, which are the effects of dietary sources of MI such as dietary phytase or pure MI supplementation.

This thesis focused on gaining a comprehensive understanding of the potential roles of strain, productive period, and dietary sources of MI on poultry metabolism. To obtain the state of the art research on MI metabolism and its dietary sources in poultry, a comprehensive review of dietary MI was written (manuscript 1, chapter 3). This review revised information about MI in poultry such as feed sources, transport and cell metabolism, physiological meaning, and the influence of dietary MI in poultry. The revision indicated that MI appears to play critical roles in several different metabolic pathways so that understanding them could be an essential approach for future research in poultry.

The second study was performed to study the effects of phytase and pure MI supplementation on the metabolite profile of broilers (manuscript 2, chapter 3). It was observed that phytase supplementation affected differently the metabolite profile than the supplementation of pure MI. Metabolites affected by phytase comprised several groups of metabolites such as acylcarnitines, phosphatidylcholines, sphingomyelins, lysophosphatidylcholines, and biogenic amines, whereas pure MI supplementation increased plasma concentrations of dopamine and serotonin.

The third study was performed to get preliminary information about the effects caused by dietary phytase on systemic MI on the gastrointestinal tract, blood, and organs MI of broiler chickens (manuscript 3, chapter 3). Supplementation of 1500 FTU phytase/kg feed increased plasma and kidney MI concentrations. Plasma MI correlated negatively with $InsP_6$ and positively with intestinal MI concentrations.

A fourth study gave a general description of MI concentrations and general metabolite profile during the productive life of Lohmann Classic Brown and Lohmann LSL Classic laying hens. It was found that productive period affected MI and MI key enzymes expression. Moreover, the analyses showed differences in metabolite profiles being the onset of egg production, a determinant point. Differences were attributed to different groups of metabolites such as amino acids, biogenic amines, phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins. The above mentioned, indicated each strain could express different MI concentrations and metabolite profiles during distinct productive periods what should be considered to future interventions.

To conclude, findings from these investigations suggested intrinsic traits such as breed and stage of production and diet could affect MI and MI key enzymes expression as well as metabolite profiles. Future studies are needed to establish the roles of MI on poultry metabolism.

7. Zusammenfassung

Die Geflügelproduktion hat im letzten Jahrzehnt erheblich zugenommen. Das schnelle Wachstum der Fleisch- und Eierindustrie impliziert eine Beschleunigung des Stoffwechsels und der allgemeinen Leistung von Geflügel. Um ein hohes Produktionsniveau aufrechtzuerhalten, wurden verschiedene Strategien zur Erzielung einer optimalen Aufzucht und Fütterung umgesetzt. Frühere Studien haben die Bedeutung des MI-Metabolismus für die Tierphysiologie aufgezeigt. Derzeit fehlen jedoch wesentliche Informationen über die Rolle des MI und seinem Metabolismus bei Geflügel. Beispielsweise sind keine Informationen über die MI-Konzentration in Geflügelorganen verfügbar. Darüber hinaus ist es nicht geklärt, welche Auswirkungen diätetische MI-Quellen wie z.B. durch Phytase-basierte Freisetzung aus der Nahrung oder reine MI-Supplementierung haben.

Diese Dissertation fokussierte sich auf ein umfassendes Verständnis der möglichen Rolle von Zuchtlinie, Lebensdauer und diätetischem MI für den Stoffwechsel des Geflügels. Mit dem Ziel, den neuesten Stand der Forschung zum MI-Metabolismus und seinen Nahrungsquellen bei Geflügel zu erhalten, wurde eine umfassende Übersicht über das MI in der Nahrung verfasst (Manuskript 1, Kapitel 3). Diese Übersichtsarbeit fasst Informationen über MI bei Geflügel zusammen, wie etwa Futterquellen, Transport und Zellstoffwechsel, physiologische Bedeutung und den Einfluss von MI in der Nahrung bei Geflügel. Es wurde erfasst, dass MI eine Schlüsselrolle in einer Reihe verschiedener Stoffwechselwege zu spielen scheint, so dass das Verständnis dieser Stoffwechselwege ein wichtiger Ansatz für die künftige Geflügelforschung sein könnte.

Die zweite Studie wurde durchgeführt, um die Auswirkungen von Phytase und reiner MI-Supplementierung auf das Metabolitenprofil von Broilern zu untersuchen (Manuskript 2, Kapitel 3). Es wurde beobachtet, dass eine Phytase-Supplementierung das Metabolitenprofil

anders beeinflusste als die Supplementierung von reinem MI. Unter Phytasefütterung betroffene Metaboliten umfassten mehrere Gruppen von Metaboliten wie Acylcarnitines, Phosphatidylcholines, Sphingomyelines, Lysophosphatidycholines und biogene Amine, während eine reine MI-Supplementierung die Plasmakonzentrationen von Dopamin und Serotonin erhöhte. Die dritte Studie wurde durchgeführt, um vorläufige Informationen über die Auswirkungen der diätetisch supplementierten Phytase auf den systemischen MI auf den Magen-Darm-Trakt, den Blut- und Organ-MI von Masthühnern zu erhalten (Manuskript 3, Kapitel 3). Die Ergänzung von 1500 FTU Phytase / kg Futter erhöhte die Plasma- und Nieren-MI-Konzentrationen. Die Plasma-MI-Konzentration korrelierte negativ mit $InsP_6$ und positiv mit den intestinalen MI-Konzentrationen. Eine vierte Studie lieferte eine allgemeine Beschreibung der MI-Konzentrationen und der Metabolitenprofile während des produktiven Lebens von Legehennen der Linien Lohmann Classic Brown und Lohmann LSL Classic. Es wurde festgestellt, dass die Produktionsperiode die Expression von MI- und MI-Schlüsselenzymen beeinflusste. Darüber hinaus zeigten die Analysen Unterschiede in den Metabolitenprofilen, wobei der Beginn der Eiproduktion ein bestimmender Punkt war. Unterschiede wurden verschiedenen Gruppen von Metaboliten wie Aminosäuren, biogenen Aminen, Phosphatidylcholinen, Lysophosphatidycholinen und Sphingomyelinen gefunden. Dies zeigte deutlich, dass jede Linie unterschiedliche MI-Konzentrationen und Metabolitenprofile entlang der Lebensdauerzeigte, was bei zukünftigen Interventionen berücksichtigt werden sollte. Zusammenfassend lässt sich sagen, dass die Ergebnisse dieser Untersuchungen darauf hindeuten, dass intrinsische Merkmale wie Zuchtlinie, Produktionsstadium und Ernährung die Expression von MI- und MI-Schlüsselenzymen sowie die Metabolitenprofile beeinflussen könnten. Zukünftige Studien sind erforderlich, um die Rolle des MI für den Geflügelstoffwechsel zu bestimmen.

8. Annexes

8.1. Acknowledgements

The last three years as a Ph.D. student have taught me precious ways to strengthen myself personally and academically. Many people contributed to my personal development during these years. In the first place, I would like to thank my supervisor Prof. Dr. Korinna Huber, for her encouragement and excellent guidance. Her positive attitude and patience permitted me to deepen my academic skills. Every discussion with her gave me the enthusiasm to continue my academic work.

I wish to express my gratitude to Prof. Dr. Markus Rodehutscord, whose continuous advice and constructive comments were crucial for my academic progress. I appreciated his doors were always open for academic discussion. I also thank Dr. Vera Sommerfeld and, in general, to all the P-FOWL members for their committed work in the project and their valuable input in each discussion.

I want to thank all my colleagues from the Department of Functional Anatomy of Livestock, whose assistance was highly valuable in the completion of this project. Many thanks to Edwin Molano and Luisa Schreyer for their contribution to my lab work. Many thanks to my doctoral colleagues with whom academic sessions, conferences, and trips were delightful.

My particular regards to all the friends I gained during these years. These include all the ones I shared my hard and comfortable moments, the ones with whom I learned German.

Finally and heartily, all my gratitude to my family for their love and support. To my grandmother, Carmen who takes care of me from the heaven. And naturally to Liliana, whose love, patience, and understanding gave me the strength to achieve this purpose.

8.2. CURRICULUM VITAE

1. PERSONAL DATA

Name:	Duvan Fernando Gonzalez Uarquin
Date of Birth:	September 23, 1986.
Place of Birth:	Bogota D.C., Colombia

2. EDUCATION

• PhD student, Department of Animal Science University of Hohenheim (Stuttgart, Germany).	Since 10/2017.
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3. ACADEMIC EXPERIENCE

• Department of Functional Anatomy of Livestock. University of Hohenheim. Stuttgart, Germany.	Scientific staff Since 10/2017
• School of Veterinary Medicine and Animal Sciences. National University of Colombia (Bogotá D.C., Colombia).	Lecturer 02/2012-12/2015
• Department of Psychological and Brain Sciences University of Massachusetts (Amherst Amherst, Massachusetts, USA).	Visiting research Student. 2014
• Cell death research group. Institute of Genetics. National University of Colombia (Bogotá D.C., Colombia).	Young Research Scholar 2011-2012
• School of Animal Sciences and food engineering. University of Sao Paulo (Pirassununga, Sao Paulo, Brazil).	Visiting research Student 2008-2009

Duvan Fernando González Uarquin
June the 2nd, 2020

8.3. Affidavit

Pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. I hereby declare that I independently completed the doctoral thesis submitted on the topic "Studies of strain, lifespan and dietary myo-inositol interventions on poultry metabolism".

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphrasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

……..……

Stuttgart, June the 2nd, 2020 Duvan Fernando Gonzalez Uarquin

8.4. Affidavit

Information

The University of Hohenheim requires an affidavit declaring that the academic work was done independently in order to credibly claim that the doctoral candidate independently completed the academic work.

Because the legislative authorities place particular importance on affidavits, and because affidavits can have serious consequences, the legislative authorities have placed criminal penalties on the issuance of a false affidavit. In the case of wilful (that is, with the knowledge of the person issuing the affidavit) issuance of a false affidavit, the criminal penalty includes a term of imprisonment for up to three years or a fine.

A negligent issuance (that is, an issuance although you should have known that the affidavit was false) is punishable by a term of imprisonment for up to one year or a fine.

The respective regulations can be found in Sec. 156 StGB (Criminal Code) (false affidavit) and in

Sec. 161 StGB (negligent false oath, negligent false affidavit).
Sec. 156 StGB: False Affidavit

Issuing a false affidavit to an authority body responsible for accepting affidavits or perjury under reference to such an affidavit shall be punishable with a term of imprisonment up to three years or with a fine.

Sec. 161 StGB: Negligent False Oath, Negligent False Affidavit:
Subsection 1: If one of the actions described in Secs. 154 and 156 is done negligently, the action shall be punishable by a term of imprisonment of up to one year or a fine.
Subsection 2: Impunity shall apply if the perpetrator corrects the false information in a timely manner. The regulations in Sec. 158 (2) and (3) apply mutatis mutandis.

The German original version of this affidavit is solely valid; all other versions are merely informative.

I have taken note of the information on the affidavit.

……
Stuttgart, June the 2nd, 2020 Duvan Fernando Gonzalez Uarquin