Expression of glucose transporters *SLC2A1*, *SLC2A8*, and *SLC2A12* in different chicken muscles during ontogenesis

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ABSTRACT: Glucose transport into cells is the first limiting step for the regulation of glucose homeostasis. In mammals, it is mediated by a family of facilitative glucose transporters (GLUTs) (encoded by *SLC2A** genes), with a constitutive role (GLUT1), or insulin-sensitive transporters (GLUT4, GLUT8, and GLUT12). Compared to mammals, the chicken shows high levels of glycemia and relative insensitivity to exogenous insulin. To date, only GLUT1, GLUT8, and GLUT12 have been described in chicken skeletal muscles but not fully characterized, whereas GLUT4 was reported as lacking. The aim of the present study was to determine the changes in the expression of the *SLC2A1*, *SLC2A8*, and *SLC2A12* genes, encoding GLUT1, GLUT8, and GLUT12 proteins respectively, during ontogenesis and how the respective expression of these three genes is affected by the muscle type and the nutritional or insulin status of the bird (fed, fasted, or insulin immunoneutralized). *SLC2A1* was mostly expressed in the glycolytic pectoralis major (PM) muscle during embryogenesis and 5 d

posthatching while *SLC2A8* was mainly expressed at hatching. *SLC2A12* expression increased regularly from 12 d in ovo up to 5 d posthatching. In the mixed-type sartorius muscle, the expression of *SLC2A1* and *SLC2A8* remained unchanged, whereas that of *SLC2A12* was gradually increased during early muscle development. The expression of *SLC2A1* and *SLC2A8* was greater in oxidative and oxidoglycolytic muscles than in glycolytic muscles. The expression of *SLC2A12* differed considerably between muscles but not necessarily in relation to muscle contractile or metabolic type. The expression of *SLC2A1*, *SLC2A8*, and *SLC2A12* was reduced by fasting and insulin immunoneutralization in the PM muscle, while in the leg muscles only *SLC2A12* was impaired by insulin immunoneutralization. Our findings clearly indicate differential regulation of the expression of three major GLUTs in skeletal muscles, with some type-related features. They provide new insights to improve the understanding of the fine regulation of glucose utilization in chicken muscles.

Key words: chicken, glucose, GLUT, metabolism, muscle

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INTRODUCTION

Glucose transport into cells is the first limiting step in the regulation of glucose utilization. It

is mediated by a family of facilitative glucose transporters (GLUTs; encoded by *SLC2A** genes; Augustin, 2010; Mueckler and Thorens, 2013). GLUT4 has been one of the most intensively studied in mammals (Huang and Czech, 2007) because it is responsible for the insulin-mediated increase in glucose uptake that occurs in response to elevated plasma glucose and insulin levels in

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the postprandial state. This pathway dominates in skeletal muscle, contributing largely to glucose homeostasis (Scheepers et al., 2004) although recent findings suggest that other isoforms such as GLUT1, GLUT12 (Rogers et al., 2002; Stuart et al., 2009; Purcell et al., 2011), and GLUT8 (de Laat et al., 2015) might also participate in glucose uptake in insulin-sensitive tissues. Phylogeny and synteny analyses confirmed the lack of GLUT4 in chickens (Coudert et al., 2015). Nevertheless, the immunoneutralization of insulin in young chickens induces an increase in plasma glucose levels (Dupont et al., 2008) and glucose transport increases in chicken skeletal muscle in response to exogenous insulin (Tokushima et al., 2005), which suggests that alternative mechanisms of insulin-responsive glucose transport exist in this species. Among the GLUTs reported in chickens, only GLUT1 (Kono et al., 2005), GLUT8 (Seki et al., 2003), and GLUT12 (Coudert et al., 2015) are expressed in skeletal muscles, but the understanding of their contribution and regulation remains incomplete in this species. The first aim of the present study was to compare the expression of these three major GLUTs during the early phase of chick development, e.g., when intense changes in energy requirements and supply occur. The second aim was to determine the effects of the muscle type and function (i.e., glycolytic vs. oxidative; fast vs. slow contraction) as well as the nutritional or insulin status of the animal on their pattern of expression.

MATERIALS AND METHODS

Animals and Muscle Tissue Collection

All investigators were certified by the French government to carry out animal experiments and this study was conducted under authorizations 37–105 (delivered to A. Collin) and 37–085 (delivered to J. Dupont) from the French Ministry of Agriculture. All procedures were approved by the French Agricultural Agency and the Scientific Research Agency and were conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

For the study of expression of *SLC2A1*, *SLC2A8*, and *SLC2A12* during ontogenesis, 10 broiler embryos and chicks originating from fertilized eggs incubated conventionally at 37.8 °C and 56% relative humidity in the experimental hatchery of PEAT experimental unit (INRA, Centre Val de Loire, Nouzilly, France) were killed by cervical dislocation and muscle tissues were immediately

sampled, snap-frozen, and stored at –80 °C until use. Both pectoralis major (PM) and sartorius (SART) muscles were collected at different stages: 12 (E12) and 19 days (E19) in ovo, hatching (D0) and 5 d posthatching (D5, fed chicks).

The expression of *SLC2A1*, *SLC2A8*, and *SLC2A12* in relation to muscle metabolic and contractile type was evaluated on tissues collected from six fed 9-wk-old broiler chickens killed by cervical dislocation. Six muscle types diverging for contractile and metabolic type according to classification of Barnard et al. (1982) were collected: PM, posterior latissimus dorsi (PLD), iliotibialis (ILIO), SART, adductor profundus (ADP), and anterior latissimus dorsi (ALD).

The effects of nutritional and/or insulin status on *SLC2A1*, *SLC2A8*, and *SLC2A12* gene expression in skeletal muscles were analyzed using an insulin immunoneutralization model previously described and characterized by Dupont et al. (2008). As described, male broiler chickens were housed in an environment-controlled room and they were fed ad libitum with a conventional balanced diet based on corn, wheat, peas, soybean meal, corn gluten, and rapeseed oil. Three groups of seven chickens of similar body weights were constituted at 16–17 days of age. The fed immunoneutralized group received three i.v. injections of anti-porcine insulin guinea pig serum (1.5 mL/kg, PromoCell, Heidelberg, Germany) at 0, 2, and 4 h. Guinea pig serum was used as the vehicle for the anti-porcine insulin. The fed control group received three i.v. injections delivering only normal guinea pig serum (1.5 mL/kg) at 0, 2, and 4 h. The last group, which served as control to measure the extent of changes induced by insulin immunoneutralization, was fasted for 5 h and given three i.v. injections delivering only normal guinea pig serum (1.5 mL/kg) at 0, 2, and 4 h. Broiler chickens were killed by cervical dislocation 5 h post-first-injection and the fast-twitch glycolytic PM muscle and leg muscles were sampled.

RNA Isolation and RT-qPCR

Total RNA was extracted from 100 mg tissue samples using RNA Now (Biogentec, Seabrook, TX, USA) according to the manufacturer's recommendations. After RNase-Free DNase treatment (Ambion, Clinisciences, Montrouge, France), RNA was reverse-transcribed using Super Script II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with Random Primers (Promega, Charbonnières-les-Bains, France). The

sequences of forward and reverse primers used to amplify chicken *SLC2A1*, *SLC2A8*, *SLC2A12*, β -hydroxyacyl-CoA dehydrogenase (*HAD*), citrate synthase (*CS*), lactate dehydrogenase (*LDH*), and housekeeping genes *β -actin*, *EIF3F*, Cytochrome b (*Cyt b*), and TATA Box Binding Protein (*TBP*) were specifically designed or reproduced from the literature (Dupont et al., 2008; Joubert et al., 2010; Coudert et al., 2015). They are presented in Table 1. The cDNA samples were amplified in triplicate by real-time PCR using Sybr Green I Master kit (Roche, Mannheim, Germany) and the LightCycler 480 II apparatus (Roche, Meylan, France). Gene expression levels were estimated on the basis of PCR efficiency and threshold cycle (Ct) deviation of an unknown sample vs. a control, as previously described (Pfaffl, 2001). Their expression was normalized with *β -actin*, *EIF3F*, *Cyt b*, or *TBP* housekeeping genes according to the experiment and/or the muscle. Data sets were normalized by the housekeeping gene that was the more stable between treatments.

Western Blotting

For myosin analysis, muscles were homogenized in myosin extraction buffer which is composed of NaCl (300 mM), NaH_2PO_4 (0.1 M), Na_2HPO_4 (0.05 M), $\text{Na}_4\text{P}_2\text{O}_7$ (0.01 M), MgCl_2 (1 mM), EDTA (10 mM), and DTT (1 mM), pH 6.5, and complete protease inhibitor cocktail (Roche, Mannheim, Germany) as previously described by Cosper and Leinwand (2012). Lysates were centrifuged at 31,000 g for 45 min at 10 °C. Solubilized lysates (5 μ g of proteins) were subjected to SDS-PAGE and Western blotting using monoclonal antibodies directed against major avian fast and slow Myosin Heavy Chain isoforms

(**MyHC**), i.e., the adult, neonatal and embryonic fast MyHC and the slow MyHC Heavy Chain isoforms. The F59 and S46 antibodies, developed by F.E. Stockdale, and the 2E9 and B103 antibodies, developed by E. Bandman, were obtained from the Developmental Studies Hybridoma Bank, created by the NIHCD of the NIH and maintained at the University of Iowa (Department of Biology, Iowa City, IA 52242. F59, 2E9, B103, and S46 are respectively directed against the fast adult MyHC, the fast neonatal MyHC, the neonatal and embryonic MyHC and against the slow 1, 2, 3 MyHC). The anti-vinculin antibody was used as a loading control (Sigma, Saint Louis, USA). After washing, membranes were incubated with a DyLight 680 conjugate antibody. Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Inc. Biotechnology, Lincoln, NE) and quantified by Odyssey infrared imaging system software (Application software, version 1.2).

Statistical Methods

Values are presented as means $\pm$ SEM. Data were analyzed using Statview software, version 5 (SAS Institute, Cary, NC, USA). Data were subjected to a one-way analysis of variance to detect significant intergroup differences. For all studies, the analysis has been realized with one factor: age (in ontogenesis experiment with four levels), muscle type (in muscle metabolic and contractile type experiment with 6 levels), or treatment (in the effects of nutritional and/or insulin status experiments with three levels). Comparisons of means for each significant effect were performed using the Tukey–Kramer test. The accepted type I error was set at 5%.

Table 1. Primers used for real-time PCR analysis

Genes ^a	Forward	Reverse	Accession numbers
β -actin	CTG GCA CCT AGC ACA ATG A	CTG CTT GCT GAT CCA CAT CT	NM_205518
Cytb	CGG ACG AGG CCT ATA CTA CG	GGG AGA ACA TAG CCC ACA AA	AB044986.1
CS	AGG GAT TTC ATC TGG AAC ACA CT	CAC CGT GTA GTA CTT CAT CTC CT	XM_015300289.1
EIF3F	GCA CAA CGA GTC CGA GGA T	CTT CCC GGC TGT AGT ACT CG	XM_421624
HAD	ATC CTT GCA AAT CAC GCA GTT	AAT GGA GGC CAC CAA ATC G	NM_001277897.1
LDH	TTA ACT TGG TCC AAC GCA ACG TCA AT	TCC ACT GGG TTT GAG ACA ATC AG	XM_015864180
SLC2A1	ACA ACA CCG GCG TCA TCA A	TTG ACA TCA GCA TGG AGT TAC G	NM_205209.1
SLC2A8	CTG GAG GAA TAC TGG GAG GC	CAC CAC CAT CAA CTG GAC AA	NM_204375.1
SLC2A12	AGA GAG TGG GGA GGT TCC C	TCA GGA CGA GCC AAG ACA	XM_419733.4
TBP	GCG TTT TGC TGC TGT TAT TAT GAG	TCC TTG CTG CCA GTC TGG AC	NM_205103.1

^aGenes: *Cytb*: cytochrome b; *CS*: citrate synthase; *EIF3F*: eukaryotic translation initiation factor 3 subunit F; *HAD*: β -hydroxyacyl-CoA dehydrogenase; *LDH*: lactate dehydrogenase; *SLC2A1*: solute carrier family 2 member 1; *SLC2A8*: solute carrier family 2 member 8; *SLC2A12*: solute carrier family 2 member 12; *TBP*: TATA box binding protein.

RESULTS

Changes in GLUT Expression During Ontogenesis in the PM and SART Skeletal Muscles

The *SLC2A1*, *SLC2A8*, and *SLC2A12* genes were all expressed during embryonic development in the two avian skeletal muscles studied. However, their respective expression showed different patterns during development and were either similar or different in the breast and leg muscles (Figure 1). The pattern of expression of *SLC2A1* and *SLC2A8* during ontogenesis

varied according to the muscle. In the PM muscle, *SLC2A1* expression decreased between E12 and E19 then remained stable until hatching before increasing posthatching (Figure 1A, $P < 0.0001$), while *SLC2A8* expression showed a significant peak of expression at hatching (Figure 1B, $P < 0.0001$). The expression of these two genes remained unchanged from E12 to D5 in the SART muscle (Figures 1D and E). *SLC2A12* expression increased between E12 and E19 then remained stable in both muscles until hatching before increasing at D5 of age (Figures 1C and F, $P < 0.0001$ and $P = 0.001$, respectively).

Figure 1. Relative mRNA abundance of *SLC2A1* (A, D), *SLC2A8* (B, E), and *SLC2A12* (C, F) during early ontogenesis in pectoralis major and sartorius skeletal muscles. Relative expression of genes (corrected for Cytochrome b (*Cyt b*) and TATA Box Binding Protein (*TBP*) mRNA in the pectoralis major and sartorius muscle, respectively) was determined by real-time PCR during embryogenesis (E12 and E19), at hatching (D0) and 5 days posthatching (D5). Data are expressed as means $\pm$ SEM ($n = 10$). Mean values without a common letter (a, b, c) differ between groups ($P \leq 0.05$). a.u.: arbitrary unit.

Levels of Expression of Genes Encoding GLUTs GLUT1, GLUT8, and GLUT12 in Relation to Contractile and Metabolic-Type Skeletal Muscle

A wide range of muscles collected from 9-wk-old chickens were selected to evaluate how the levels of the *SLC2A1*, *SLC2A8*, and *SLC2A12* mRNA expression vary in relation to muscle typology and metabolism. These muscles are located in the thigh (ILIO, SART, ADP), breast (PM), or back (ALD, PLD), and are involved in different functions such as locomotion (for leg muscles), posture, and/or wing flapping (for back and pectoral muscles). The selected muscles potentially covered a large range of contractile and metabolic types but their precise characteristics were determined by focusing on key enzymes of the main energy-supplying pathways, like CS (Krebs cycle oxidative potential), HAD (β -oxidation), LDH (glycolytic capacity) (metabolic properties), and MyHC isoforms (contractile properties). The expression of genes coding the *HAD*, *CS*, and *LDH* enzymes were measured in all studied muscles (Figure 2). The gene encoding *LDH* was the most expressed in the PM and PLD muscles, confirming their predominant glycolytic metabolism, compared to the other muscles. The lowest level of *LDH* expression was observed in the ALD muscle. The expression of *HAD* was the lowest in the PM, PLD, and ILIO muscles and the highest in the ADP muscle, SART, and ALD muscles being intermediate for this trait. Similar trends were observed for the gene encoding *CS*, except for the ALD that showed *CS* expression level similar to those observed in PM, PLD, and ILIO muscles. Based on the calculation of the ratio LDH/CS (glycolysis with pyruvate reduction/aerobic potential of the Krebs cycle, Hochachka et al., 1983), muscles can be classified according to their glycolytic capacity as follow: PM > PLD > ILIO > SART > ADP > ALD (data not shown).

The contractile type of muscles was defined by analyzing their content in slow and fast MyHC isoforms by Western Blot (Figure 3). As expected, the adult, neonatal, and/or embryonic forms of the fast MyHC were expressed in fast-twitch and mixed muscles (PM, PLD, ILIO, SART, ADP), but almost not in the slow-twitch ALD. The adult form was expressed in all fast-twitch muscles, whereas the neonatal form was only expressed in the ILIO muscle. Compared to other fast-twitch or mixed muscles, the PM did not express the embryonic and neonatal forms of the MyHC. The PLD, SART, and ADP muscles expressed the embryonic MyHC isoform but we cannot conclude whether the ILIO

muscle expresses the embryonic form because the antibody directed against this isoform also recognizes the neonatal form widely expressed in this muscle. The ALD muscle expressed almost exclusively the slow MyHC isoforms. The PLD and SART are mixed muscles since they expressed both slow and fast MyHC isoforms.

The mRNA expression of *SLC2A1* appeared to be clearly related to the metabolic characteristics of muscles ($P < 0.0001$, Figure 4A). It was the lowest in the most glycolytic muscles (PM, PLD, and ILIO) and the greatest in the oxidative muscle (ALD). On average, the expression of *SLC2A1* was about three times greater in oxidative than in glycolytic muscles. *SLC2A1* expression was intermediate in the oxidoglycolytic SART and ADP muscles. The mRNA expression of *SLC2A8* was the greatest in the oxidative ALD muscle and the lowest in the fast-twitch glycolytic PM muscle, all other fast-twitch or mixed-type oxidoglycolytic muscles showing intermediate expression ($P < 0.0001$, Figure 4B). The mRNA expression of *SLC2A12* varied considerably according to the muscle type but not necessarily in relation to the contractile and metabolic properties ($P < 0.0001$, Figure 4C). *SLC2A12* was 5 to 10 times more expressed in the breast PM muscle than in the back-mixed glycolytic PLD and thigh slow-twitch ADP muscles. The other muscles under study showed intermediate expression levels for this gene, however closer to levels observed in PM at least for ILIO and SART than to levels measured in both PLD and ADP muscles.

Effects of Nutritional Status and Insulin Immunoneutralization on GLUT Expression in Breast and Leg Skeletal Muscles

The effects of fasting and insulin-deprivation on the mRNA expression of the *SLC2A1*, *SLC2A8*, and *SLC2A12* genes were studied in breast and leg skeletal muscles using a chicken model previously described and characterized by Dupont et al. (2008). The expression of the *SLC2A1*, *SLC2A8*, and *SLC2A12* genes was measured 5 h postinjection or postfasting (Figure 5). In the PM, both fasting and insulin immunoneutralization lowered the expression of *SLC2A1*, *SLC2A8*, and *SLC2A12* (by 30 to 50% according to the transporter compared to the levels observed in fed animals). In the leg muscles, only insulin immunoneutralization significantly decreased the expression of *SLC2A12*, the mRNA expression of *SLC2A12* after fasting being intermediate between those observed in fed and insulin-immunoneutralized chickens. Levels of

Figure 2. Relative mRNA abundance of lactate dehydrogenase (*LDH* in A), β -hydroxyacyl-CoA dehydrogenase (*HAD* in B) and citrate synthase (*CS* in C) in six chicken skeletal muscles. Relative expression of genes (corrected for *EIF3F* mRNA) was determined by real-time PCR in six muscles sampled from 9-wk-old chickens. Data are expressed as means $\pm$ SEM ($n = 5$). Mean values without a common letter (a, b, c) differ between groups ($P \leq 0.05$). a.u.: arbitrary unit; ADP = adductor profundus; ALD = anterior latissimus dorsi; ILIO = iliotibialis; PM = pectoralis major; PLD = posterior latissimus dorsi; SART = sartorius.

Figure 3. Western Blots illustration of the myosin heavy chain contents in six chicken skeletal muscles. Membranes were incubated with monoclonal antibodies F59, 2E9, B103, S46 purchased by the Developmental Studies Hybridoma Bank and directed respectively against fast adult (F59), fast embryonic and neonatal (B103), fast neonatal (2E9), and slow 1, 2, and 3 (S46) myosin heavy chain (MyHC) isoforms. The anti-vinculin antibody was used as a loading control. ADP = adductor profundus; ALD = anterior latissimus dorsi; ILIO = iliotibialis; PM = pectoralis major; PLD = posterior latissimus dorsi; SART = sartorius.

mRNA expression of *SLC2A1* and *SLC2A8* in leg were insensitive to the nutritional and insulin status.

DISCUSSION

As in mammals, the striated skeletal muscle in birds is a major glucose-utilizing tissue. However, chicken glucose metabolism presents some particular features such as high glycemia, a relative insensitivity to exogenous insulin (Simon, 1989; Akiba et al., 1999; Braun and Sweazea, 2008) and the absence of GLUT4 (Carver et al., 2001; Seki et al., 2003; Coudert et al., 2015). In mammals, the tissue expression of each GLUT varies throughout development (Santalucia et al., 1992; Macheda et al., 2002). At the mRNA and/or protein level, GLUT1 has been shown to be the primary GLUT expressed in the rat heart and skeletal muscle, with levels declining after birth, while GLUT4 expression increases during late gestation (Santalucia et al., 1992). The expression of GLUT12 also increases during late gestation in rat fetal insulin-sensitive tissues, including skeletal muscles (Macheda et al.,

2002). It was suggested that GLUT12 could act as a “substitute” for GLUT4 in fetal tissues and allow the increased uptake of the glucose into the cells in the presence of insulin. In chickens, the skeletal muscle expression of each GLUT studied here also varied during development. Indeed, *SLC2A1* mRNA expression was high during mid-embryogenesis (E12) then was lower at E19 before increasing to reach similar levels to those observed at E12 after hatch. Carver et al. (2001) similarly reported that the mRNA level of *SLC2A1* decreased progressively from day 10 to day 19 during chick embryogenesis. Interestingly, our result pointed out that this trend is only observed in the glycolytic (PM) muscle but not in the mixed oxidoglycolytic SART muscle, where the mRNA expression of the *SLC2A1* gene did not change during muscle ontogenesis. Originally, the present study demonstrated that the expression of *SLC2A12* increased continuously during embryogenesis and after hatching, this trend being observed whatever the muscle type.

The mRNA expression *SLC2A8* showed a peak at hatching, at least in the fast-twitch glycolytic PM muscle, suggesting a particular role for this transporter at this stage of development which represents a high-consuming energy period for chicks to achieve pipping and hatch. Unlike other transporters such as GLUT1 that resides in the cell or GLUT4 that is shuttled to the plasma membrane, GLUT8 appears to be mainly found intracellularly (Augustin et al., 2005). GLUT8 may play a role in the hexose transport, facilitating the recycling of sugars during processes requiring an abundance of energy (Schmidt et al., 2009; Adastra et al., 2012). Because of the limited carbohydrate content in the egg, maintenance of glucose homeostasis during late-term embryonic development depends on the amount of glucose kept in reserve, primarily as glycogen in the liver, and glucose generated by gluconeogenesis from proteins (proteolysis and amino acid metabolism) mobilized first from albumen and then from muscles. At the end of incubation, the glycerol issued from the hepatic metabolism of triglycerides and originating from egg yolk is the predominant substrate for the synthesis of glycogen in the liver and muscle (Sunny and Bequette, 2011). Between 15 and 19 d of incubation, active metabolism takes place in the liver to transfer the glucose and fatty acids to the cervical pipping muscle that is progressively enriched in glucose, glycogen, and protein to sustain hatching activities (Pulikanti et al., 2010). Insufficient energy supply from glycogen or albumen means that the embryo mobilizes more muscle protein for

Figure 4. Relative mRNA abundance of *SLC2A1* (A), *SLC2A8* (B), and *SLC2A12* (C) in six chicken skeletal muscles. Relative expression of genes (corrected for *EIF3F* mRNA) was determined by real-time PCR in six muscles sampled from 9-wk-old chickens. Data are expressed as means $\pm$ SEM ($n = 6$). Mean values without a common letter (a, b, c) differ between groups ($P \leq 0.05$). a.u.: arbitrary unit; ADP = adductor profundus; ALD = anterior latissimus dorsi; ILIO = iliobtibialis; PM = pectoralis major; PLD = posterior latissimus dorsi; SART = sartorius.

gluconeogenesis, which can explain why during late embryogenesis the PM muscle yield significantly decreased (Guerneq et al., 2003). Moreover, the increased expression of the atrophy-related genes, atrogin-1 and MuRF1, observed between E18 and hatching (Everaert et al., 2013) is consistent with the negative allometric development of the PM muscle. Chicken GLUT8 could therefore act as a lysosomal hexose transporter, as already suggested in mammals (Adastra et al., 2012), to provide sufficient carbohydrate supply to the chick's skeletal muscle during the hatching process that requires a high level of energy. The rapid decrease in *SLC2A8* expression observed in PM muscle after hatching supports this hypothesis. In contrast, the expression of both *SLC2A1* and *SLC2A12* clearly increased after hatching at least in the PM muscle. During this period, there is a transition of nutrient supply in newly hatched chicks from residual yolk to exogenous feeding, corresponding to chick's ingestion of carbohydrate-rich diets. Glucose becomes the main energy source for chicks that need to reconstitute their glycogen store and initiate their growth. In the absence of GLUT4 (Coudert et al., 2015), it is likely that the chicken GLUT12 may have a major

role in providing energy to muscle cells. Indeed, a compensation process has already been described in genetically altered mice lacking GLUT4 in which an increase in the expression of GLUT12 was reported (Aerni-Flessner et al., 2012). Only GLUT4 shows increased expression after birth in mammalian skeletal muscle since GLUT1 is mainly expressed in fetal muscle but progressively decreases after birth (Santalucia et al., 1992). In mammals, the only condition in which expression of GLUT1 can be induced again in skeletal muscle fibers is the muscle regeneration process (Gaster et al., 2000a). The reason why mRNA expression of chicken GLUT1 increased in the PM muscle after hatching remains to be investigated, but these results may highlight a specific role of this transporter in the chicken different from that described in mammals.

Our study clearly indicates that the regulation of GLUT mRNA expression during early development (ontogenesis) and according to the physiological status of chickens is muscle type-dependent. The expression of all GLUTs (1, 8, 12) was regulated during ontogenesis and by fasting or immunoneutralization in the PM muscle, whereas GLUT1 and GLUT8 expressions were not affected by any of

Pectoralis major

Figure 5. Relative mRNA expression of *SLC2A1* (A, D), *SLC2A8* (B, E), and *SLC2A12* (C, F) in pectoral and leg muscles from fasted, insulin immunoneutralized and fed chickens. Relative expression of genes (corrected for Cytochrome b (*Cyt b*) and β -actin mRNA in pectoral and leg muscles, respectively) was determined by real-time PCR in muscles sampled from 17-day-old chickens. Chickens were either fed (fed), fasted for 5 h (fasted) or insulin immunoneutralized by three consecutive injections of an anti-insulin antibody carried out in fed chickens (Ins immunoneutralized group). Data are expressed as means $\pm$ SEM ($n = 7$). Mean values without a common letter (a, b) differ between groups ($P \leq 0.05$). a.u.: arbitrary unit.

these treatments in the leg muscles. Such tissue-specific physiological regulation (fasting, diet, insulin, etc.) has previously been reported but with some variations due to the muscle features linked to the species. Indeed, changes in GLUT1 and GLUT4 mRNA expressions were observed in red but not in white muscles in fish (Capilla et al., 2002), whereas changes in GLUT4 mRNA expression were reported in white but not in red muscles in rats (Camps et al., 1992). In comparison with GLUT1 and 8, the mRNA expression of GLUT12 was regulated during ontogenesis or in relation to nutritional or hormonal (insulin) status in both breast and leg muscles. The PM muscle has been described as a pure glycolytic white muscle that is composed exclusively of type IIB fibers, poor in mitochondria content and enzymes involved in oxidative pathways, like CS and HAD, but rich in glycogen and enzymes involved in glycolytic activity, like

LDH (Rémignon et al., 1994). This muscle mainly depends on glucose provision by glucose transport via GLUTs after a meal or by glycogenolysis in the fasted state for functioning. Leg muscles, such as the SART muscle, are generally described as oxidoglycolytic type muscles and are composed of type IIB, IIA with or without I fibers, these two last type of fibers being richer in mitochondria and oxidative enzymes but poorer in glycolytic enzymes. This typology makes these muscles more adapted to oxidize glucose and fatty acids to produce ATP than glycolytic muscles. The greater sensitivity of GLUTs to nutritional, hormonal, and more generally physiological states during ontogenesis of the breast PM muscle is therefore consistent with its predominant glucose-dependence to optimize glucose uptake and stimulate glycogen storage compared to leg muscles.

The impact of muscle type on GLUT expression was further explored in a wide panel of muscle types. The three genes encoding GLUTs (GLUT1, GLUT8, GLUT12) were all expressed in the different types of muscle. However, there was a gradient of expression for both *SLC2A1* and *SLC2A8* that were more highly expressed in slow oxidative than in fast glycolytic muscles, oxidoglycolytic muscles generally showing intermediate expression levels. These results are consistent with previous findings obtained in human muscle showing that GLUT1 is predominantly expressed in type I slow oxidative fibers (Stuart et al., 2006). Regarding GLUT12 (as for GLUT4 in mammals), the link between its mRNA expression and muscle fiber typology depends on the species. Indeed, GLUT4 and GLUT12 are predominantly expressed in type I slow oxidative fibers in human muscle (Gaster et al., 2000b; Stuart et al., 2006). Expression of GLUT4 was also found to be greater in oxidative than in glycolytic muscles in the rodent (James et al., 1989; Henriksen et al., 1990; Marette et al., 1992) but not in bovine and goat, in which GLUT4 shows higher expressions in glycolytic and oxidoglycolytic than in oxidative muscles (Hocquette et al., 1995). Our study indicated a more complex relationship between GLUT12 expression and muscle typology in the chicken. For instance, expression of *SLC2A12* was five times greater in the PM than in the PLD muscles, which are both glycolytic muscles, despite differing in MyHC isoform expression (fast-twitch and mixed muscles, respectively). The expression of *SLC2A12* was almost eight times greater in SART and ILIO than in ADP muscles, all classified as oxidoglycolytic muscles.

In avian species, at least three slow and seven fast myosin heavy chain genes have been identified (Bandam and Rosser, 2000), which cannot be unambiguously assigned to the different subtypes of slow or fast fibers. Depending on the muscle, fast fibers of adult muscles can express one of the embryonic, neonatal, or adult fast myosin heavy chain genes. In fact, the persistence of embryonic or neonatal isoforms and the expansion of adult isoforms during late development differ according to muscles even belonging to the same type according to the classical nomenclature (Barnard et al., 1982). Indeed, the embryonic and neonatal myosin forms have been reported to be replaced by the adult form more rapidly in the PM than in the PLD muscle in the chicken in which both embryonic and neonatal myosin forms were still expressed up to at least 60 d posthatching (Gauthier and Orfanos, 1993). It has been shown that differences in contractile

velocity even exist within avian fast myosin heavy chain isoforms, the embryonic form having the lowest velocity and the adult the highest (Lowey et al., 1993a, Reiser et al., 1996). The diversity of myosin isoforms in developing and adult muscle fibers provides the continuum of shortening velocities needed to meet changing functional demands (Lowey et al., 1993a). The mRNA expression of GLUT12 in the chicken could therefore be closely related to the myosin expression pattern within muscle fibers that itself is adapted to several specialized functions including locomotion, maintenance, and posture. Among fast-twitch glycolytic muscles, the very high expression of GLUT12 in the PM muscle is consistent with its high velocity rate, which requires rapid energy provision, and its primordial role in wing flapping, thermogenesis, and protein reserve functions compared to the back PLD muscle that is mostly solicited for wing flapping, a function barely required in modern commercial broiler lines. It could be also hypothesized from our results that according to its low GLUT12 expression the ADP would be less often solicited than other oxidoglycolytic leg muscles studied here (SART, ILIO).

This study showed for the first time in the chicken the differential regulation of the expression of three major GLUTs during early muscle development and in response to nutritional or insulin status. It also highlighted a specificity of GLUT expression and regulation according to the muscle type or function, with some specific features related to the species, thus providing new insights to improve understanding regarding the role of GLUT transporters in the fine regulation of glucose utilization in the chicken muscle.

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