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Possible roles of parathyroid hormone, 1.25(OH)₂D₃ and Fibroblast Growth Factor 23 on genes controlling calcium metabolism across different tissues of the laying hen.

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ABSTRACT

This study provides an integrative description of candidate gene expression across tissues involved in Ca metabolism during the egg laying cycle, using the well-defined model of Ca supply as fine or coarse particles of calcium carbonate (CaCO_3). Plasma and tissue samples were collected from hens at the peak of laying at 0 to 1, 9 to 10 and 18 to 19 h post ovulation (PO). After mRNA preparation from the parathyroid gland, medullary bone, liver, kidney, duodenum and jejunum, gene expressions were quantified using RT-qPCR. The highest levels of Parathyroid Hormone (PTH) mRNA in the parathyroid gland ($P < 0.05$), and of the active form of vitamin D_3 $1,25(\text{OH})_2\text{D}_3$ in the plasma ($P < 0.01$) were observed at 18 to 19 h PO. During this active phase of eggshell formation, bone resorption was attested by high levels of plasma inorganic Phosphorus ($i\text{P}$) and the receptor activator of NF- κB (RANK) expression in the bone ($P < 0.001$ and $P < 0.05$, respectively). At this stage, five genes of the transcellular and the paracellular Ca absorption pathways in the intestine ($P < 0.05$) and the Ca channel transient receptor potential cation channel subfamily V member 5 (TRPV5; $P < 0.05$), involved in its reabsorption in the kidney, were overexpressed. At 0 to 1 h PO during the subsequent daylight period, two candidates of the transcellular and the paracellular Ca pathways ($P < 0.05$) remained at high levels in the intestine, while calbindin D 28K (CALB1) expression was the highest in the kidney ($P < 0.05$). As PTH mRNA and $1,25(\text{OH})_2\text{D}_3$ were low, bone accretion was likely active at this stage. The phosphaturic hormone Fibroblast Growth Factor 23 (FGF23) was overexpressed at 18 to 19 h PO ($P < 0.05$) in the bone when plasma $i\text{P}$ was high, which suggested a role in the subsequent reduction of P reabsorption in the kidney, as attested by the decreased expression of P cotransporters, leading to $i\text{P}$ clearance from the plasma at 0 to 1 h PO ($P < 0.05$). The low levels of $1,25(\text{OH})_2\text{D}_3$ at this stage coincided with increased expression of the 24-hydroxylase gene in the kidney ($P < 0.05$). In hens fed fine particles of CaCO_3 , the higher plasma levels of $1,25(\text{OH})_2\text{D}_3$ and the higher expression of several genes involved in bone turnover reflected a stronger challenge to Ca homeostasis. Altogether, these data support the hypothesis that FGF23 could drive vitamin D metabolism in the laying hen, as previously documented in other species, and explain the tight link between P and Ca metabolisms.

Keywords : 6 Calcium homeostasis – laying hens – eggshell formation – bone remodeling – gene expression – dietary calcium

1. Introduction

Eggshell formation in laying hens is a rapid mineralization process, which takes place daily in the uterine gland [1]. This process involves three steps: the initiation of calcite crystal formation from 5 to 9 h post-ovulation (PO), the active phase of calcium carbonate (CaCO_3) deposition from 9 to 22 h PO, and the arrest of mineralization about 1h30 before the egg expulsion [2]. During the active phase, as Ca^{2+} is not stored in the uterus, high amounts are exported at a rate of 0.33 g of CaCO_3 per h, which leads to a total daily exportation of 2 g of Ca [2]. The lack of synchronization between the intake of dietary calcium (Ca) during the day and the deposition of Ca into the eggshell mainly during the night, imposes severe demands on ionic Ca homeostasis [3]. Some nutritional strategies have been developed in order to enhance the provision of soluble Ca in the digestive tract and to sustain eggshell formation [1]. Providing CaCO_3 as coarse instead of fine particles enhances Ca availability due to a longer retention time in the gizzard and a progressive and extended period of Ca solubilization during the dark period [4,5]. This strategy is potentiated by the specific appetite of the laying hen for Ca before dark [6], and by an increase of intestinal Ca retention during eggshell formation [3,7], thus improving eggshell solidity and reducing bone resorption [1,4]. The absorption of Ca relies on the transcellular (saturable) and paracellular (non-saturable) pathways, previously identified in laying hens [8]. During the active phase of calcification, hypocalcemia increases parathyroid hormone (PTH) synthesis, and in turn enhances the production of the active form of Vitamin D_3 ($1,25(\text{OH})_2\text{D}_3$) by the $1-\alpha$ hydroxylase (CYP27C1) enzyme in the kidney [9,10]. Both hormones act coordinately to increase the circulating levels of Ca^{2+} [1,11]. Both stimulate bone resorption, and $1,25(\text{OH})_2\text{D}_3$ also acts on the intestine and the kidney to increase Ca absorption and reabsorption, respectively [1,11]. These mechanisms are well described at molecular level in humans [10,12]. Bone resorption is a multistep process which involves osteoblast and osteoclast cells [13]. First, the osteoblasts induce osteoclastogenesis under the stimulation of PTH and $1,25(\text{OH})_2\text{D}_3$, leading to the differentiation and proliferation of mature osteoclasts [13]. Then, the osteoclasts resorb the organic and mineral matrices of bone through enzymatic reactions [13], which increases blood levels of Ca^{2+} and inorganic phosphorus (iP) [10]. The return to normal phosphatemia is regulated by a bone phosphaturic hormone, Fibroblast Growth Factor 23 (FGF23), which acts on the kidney to increase iP excretion by reducing its reabsorption [14]. It

also reduces $1.25(\text{OH})_2\text{D}_3$ levels, by increasing the activity of the 24-hydroxylase enzyme and decreasing that of $1-\alpha$ hydroxylase [14]. As a consequence, bone resorption is reduced and bone accretion takes place under the control of osteoblast cells [14]. This bone remodeling cycle takes several months in humans [15]. In laying hens, the medullary bone formed in the marrow cavities of long bones during sexual maturity [16], is the site of a rapid bone turnover and serves as a labile source of Ca^{2+} [17], providing up to 40% of the Ca required for the daily shell deposition [11,18]. Bone resorption also liberates high levels of *i*P, which are not required for eggshell formation and must be cleared from the blood stream [19] prior to a new phase of bone accretion during the subsequent daylight period [1]. Although FGF23 is suspected to exert a phosphaturic action in the domestic hen, as its inactivation by immunization lowers P excretion [20], its possible involvement during the ovulatory cycle remains to be documented.

Therefore, the present study aimed to identify the molecular mechanisms underlying the daily cycle of bone turnover, and its regulation in the laying hens, using the well-defined model of hens fed diets with fine or coarse particles of CaCO_3 . The first hypothesis was that the high demand of Ca by the uterus for eggshell formation would activate the PTH gene and the genes under its control involved in bone resorption, while the resulting increase of phosphatemia should trigger FGF23 expression for a return to basal levels. The second hypothesis relied on the dietary supply of coarse instead of fine particles of CaCO_3 which provides more soluble Ca during the active phase of eggshell calcification, and should reduce bone resorption through alterations of vitamin D metabolism and actions.

2. Materials and methods

2.1. Animals and housing conditions

The experiment was conducted under the guidelines of the French Ministry of Agriculture for Animal Research at the Experimental Poultry Unit of Tours, INRA, Nouzilly, France (PEAT INRA 1295). The experimental design was approved by the Regional Ethics Committee on animal experimentation (Tours, France) and the French Ministry of Higher Education and Research (Paris, France; authorization: 10043). Two groups of 20 weeks old laying hens (Isa Brown strain, Hendrix Genetics Layers, Ploufragan, France) were allocated to individual cages in order to record the oviposition time. They were fed a diet supplemented with 100% of fine (76% of the particles below 0.5 mm) or a diet containing a mixture of 30% fine (30%) and 70% coarse (89% of the particles between 2 and 4 mm) particles of CaCO₃. Both diets were formulated with similar nutritional values (2710 kcal/ kg of metabolized energy, 17.3% of proteins, 3.5% of total Ca, 0.45% of total P and 2000 UI/ kg). All animals had free access to water and feed, and were subjected to a photoperiodic program of 14L:10D. They were kept until 23 weeks old, corresponding to the beginning of the peak of lay, and their laying activity was recorded by the regular monitoring of oviposition for their last week (every 15 minutes during the first 3 hours of light). The determination of the precise stage in the ovulatory cycle was confirmed based on the weight on the dried eggshell, as previously described by Nys et al. [2].

2.2. Sampling of tissues

Six or seven laying hens per diet and PO stage (0 to 1, 9 to 10 and 18 to 19 h PO) were submitted to blood collection in the occipital sinus using heparin-lithium coated tubes (BD Vacutainer). These hens were then euthanized by an intravenous injection of Dolethal® (182 mg/ml, pentobarbital sodium, Vetoquinol, France). Blood was centrifuged immediately at 1000 g x 15 min for plasma collection and stored at - 20°C for further analysis. For each hen, the duodenum and jejunum were immediately removed and thoroughly washed with a cold PBS solution. A 4-cm proximal sample was isolated, scraped and snap-frozen in liquid nitrogen. The left kidney, parathyroid gland and a piece of liver were isolated and immediately snap-frozen

118 in liquid nitrogen. The left tibiae was removed, cleaned and the medullary bone was isolated, according to
119 the procedure described by Wang et al. and immediately snap-frozen in liquid nitrogen [21]. All samples
120 were stored at -80°C until further analysis.

121 122 2.3. Blood sample analysis

123 Plasma *iP* was analyzed using a commercial kit (Kit Phosphore UV 61571; bioMérieux, 69280 Marcy
124 l'Etoile, France) as previously described [22]. In addition, determination of 25(OH)D₃ and 1.25(OH)₂D₃
125 concentrations were performed by a chemiluminescent immunoassay (Diasorin, Saluggia, Italy, Spanaus and
126 von Eckardstein, 2017) at the Liaison XL platform “Service des Explorations Fonctionnelles” (G.H. Necker
127 Enfants Malades, 75743, Paris cedex 15, France) , as previously described [8].

128 129 2.4. Selection of candidate genes and primer design

130 The candidate genes were selected after a literature review of mammalian and chicken studies. The list of
131 genes with the corresponding primers, designed with the aid of Primer-BLAST [23], is available in the
132 supplementary Table 1. For bone, a list of 10 candidate genes was selected based on their roles in
133 osteoclastogenesis [13,24], bone resorption [13,24–26], bone accretion [27], or their regulation [28,29].
134 Additionally, specific primers were selected for the phosphaturic factor FGF23 [30,31]. In the parathyroid
135 gland, the targeted genes were calcium sensing receptor (CASR) and parathyroid hormone (PTH), a major
136 regulator of Ca homeostasis under hypocalcaemia [10,32]. In the liver, the targeted genes were cytochrome
137 P450 family 27 subfamily A member 1 (CYP27A1) which encodes a 25-hydroxylase enzyme [33], and GC
138 vitamin D binding protein (GC) [10]. In the kidney, 11 genes were selected based on their response to PTH,
139 1.25(OH)₂D₃ or FGF23 [10,14,30,34–36]. In the intestine, 16 genes identified in a previous study [8] were
140 selected in relation to their role in Ca and P absorption [11,37–41]. A list of nine housekeeping genes
141 (HKGs) with their validated primers in each of the five tissues or organs was also chosen. The validation of
142 all primers was performed by RT-qPCR, using a LightCycler® 480 Instrument II (Roche Applied Science,
143 Meylan, France), considering primer efficiencies between 80 and 120% as acceptable, and sequencing of the
144 PCR product (Genewiz, United Kingdom).

2.5. RNA extraction and RT-PCR analysis

Prior to RNA extraction, samples of medullary bone, kidney, liver and intestinal segments were ground in liquid nitrogen. All samples were extracted using methods adapted from Chomczynski and Sacchi [42].

For the liver, kidney and intestinal samples, total RNA was extracted using the RNeasy kit (Qiagen, Saint-Quentin en Yvelines, France). The total RNA of medullary bone and parathyroid gland was extracted using the Trizol reagents (TRI Reagent T9424 and TRI Reagent LS T3934, and TRI Reagent T9424 respectively, Sigma Aldrich, St Louis, USA).

Concentration and quality of the extracted RNA were assessed by spectrophotometry and agarose gel electrophoresis, prior to reverse transcription as described previously [8].

Following the primer validation step, gene expression was quantified by high throughput real-time quantitative PCR using a microfluidic system (BMK-M-96.96, Fluidigm®), as previously described [8].

2.6. Calculation of relative gene expression

The relative mRNA quantification was performed according to the $\Delta\Delta C_t$ calculation method proposed by Pfaffl, (2001) [43]. For a given tissue, the relative expression ratio (R) of a candidate gene was calculated, based on the Efficiency (E) and the cycle threshold (C_t) deviation of a cDNA sample (bone, parathyroid gland, kidney, liver, duodenum or jejunum of individuals) versus a control (cDNA mix of all samples for each tissue at a similar concentration) and expressed in comparison to the geometric average of a set of HKGs according to the equation:

$$R = (1+E \text{ candidate})^{\Delta C_t \text{ candidate (control - sample)}} / \text{Geometric Average} [(1+E \text{ HKGs})^{\Delta C_t \text{ HKGs (control - sample)}}]$$

The most stable HKGs (out of nine) were chosen for each tissue using GeNorm [44]:

- Medullary bone: GAPDH, STAG2, MATR3, PPIA, EIF3I, EIF3F and B2M
- Parathyroid gland: EIF3F, PPIA, SDHA, EIF3I, MATR3, STAG2 and TBP
- Liver: GAPDH, B2M, EIF3F, PPIA, SDHA, EIF3I and TBP
- Kidney: TBP, MATR3, EIF3I, PPIA, GAPDH, SDHA and STAG2

172 - Duodenum and jejunum: GAPDH, EIF3F, PPIA, SDHA, EIF3I, MATR3 and TBP

173

174 2.7. Statistical analysis

175 All statistical analyses were performed using the R 3.4.0 software (R Core Team, 2017, Vienna, Austria).

176 For plasma levels of *i*P, vitamin D metabolites and the mRNA expression of genes in the bone, the

177 parathyroid gland, the liver and the kidney, the normality of the data was checked by a quantile-quantile

178 plot, which identified outliers based on the linear relationship between theoretical and sample percentiles.

179 Therefore, the data were analyzed using a linear model by a robust regression (R Package MASS version

180 7.3-51.1), which reduced the weight of outliers in the analysis. The effects of the main factors PO stage, diet

181 and the interaction were tested. In case of significant effects in the model ($P < 0.05$), all pairwise

182 comparisons were performed using the least-square means (LSMeans) method (R Package emmeans version

183 1.3.0) and a Tukey adjustment.

184 To evaluate the effects of the PO stage and the diet on the expression pattern of genes involved in Ca and P

185 absorption across the duodenum and the jejunum the statistical model was adapted. The normality of the

186 data was checked by a quantile-quantile plot, and the outliers were removed from the dataset, which resulted

187 in a slight reduction in the number of samples for some genes in their experimental group. The

188 corresponding data were analyzed with a linear mixed model (R Package nlme version 3.1-137) including

189 the main factors: intestinal segments, PO stage, diet, and all interactions of order 2 and 3. The hen,

190 considered as the experimental unit, was included as a random effect in the model. The interaction of order 3

191 was removed from the model when it did not significantly affect mRNA expression. In the case of

192 significant effects in the model ($P < 0.05$), all pairwise comparisons were performed using the least-square

193 means (LSMeans) method (R Package emmeans version 1.3.0) and a Tukey adjustment.

194

3. Results

3.1. Plasma parameters and candidate genes in the parathyroid gland

The effect of the PO stage and the diet on plasma levels of *iP*, 25(OH)D₃ and 1.25(OH)₂D₃ are shown in Table 1. No interaction was observed between the PO stage and the diet for either parameter. All recorded plasma parameters varied with the stage and essentially between 0 to 1 and 18 to 19 h PO. Plasma levels of *iP* were higher at 18 to 19 h PO than at 0 to 1 h PO ($P < 0.001$). For vitamin D metabolites, differences in plasma levels were observed with the PO stage, with a reduction between 0 to 1 and 18 to 19 h PO for 25(OH)D₃, and an increase for 1.25(OH)₂D₃ ($P < 0.05$ and $P < 0.01$, respectively). Only 1.25(OH)₂D₃ plasma levels were affected by the diet, a higher level of 1.25(OH)₂D₃ being observed for hens fed fine particles of CaCO₃ compared to hens fed coarse particles ($P < 0.001$). In the parathyroid gland, no interaction between the PO stage and the diet was observed for the mRNA levels of the PTH gene responsible for the synthesis of the parathyroid hormone (PTH). They were higher at 18 to 19 h PO than at 0 to 1 and 9 to 10 h PO, and higher in hens fed coarse particles of CaCO₃ compared to those fed fine particles. No variation of the calcium sensing receptor (CASR) expression was observed (data not shown).

3.2. Candidate genes in the medullary bone

For candidate genes in charge of bone resorption, accretion and their regulation (described in the supplementary Table 1) no interaction between the PO stage and the diet was observed (Fig. 1-2). Only the mRNA expressions of RANK (enhancing osteoclastogenesis) and FGF23 (phosphaturic hormone) were affected by the PO stage. The expression of RANK was higher at 18 to 19 compared to 0 to 1 and 9 to 10 h PO (Fig. 1A). The mRNA levels of FGF23 were not measurable at 0 to 1 h PO due to very low levels of gene expression. This increased between 9 to 10 and 18 to 19 h PO (Fig. 1B). The mRNA expressions of ACP5, CTSK, OPG (involved in bone resorption), COL1A1 (bone accretion), ITGB1 and MMP2 (bone remodeling) were affected by the diet (Fig. 2A-F). Hens fed fine particles of CaCO₃ showed a higher mRNA expression than hens fed coarse particles, for all those genes. No significant effects of the PO stage or the diet were observed for CA2, the RANK ligand (RANKL) and VDR (data not shown).

3.3. Candidate genes in the kidney and the liver

The mRNA expression of candidate genes quantified in the kidney is shown in Fig. 3 and 4. Out of the eleven genes studied in the kidney and described in the supplementary Table 1, four of them (KL, FGFR3, CYP27C1 and SCL34A1) did not vary with either the PO stage, or the diet (data not shown). The mRNA expressions of the Sodium-phosphate symporters SLC20A1 and SLC20A2 were influenced by the PO stage in the same manner. They showed higher levels at 18 to 19 h PO than at the other PO stages (Fig. 3A-B). The mRNA expression of the Ca channel TRPV5 was higher at 18 to 19 than at 9 to 10 h PO, and intermediate at 0 to 1 h PO (Fig. 3C). The expression of the Ca binding protein CALB1 was higher at 0 to 1 than at 9 to 10 h PO and intermediate at 18 to 19 h PO (Fig. 3D). The mRNA expression of FGF receptor 2 (FGFR2) was higher at 9 to 10 compared to 0 to 1 h PO, and intermediate at 18 to 19 h PO (Fig. 3E). Only the $\text{Ca}^{2+}/\text{H}^{+}$ exchange pump ATP2B1 mRNA expression was affected by the diet, and was higher in hens fed coarse particles of CaCO_3 than in hens fed fine particles (Fig. 4F).

Genes involved in the vitamin D metabolism expressed in the liver and the kidney were also evaluated, and the results are shown in Fig. 4. In the liver, the expression of GC, encoding the vitamin D binding protein, did not vary with the PO stage, or the diet (data not shown). The mRNA expression of CYP27A1 (25-hydroxylase) was higher at 18 to 19 h PO than at 9 to 10 h PO (Fig. 4A). In the kidney, the mRNA expression of CYP24A1, encoding the 24-hydroxylase enzyme responsible for the inactivation of $1.25(\text{OH})_2\text{D}_3$, was affected by the interaction between the PO stage and the diet (Fig. 4B). Its mRNA expression was the highest at 0 to 1 h PO in hens fed coarse particles of CaCO_3 compared to all other groups. The mRNA expression of CYP24A1 in hens fed fine particles of CaCO_3 was also higher at 0 to 1 h PO than at the other stages and for both diets.

3.4. Candidate genes in the duodenum and the jejunum

Out of the 13 candidate genes involved in Ca absorption, the mRNA expression of two genes, CALB1 and TJP1, did not vary with the PO stage or with the diet (data not shown). The results for five genes, which were affected by one interaction or more, are presented in Table 2, while the results for six genes affected by the main factors without interactions are presented in Table 3.

249 The mRNA expression of the $\text{Ca}^{2+}/\text{H}^{+}$ exchange pump ATP2B2 was affected by three interactions. Its
250 highest gene expression was observed in hens fed coarse particles of CaCO_3 , at 9 to 10 h PO across
251 segments ($P < 0.01$), and in the duodenum across stages ($P < 0.01$). It was also highest at 9 to 10 h PO in the
252 duodenum, across diets ($P < 0.01$).

253 The mRNA expression of the cation channel TRPV2 was affected by the interactions diet by stage, or stage
254 by segment. Its mRNA expression was higher in hens fed coarse particles at 0 to 1 h PO than at 9 to 10 h
255 PO, and higher than in hens fed fine particles of CaCO_3 at all stages ($P < 0.05$). Its mRNA expression was
256 also higher in the jejunum at 0 to 1 and at 18 to 19 h PO than in the duodenum at 18 to 19 h PO and in both
257 segments at 9 to 10 h PO ($P < 0.05$). The other three candidates were affected by a single interaction, stage
258 by segment, diet by stage, respectively for the Ca specific claudins CLDN2 and CLDN12, and diet by
259 segment for the transmembrane protein occludin (OCLN). The mRNA expression of CLDN2 was higher in
260 the duodenum at 0 to 1 and 18 to 19 h PO, than at 9 to 10 h PO ($P < 0.05$). Gene expression of CLDN12 was
261 higher in hens fed coarse particles of CaCO_3 at 9 to 10 h PO than in all other groups ($P < 0.001$). In addition,
262 its expression was higher in the jejunum than in the duodenum ($P < 0.001$). A lower mRNA expression of
263 OCLN was observed in the duodenum of hens fed fine particles of CaCO_3 compared to the other groups ($P <$
264 0.01).

265 For P transporters, the expressions of the Sodium-phosphate symporters SLC20A1 and SLC34A2 were
266 affected by the interaction segment by stage (Table 2). The mRNA expression of SLC20A1 was higher in
267 the jejunum at 0 to 1 h PO than in the duodenum at all PO stages ($P < 0.05$). Gene expression of SLC34A1
268 was higher at 0 to 1 h PO in the duodenum than in the jejunum at all stages and in the duodenum at 9 to 10 h
269 PO ($P < 0.05$). Their mRNA expressions were higher in hens fed coarse particles of CaCO_3 than in hens fed
270 fine particles ($P < 0.05$).

271 The expression of the remaining six genes was altered by one (TRPM7), two (TRPC1, ATP2B1, ATP2B4,
272 TJP2 and TJP3) or three factors (VDR) without interaction (Table 3).

273 The mRNA expression of the $\text{Ca}^{2+}/\text{H}^{+}$ exchange pump ATP2B4 and the vitamin D receptor VDR were
274 higher at 18 to 19 h than at the other PO stages ($P < 0.05$ and $P < 0.001$, respectively). Gene expression of
275 the $\text{Ca}^{2+}/\text{H}^{+}$ exchange pump ATP2B1 was higher at 18 to 19 h than at 0 to 1 h PO, and gene expression of

276 the anchoring protein TJP3 was higher at 9 to 10 h and 18 to 19 h, than at 0 to 1 h PO ($P < 0.05$). The
277 jejunum showed higher mRNA expression than the duodenum for the Ca^{2+} channel TRPC1, the $\text{Ca}^{2+}/\text{H}^{+}$
278 exchange pump ATP2B4, the anchoring proteins TJP2, TJP3, and the vitamin D receptor VDR ($P < 0.001$, P
279 < 0.001 , $P < 0.05$, $P < 0.001$, $P < 0.05$, respectively). In contrast, the $\text{Ca}^{2+}/\text{H}^{+}$ exchange pump ATP2B1 was
280 expressed at higher levels in the duodenum than in the jejunum ($P < 0.01$). The mRNA expressions of the
281 cation channel TRPM7, the Ca^{2+} channel TRPC1, the anchoring proteins TJP2, and the vitamin D receptor
282 VDR were higher in hens fed coarse particles of CaCO_3 than hens fed fine particles ($P < 0.01$, $P < 0.001$, $P <$
283 0.01 , $P < 0.01$, respectively).

284

4. Discussion

In the laying hen, the active phase of Ca transfer from the blood to the uterine gland for eggshell calcification lasts 12 hours (10 to 22 h PO) [2]. This process, which imposes a severe demand of Ca and challenges the homeostasis of ionic Ca^{2+} , activates very efficient mechanisms to transfer large quantities of Ca^{2+} [11]. They are controlled by two major Ca regulating hormones, $1,25(\text{OH})_2\text{D}_3$, the active metabolic form of vitamin D, and the parathyroid hormone (PTH) [1,11]. In the present study, the increased gene expression of PTH in the parathyroid gland during the active phase of eggshell biomineralization at 18 to 19 h PO in hens from both groups is consistent with the reported increase of circulating PTH [9]. It is also the hallmark of the documented decrease of plasma ionic Ca^{2+} occurring from 10 h until 22 h PO [45]. Its downstream effects, the elevation of $1,25(\text{OH})_2\text{D}_3$ plasma levels observed at 18 to 19 h PO, must be driven by the action of PTH on the 1α -hydroxylase activity in the kidney [10]. In the chicken, this enzymatic activity has been attributed to CYP27C1 [35], but its mRNA expression was not regulated in the present study. To overcome the lowering of plasma ionic Ca^{2+} , PTH and $1,25(\text{OH})_2\text{D}_3$ act concomitantly on bone and on the kidney to increase blood Ca^{2+} [11]. Both hormones trigger bone resorption under hypocalcemia [11,13,46], which involves osteoclastogenesis followed by the degradation of the bone organic and inorganic matrices by osteoclasts [11,13]. Osteoblasts, when stimulated by PTH and $1,25(\text{OH})_2\text{D}_3$, release RANKL, to activate osteoclastogenesis. The immature osteoclast commits to the mature osteoclast phenotype, including the expression of RANK, a receptor which binds RANKL enhancing bone resorption [13,24]. In the present study, the high level of gene expression of RANK at 18 to 19 h PO indicated that osteoclastogenesis was stimulated in both groups of hens. Although the dietary supply of coarse particles of CaCO_3 should have delayed osteoclastogenesis [1,4], this study highlighted that this process was initiated for both groups of hens only 4 hours after the beginning of the dark period. The absence of variation of RANKL gene expression does not exclude variations at the protein level.

Bone resorption is taken over by mature osteoclasts, which express different enzymes to resorb the bone organic and mineral matrices [13,24]. Acidic conditions are required to initiate bone resorption. Osteoclasts acidify the extracellular environment by the secretion of protons, and the maintenance of an acidic pH is facilitated by the anion exchange of HCO_3^- and Cl^- , which is responsible for the bicarbonate efflux regulated

312 by the Carbonic Anhydrase enzyme CA2 [13,25]. This environment favors bone mineral matrix
313 mobilization, prior to organic matrix degradation by CTSK and ACP5 [13,26]. In the absence of variation of
314 CA2 expression, the higher expression of ACP5 and CTSK in the medullary bone of hens fed fine particles
315 of CaCO₃ strengthens the hypothesis that bone resorption was more severe in those hens. When eggshell
316 formation is completed, bone resorption ends and bone restauration occurs during the subsequent daylight
317 period [3,18]. In the mouse, this process results from an inhibition of osteoclastogenesis by osteoprotegerin
318 (OPG), an inhibitor of RANKL and RANK interaction [24]. Subsequently, the osteoblasts synthetize type I
319 collagen (COL1A1) as the most important collagenic protein, and osteocalcin (OC) a non collagenic protein
320 driving matrix mineralization [27]. Regulatory proteins including ITGB1 and MMP2 contribute to bone
321 formation and maintenance. ITGB1 is a receptor for mechanical loading in bone, which promotes osteoblast
322 proliferation in vitro and collagen 1 synthesis [28]. The matrix metalloproteinase-2 (MMP2) is a key factor
323 of bone remodeling, as indicated in mice invalidated for MMP2, which shows a reduction in bone density
324 [29]. Hens fed fine particles of CaCO₃ exhibited higher levels of COL1A1, ITGB1 and MMP2 expressions,
325 which indicated that the higher challenge to Ca metabolism increased bone accretion and remodeling. In
326 addition, OPG was overexpressed in the medullary bone of those hens. The invalidation of OPG in mice
327 induces excessive bone loss [13]. This suggested OPG overexpression as a protective mechanism in hens
328 exhibiting an intense Ca demand. Several studies have discussed the link between bone loss and eggshell
329 solidity [16]. In the present study, dietary supply of fine particles of CaCO₃ enhanced the expression of
330 candidate genes involved in bone remodeling activity, but had no detrimental effect on eggshell strength
331 (data not shown).

332 It is known that PTH and 1.25(OH)₂D₃ act on the kidney to increase Ca reabsorption [10,11], namely trough
333 the induction of TRPV5, CALB1 and ATP2B1 [34]. The high levels of TRPV5 and CALB1 expression,
334 observed at 18 to 19 and 0 to 1 h PO, respectively, may contribute to increase renal Ca²⁺ reabsorption and
335 consequently the maintenance of blood Ca²⁺ levels. In hens fed coarse particles of CaCO₃, the elevated
336 levels of ATP2B1 mRNA in the kidney could lead to increase Ca reabsorption, and thus prevent excessive
337 bone mobilization. Bone mobilization leads to *i*P release in the blood flow, thus plasma *i*P is a satisfactory
338 estimator of bone resorption during eggshell formation [19]. Its increase at 18 to 19 h PO strengthened the

hypothesis that the induction of osteoclastogenesis marked by RANK overexpression, resulted in bone resorption in both groups of hens at 18 to 19 h PO. Quantifying the expression of genes responsible for bone resorption, accretion and remodeling helped to differentiate the involvement of bone in eggshell formation between the different Ca sources. Phosphatemia is controlled by Fibroblast growth factor 23 (FGF23), a phosphaturic hormone synthesized in the bone by the osteocytes and osteoblasts in response to elevated plasma *iP* and 1.25(OH)₂D₃ [14]. In the medullary bone, FGF23 expression was enhanced during the active phase of eggshell mineralization at 18 to 19 h PO, when plasma *iP* and 1.25(OH)₂D₃ reached their highest levels. This confirms and extends previous observations about FGF23 mRNA expression and the detection of the immunoreactive FGF23 in the medullary bone of laying hens [31]. In this study, the ratio of medullary bone to cortical bone expression of FGF23 was maximal in laying hens at 16 h PO [31]. FGF23 mRNA expression has also been described in numerous other tissues of the laying hen, including the liver, which showed the highest expression [47]. In the present study, expression of FGF23 was also detected in the liver, where it did not vary with the PO stage (data not shown), which reinforces the specific role of bone-derived FGF23 on phosphatemia regulation. The effect of FGF23 occurs in the kidney through binding to FGFR/KL complexes in the distal tubule [14]. Four type of FGFRs have been described in mammals, while only three types were identified in chicken: FGFR1, FGFR2 and FGFR3 [14,36]. In the present study, no expression of FGFR1 was detected, as opposed to FGFR3, FGFR2 and KL gene expressions. Only FGFR2 mRNA expression was affected by the PO stage, increasing between 0 to 1 and 9 to 10 h PO. FGF23 has been shown to exert its phosphaturic action in the kidney through the downregulation of P transporters and a reduction of P reabsorption in the proximal tubule [14]. While SLC34A1 expression did not vary, both SLC20A1 and SLC20A2 exhibited a high expression at 18 to 19 h PO. Nevertheless, the decrease of plasma *iP* observed between 18 to 19 h PO and the next ovulation (0 to 1 h PO) implied a decrease of P reabsorption, which was accompanied by a decreased expression of both P transporters SLC20A1 and SLC20A2. It could result from the higher expression of FGF23 in the medullary bone at the previous stage. Indeed, the active immunization of laying hens against a synthetic FGF23 peptide results in increased plasma levels of *iP* and reduced P excretion [20]. The enzyme 24-hydroxylase (CYP24A1), which catalyzes the conversion of 1.25(OH)₂D₃ into its inactive form 1.24.25(OH)₃D₃ in the kidney, is stimulated by FGF23

[10,14]. The increased CYP24A1 expression at 0 to 1 h PO coincided with the reduction plasma of 1.25(OH)₂D₃ at this stage, and followed the increase of FGF23 at 18 to 19 h PO, suggesting a cascade similar to that described in other species. In addition, the higher expression of CYP24A1 observed at this stage in hens fed coarse particles of CaCO₃ could be responsible for the lower plasma levels of 1.25(OH)₂D₃ observed in this condition. Moreover, 1.25(OH)₂D₃ exerts a negative feedback on the levels of circulating PTH [10]. This may explain the higher levels of PTH mRNA expression in those hens. FGF23 has also been shown to decrease the expression of PTH mRNA [48], which may be responsible for its reduced level at 0 to 1 h PO in the present study.

The inverse variations observed for plasma levels of 25(OH)D₃ and 1.25(OH)₂D₃ with PO stages suggested that, in the absence of regulation of CYP27C1 mRNA expression, the hepatic hydroxylation step could be critical for vitamin D₃ turnover. In the liver of mammals, the activity of the 25-hydroxylase enzyme shows little variation, and the circulating levels of 25(OH)D₃ are mainly dependent on vitamin D intake [10]. In the present study, the differential expression of CYP27A1 in the liver over the ovulatory cycle suggested an additional level of regulation of 25(OH)D₃ turnover.

The retention of Ca is highly increased during eggshell formation, as a result of increased Ca reabsorption by the kidney, as discussed above, and of Ca absorption by the small intestine, both under the regulation of 1.25(OH)₂D₃ [1,10,11]. To overcome the absence of feed intake during the dark period, a dietary supply of Ca sources with coarse particles permits a progressive diffusion of soluble Ca in the intestine [4]. This strategy is potentiated by the specific appetite of the hens for Ca [6] and the induction of an acid secretion in the proventriculus before light extinction [49]. Consequently, the intestine plays a role of the utmost importance in Ca homeostasis [50]. In a previous study, we identified a set of candidate genes for Ca absorption in the small intestine, many of which were stimulated during sexual maturity of laying hens, as circulating 1.25(OH)₂D₃ levels increased [8]. We hypothesized that they mediated the increased uptake of Ca observed in the duodenum and the jejunum at this period [8]. In the present study, all the molecular actors of the transcellular and the paracellular Ca absorption pathways previously detected [8] were confirmed, and some showed variations during the ovulatory cycle. At 0 to 1 h PO, when the hen has access to the feed and Ca is requested for bone recovery [11], the high levels of expression of the Ca²⁺ selective

393 claudin CLDN2 in the duodenum and of the Ca^{2+} channel TRPV2 in the jejunum, could boost Ca absorption
394 through the paracellular and the transcellular pathways, respectively [37,38]. The absence of variation of
395 CALB1 expression during stages in the intestine confirmed an earlier study [39]. This does not preclude its
396 importance, as demonstrated by the consequences of an experimental suppression of shell formation through
397 expulsion of the egg, which decreases duodenal CALB1 levels and depresses $1.25(\text{OH})_2\text{D}_3$ levels [39].
398 Some genes were also stimulated at 9 to 10 h PO, during the initiation phase of eggshell formation, such as
399 the $\text{Ca}^{2+}/\text{H}^+$ exchange pump ATP2B2 in the duodenum [3] and the anchoring transmembrane proteins TJP3
400 in both segments [40]. During the active phase of eggshell formation at 18 to 19 h PO, the high levels of
401 expression of ATP2B1, ATP2B4, TJP3, CLDN2 and TRPV2 in the duodenum and the jejunum respectively,
402 could contribute to the well documented high Ca absorption at this stage [7,51]. This may be regulated by
403 plasma $1.25(\text{OH})_2\text{D}_3$, which increased at this stage, and be facilitated by the high levels of VDR gene
404 expression [10]. The high expression of CLDN12 in the jejunum and of CLDN2 at 0 to 1 and 18 to 19 h PO
405 in the duodenum suggested that Ca absorption through the paracellular pathway was extended to both
406 intestinal segments responsible for most of the Ca absorbed [37,52,53]. This pathway can be of great
407 importance in hens, as it facilitates the diffusion of large amounts of soluble Ca from the intestinal lumen to
408 the blood, namely when plasma level of ionic Ca^{2+} is low [54], during eggshell formation [1]. We observed
409 a high expression of transporters of the transcellular (TRPM7, TRPC1, TRPV2 at 0 to 1 h PO and ATP2B2
410 at 9 to 10 h PO) and of the paracellular (CLDN12 at 9 to 10 h PO and TJP2) pathways in hens fed coarse
411 particles of CaCO_3 . This strengthens previous observations suggesting that both pathways work
412 cooperatively in the small intestine of the laying hen [8]. Finally, the high expression of genes from both
413 pathways in the duodenum and the jejunum at 18 to 19 h PO, and across stages in hens fed coarse particles
414 of CaCO_3 , highlighted that the gut physiology is adapted to absorb high amounts of Ca during the active
415 phase of eggshell formation. As previously discussed Ca and P homeostasis are strongly and coordinately
416 regulated at all levels, including absorption. The elevated level of plasma $i\text{P}$ at 9 to 10 h PO may be the
417 consequence of dietary P absorption as observed previously [7]. The expressions of intestinal P transporters
418 SLC20A1 and SLC34A2 [41] were high which may facilitate P absorption at 0 to 1 h PO in the jejunum
419 and the duodenum, respectively.

5. Conclusion

To conclude, this study provides a simultaneous overview of gene expression across tissues involved in Ca metabolism during the egg laying cycle, using the well-defined model of Ca supplied as fine or coarse particles of CaCO₃. The results exemplify the mechanisms that satisfy the high demand of Ca by the uterus. They confirm the hypothesis that the high demand for Ca during the active phase of eggshell formation triggers the increase of PTH mRNA and plasma 1.25(OH)₂D₃, which leads to bone resorption. The higher expression of several candidate genes involved in bone turnover provides an explanation to the stronger challenge to Ca homeostasis in hens fed fine rather than coarse particles of CaCO₃. At this stage, the transcellular and the paracellular Ca pathways could drive the intestinal absorption of Ca, and the Ca reabsorption in the kidney through the Ca²⁺ channel TRPV5. Following oviposition, during the subsequent daylight, the demand of Ca by the uterus decreases, while the access to the feed and the expression of candidates of the transcellular and the paracellular Ca pathways permit intestinal Ca absorption. At the same time, increased CALB1 expression in the kidney favors reabsorption. These conditions support bone accretion, when PTH mRNA and 1.25(OH)₂D₃ levels are low. Our data also strongly suggest the involvement of FGF23 in coupling P and Ca metabolisms. This phosphaturic hormone is overexpressed in the medullary bone when plasma *iP* is elevated during eggshell formation. It could induce the subsequent reduction of *iP* and 1.25(OH)₂D₃ levels through decreased expression of the P transporters and increased expression of 24-hydroxylase in the kidney. Therefore, FGF23 could drive vitamin D metabolism in the laying hen, as previously documented in other species. The study confirms that hens fed fine particles of CaCO₃ exhibit a higher challenge to Ca homeostasis, which is marked by the elevated 1.25(OH)₂D₃ levels. However, this does not result in higher intestinal expression of VDR, and several genes of the transcellular and paracellular Ca absorption pathways are downregulated compared to hens fed coarse particles of CaCO₃. To reach similar eggshell quality, hens fed fine particles of CaCO₃ exhibit a higher bone remodeling cycle to meet the Ca demand of the uterus.

445

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571

Table 1: Effect of the post-ovulation stage and the diet on plasma levels of vitamin D metabolites and *iP*, and on mRNA expression of PTH in the parathyroid gland.

	Plasma levels			mRNA expression (AU)
	25(OH)D ₃ (ng/ ml)	1.25(OH) ₂ D ₃ (pg/ml)	<i>iP</i> (mg/l)	PTH
PO stage				
0-1	44.6 ^a ± 2.5	402.9 ^b ± 35.2	104.6 ^b ± 5.97	1.68 ^b ± 0.24
9-10	37.4 ^{ab} ± 2.4	490 ^{ab} ± 32.8	123.2 ^{ab} ± 5.35	1.62 ^b ± 0.21
18-19	36.1 ^b ± 2.4	554.7 ^a ± 33.9	134.3 ^a ± 5.53	2.55 ^a ± 0.21
Diet				
Fine	37.5 ± 1.9	563.9 ^a ± 27.1	118.6 ± 4.53	1.58 ^b ± 0.17
Coarse	41.2 ± 2.0	401.3 ^b ± 28.4	122.7 ± 4.63	2.31 ^a ± 0.19
Source of variation				
PO stage	0.022	0.004	<0.001	0.019
Diet	0.203	<0.001	0.528	0.007
PO stage x Diet	0.125	0.081	0.149	0.266

Values are presented as LSMeans ± SEM with n = 13 to 15 per post-ovulation stage and n = 20 to 22 per diet. PO stage = post-ovulation stage (hour); PTH = parathyroid; Fine = fine particles of calcium carbonate and Coarse = coarse particles of calcium carbonate. ^{a-b} Means with different superscripts for each effect tested in each model are significantly different (*P* < 0.05).

Table 2: Effect of the post-ovulation stage and the diet or the intestinal segment and their interactions on the relative mRNA expression of candidate genes for the transcellular and paracellular pathways involved in Ca absorption.

Genes		TRPV2 [*]	ATP2B2 [*]	CLDN2 [†]	CLDN12 [†]	OCLN [†]	SLC20A1 [‡]	SLC34A2 [‡]
Diet	PO stage							
	0-1	0.75^c	0.41^b	1.38	0.85^b	0.67	0.59	4.72
	9-10	0.86^{bc}	1.89^b	1.47	0.82^b	0.57	0.41	3.50
Fine	18-19	0.90^{bc}	0.91^b	1.56	0.74^b	0.68	0.64	3.84
	0-1	2.24^a	0.32^b	1.43	0.70^b	0.90	0.70	5.16
	9-10	0.87^{bc}	4.84^a	1.00	1.42^a	0.79	0.66	5.26
Coarse	18-19	1.80^{ab}	0.75^b	1.37	0.64^b	0.69	0.73	4.8
Diet	Segment							
	Duodenum	0.66	1.33^b	1.57	0.70	0.52^b	0.33	4.87
	Jejunum	1.02	0.81^b	1.38	0.91	0.75^a	0.77	3.16
Fine	Duodenum	1.17	3.36^a	1.34	0.77	0.80^a	0.43	6.13
	Jejunum	2.11	0.58^b	1.20	1.07	0.79^a	0.97	4.01
Coarse	Segment							
	Duodenum	1.08^{ab}	0.51^b	1.57^a	0.71	0.73	0.36^{cd}	6.63^a
	Jejunum	1.91^a	0.22^b	1.24^{ab}	0.84	0.83	0.93^a	3.25^b
9-10	Duodenum	0.90^b	5.55^a	1.19^b	0.97	0.64	0.22^d	4.75^b
	Jejunum	0.85^b	1.18^b	1.28^{ab}	1.26	0.72	0.85^{ab}	4.01^b
18-19	Duodenum	0.76^b	0.98^b	1.59^a	0.51	0.61	0.56^{bc}	5.13^{ab}
	Jejunum	1.94^a	0.68^b	1.35^{ab}	0.87	0.76	0.82^{ab}	3.51^b
PO stage								
0-1								
9-10								
18-19								
Segment								
Duodenum								
Jejunum								
Diet								
Fine								
Coarse								
Source of variation								
PO stage		0.048	<.0001	0.429	<.0001	0.100	0.159	0.380
Segment		0.003	0.000	0.011	<.0001	0.006	<.0001	<.0001
Diet		0.000	0.038	0.148	0.051	0.001	0.030	0.013
Diet x PO stage		0.011	0.008	0.349	<.0001	0.062	0.579	0.425
Diet x segment		0.125	0.006	0.667	0.356	0.005	0.446	0.572
PO stage x segment		0.031	0.001	0.012	0.259	0.779	0.026	0.013
PO stage x Diet x segment		0.255	0.053	0.951	0.439	0.763	0.136	0.711

^{*} genes of the transcellular pathway ; [†] genes of the paracellular pathway; [‡] P transporters

585 Relative expression values expressed as Arbitrary Units are presented as LSMeans with n = 12 to 16 per diet
586 and PO stage; n = 20 to 22 per diet and intestinal segment; n = 13 to 15 per PO stage and intestinal segment;
587 n = 26 to 30 per post-ovulation stage, n = 40 to 44 per diet and n = 42 per intestinal segment. PO stage =
588 post-ovulation stage (hour); Fine = fine particles of calcium carbonate and Coarse = coarse particles of
589 calcium carbonate.

590 ^{a-d} Means with different superscripts for each effect tested in each model are significantly different ($P <$
591 0.05). TRPV2 = Transient receptor potential cation channel subfamily V member 2; ATP2B2 = ATPase
592 plasma membrane Ca^{2+} transporting 2; CLDN2 = Claudin 2; CLDN12 = Claudin 12; OCLN = Occludin;
593 SLC20A1 = Solute carrier family 20 member 1; SLC34A2 = Solute carrier family 34 member 2.

594

Table 3: Effect of the post-ovulation stage, the diet and the intestinal segment on the relative mRNA expression of candidate genes for the transcellular and paracellular pathways of Ca absorption.

	PO stage			Segment		Diet		Source of variation		
	0-1	9-10	18-19	Duodenum	Jejunum	Fine	Coarse	PO stage	Segment	Diet
TRPM7*	0.78	0.77	0.74	0.77	0.74	0.68 ^b	0.85 ^a	0.806	0.796	0.007
TRPC1*	0.26	0.36	0.26	0.22 ^b	0.36 ^a	0.20 ^b	0.39 ^a	0.057	0.000	<.0001
ATP2B1*	0.70 ^b	0.81 ^{ab}	0.98 ^a	0.93 ^a	0.73 ^b	0.79	0.87	0.018	0.003	0.285
ATP2B4*	0.36 ^b	0.36 ^b	0.44 ^a	0.31 ^b	0.46 ^a	0.38	0.39	0.022	<.0001	0.527
TJP2 [†]	0.50	0.58	0.57	0.50 ^b	0.60 ^a	0.47 ^b	0.64 ^a	0.491	0.015	0.004
TJP3 [†]	0.77 ^b	1.02 ^a	1.03 ^a	0.80 ^b	1.08 ^a	0.92	0.96	0.020	<.0001	0.643
VDR [‡]	1.62 ^b	1.51 ^b	2.55 ^a	1.67 ^b	2.11 ^a	1.65 ^b	2.13 ^a	<.0001	0.010	0.004

* genes of the transcellular pathway ; [†] genes of the paracellular pathway; [‡] Vitamin D receptors

Relative expression values expressed as Arbitrary Units are presented as LSMeans with n = 26 to 30 per stage post-ovulation, n = 40 to 44 per diet and n = 42 per intestinal segment. PO stage = post-ovulation stage (hour); Fine = fine particles of calcium carbonate and Coarse = coarse particles of calcium carbonate. ^{a-b} Means with different superscripts for each effect tested in each model are significantly different ($P < 0.05$). TRPM7 = Transient receptor potential cation channel subfamily M member 7; TRPC1 = Transient receptor potential cation channel subfamily C member 1; ATP2B1 = ATPase plasma membrane Ca²⁺ transporting 1; ATP2B4 = ATPase plasma membrane Ca²⁺ transporting 4; TJP2 = Tight junction protein 2; TJP3 = Tight junction protein 3; VDR = Vitamin D receptor

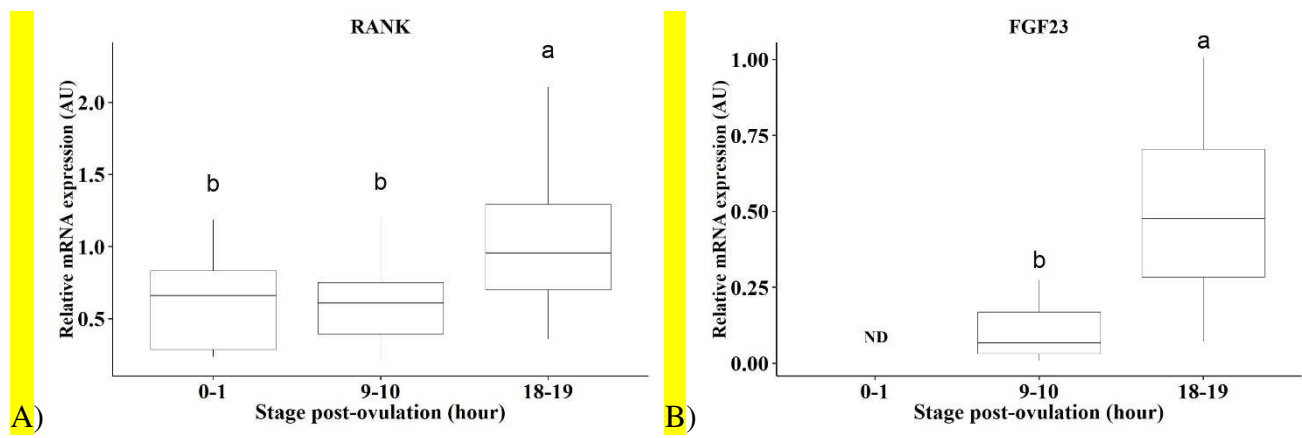


Figure 1

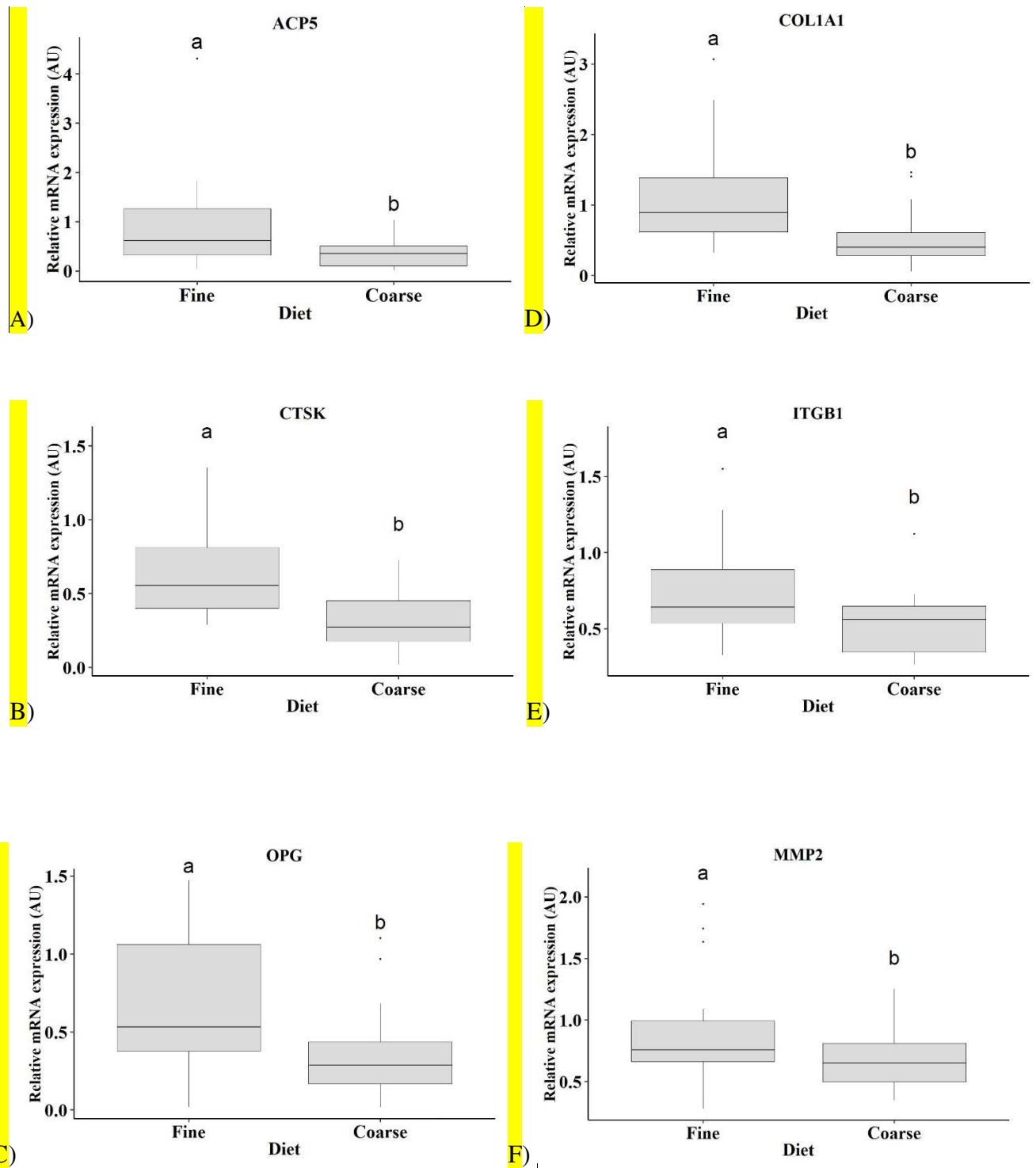


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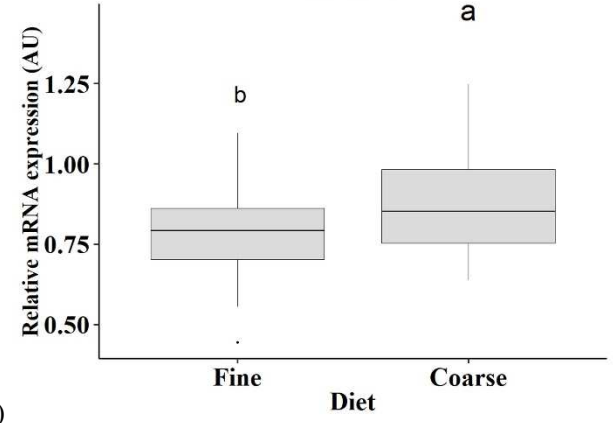
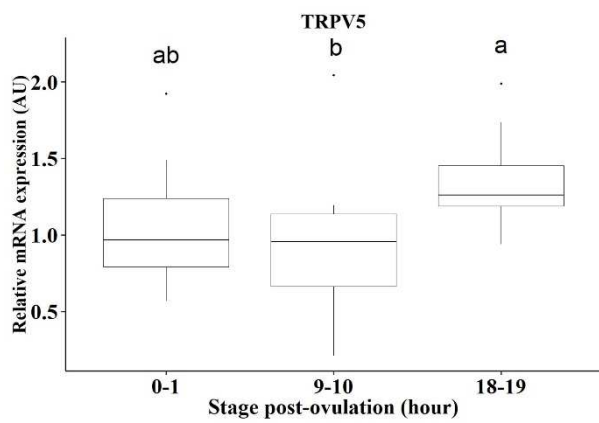
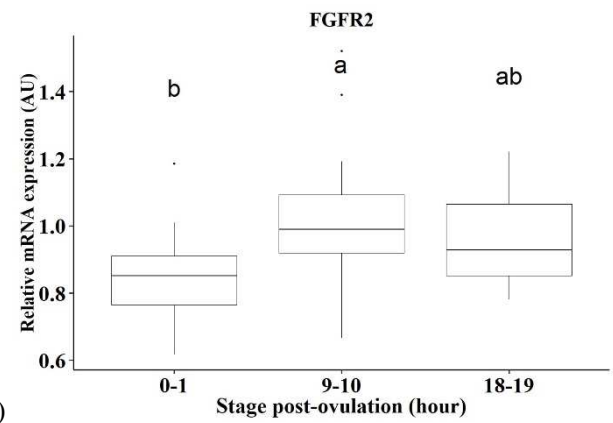
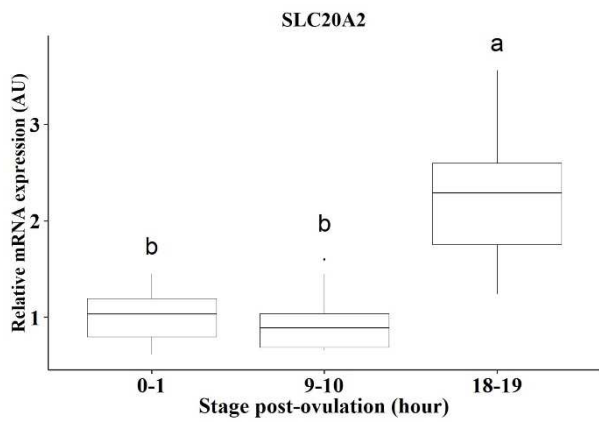
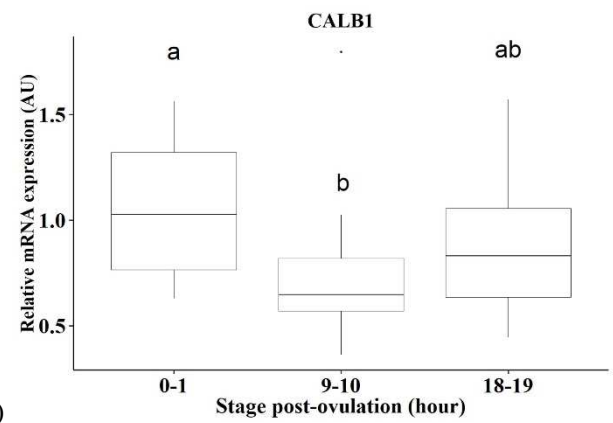
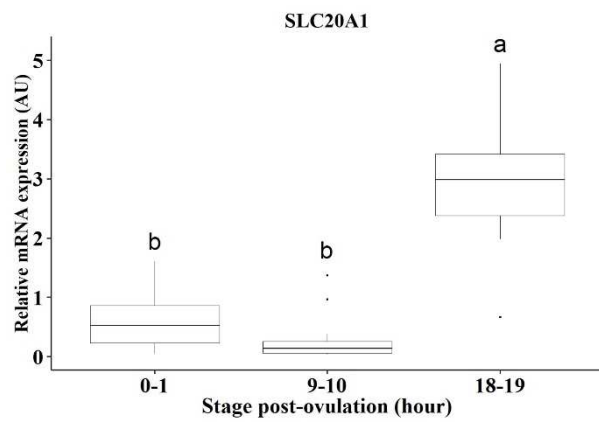


Figure 3

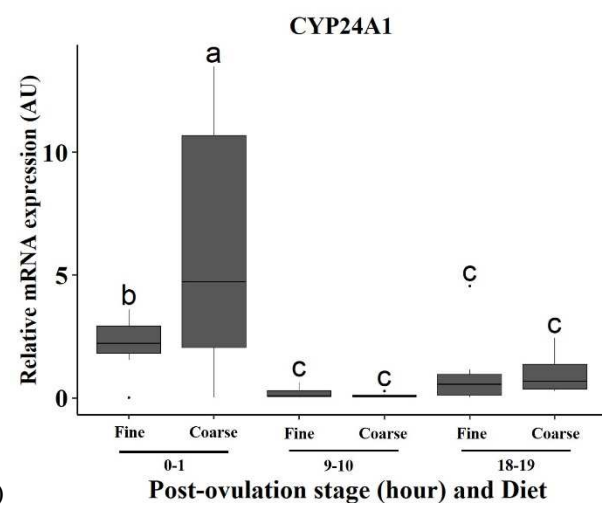
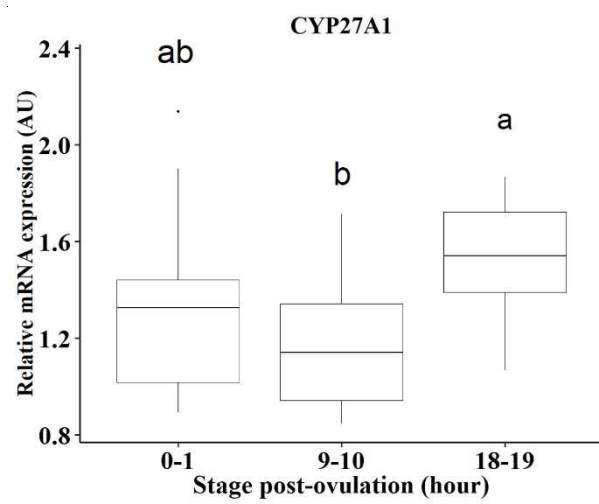


Figure 4

List of Figures

Figure 1: Effect of the post-ovulation stage on the relative mRNA expression of candidate genes in the medullary bone. (A) receptor activator of nuclear factor- κ B (RANK); (B) Fibroblast growth factor 23 (FGF23). Values are expressed as Arbitrary Units (AU) with n= 13 to 15 per post-ovulation stage; for FGF23 n= 13 to 14 per stage post-ovulation. Different letters indicate significant differences ($P < 0.05$).

Figure 2: Effect of the diet on the relative mRNA expression of candidate genes in the medullary bone involved in bone resorption (A, B, C) accretion (D), and their regulation (E,F). (A) acid phosphatase 5, tartrate resistant (ACP5); (B) cathepsin K (CTSK); (C) Osteoprotegerin (OPG); (D) collagen type I alpha 1 chain (COL1A1); (E) integrin subunit beta 1 (ITGB1); (F) matrix metalloproteinase 2 (MMP2). Values are expressed as Arbitrary Units (AU) with n = 20 to 22 per diet. Fine = fine particles of calcium carbonate and Coarse = coarse particles of calcium carbonate. Different letters indicate significant differences ($P < 0.05$).

Figure 3: Effect of the post-ovulation stage (A-E) or the diet (F) on the relative mRNA expression of candidate genes in the kidney. (A) solute carrier family 20 member 1 (SLC20A1); (B) solute carrier family 20 member 2 (SLC20A2); (C) transient receptor potential cation channel subfamily V member 5 (TRPV5); (D) calbindin 1 (CALB1); (E) fibroblast growth factor receptor 2 (FGFR2); (F) ATPase plasma membrane Ca^{2+} transporting 1 (ATP2B1). Values are expressed as Arbitrary Units (AU) with n= 13 to 15 per post-ovulation stage and n = 20 to 22 per diet. Fine = fine particles of calcium carbonate and Coarse = coarse particles of calcium carbonate. Different letters indicate significant differences ($P < 0.05$).

Figure 4: Effect of the post-ovulation stage (A) and of the interaction post-ovulation stage by diet (B) on the relative mRNA expression of candidate genes in the liver (A) and in the kidney (B). (A) cytochrome P450 family 27 subfamily A member 1 (CYP27A1); (B) cytochrome P450 family 24 subfamily A member 1 (CYP24A1); Values are expressed as Arbitrary Units (AU) with n= 13 to 15 per post-ovulation stage. For CYP24A1 n= 6 to 7 per group. Fine = fine particles of calcium carbonate and Coarse = coarse particles of calcium carbonate. Different letters indicate significant differences ($P < 0.05$).