Expression and activity of the 5′-adenosine monophosphate-activated protein kinase pathway in selected tissues during chicken embryonic development[1]

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ABSTRACT The 5′-adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved serine-threonine protein kinase and a key part of a kinase-signaling cascade that senses cellular energy status (adenosine monophosphate:adenosine triphosphate ratio) and acts to maintain energy homeostasis by coordinately regulating energy-consuming and energy-generating metabolic pathways. The objective of this study was to investigate aspects of the AMPK pathway in the liver, brain, breast muscle, and heart from d 12 of incubation through hatch in chickens. We first determined mRNA and protein expression profiles for a major upstream AMPK kinase, LKB1, which is known to activate (phosphorylate) AMPK in response to increases in the adenosine monophosphate:adenosine triphosphate ratio. Expression of LKB1 protein was greatest in the brain, which demonstrated tissue-specific patterns for phosphorylation. Next, AMPK subunit mRNA and protein expression profiles were determined. Significant changes in AMPK subunit mRNA expression occurred in all tissues from d 12 of incubation to hatch. Differences in the levels of active (phosphorylated) AMPK as well as α and β subunit proteins were observed in all 4 tissues during embryonic development. Finally, we determined the protein level and phosphorylation status of an important downstream target for AMPK, acetyl-coenzyme A carboxylase. The expression of acetyl-coenzyme A carboxylase and phosphorylated acetyl-coenzyme A was greater in the brain than the liver, but was undetectable by Western blotting in the breast muscle and heart throughout the period of study. Together, our results are the first to demonstrate the expression and activity of the AMPK pathway in key tissues during the transition from embryonic to posthatch development in chickens.

Key words: acetyl-coenzyme A carboxylase, 5′-adenosine monophosphate-activated protein kinase, LKB1, chicken embryo, energy balance

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INTRODUCTION

Cellular energy levels must be tightly regulated to meet the metabolic demands of growth and development. This is especially true during embryonic development, when nutrient demand is great and when an insufficient supply of energy can lead to developmental defects, culminating in embryo mortality. It has been proposed that every organism, from a single-cell prokaryote to a complex multicellular eukaryote, has an inherent ability to sense available energy supply and adjust internal metabolic pathway flux accordingly to maintain homeostasis (Lindsley and Rutter, 2004). How energy-sensing and metabolic activity are coordinately regulated and the mechanisms used for achieving energy balance are beginning to be elucidated at the whole-organism, cellular, and molecular levels. For example, higher multicellular organisms have developed complex integrated endocrine, neuroendocrine, and nutrient-signaling networks that interact with intracellular signal transduction pathways to bring about changes in metabolism, thus linking the sensing of energy with cellular processes involved in growth and development (Lindsley and Rutter, 2004). The involvement of a highly conserved energy sensor and metabolic transducer, 5′-adenosine monophosphate-activated protein kinase (**AMPK**), in regulating both cellular and whole-body energy balance in response to metabolic stresses that inhibit adenosine triphosphate (**ATP**) production or accelerate ATP consumption has been a focus of much investigation (Hardie et al., 2006).

The AMPK enzyme complex contains 1 catalytic (α) and 2 regulatory (β and γ) subunit proteins, each of

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which has multiple isoforms encoded by separate genes (Mitchelhill et al., 1994; Stapleton et al., 1996). As a serine-threonine kinase, AMPK phosphorylates a wide variety of downstream target proteins (e.g., enzymes, regulatory proteins, transcription factors, and coactivators) involved in controlling different metabolic and cellular functions. 5′-Adenosine monophosphate-activated protein kinase is activated by phosphorylation of a specific threonine residue (**T172**) located in the activation loop of the catalytic domain of α subunit proteins by an upstream AMPK kinase such as the tumor suppressor protein LKB1 (also referred to as serine-threonine kinase 11, or STK11) complexed with its coactivating proteins STE20-related adaptor (**STRAD**) and mouse protein 25 (**MO25**) or by the calcium-calmodulin-dependent protein kinase kinase (Hardie, 2007). Thus, AMPK can exist in both active (phosphorylated) and inactive (dephosphorylated) states. Activation of AMPK occurs in response to metabolic stresses, which cause a depletion of cellular ATP and a concomitant rise in adenosine monophosphate (Kahn et al., 2005). The activity of AMPK can also be modulated by various hormones, nutrients, and cytokines (Kola et al., 2006; Xue and Kahn, 2006; Hardie 2007). Activated AMPK promotes cellular energy conservation by increasing the activity of ATP-producing catabolic pathways such as glycolysis and fatty acid oxidation while reducing energy expenditure by inhibiting ATP-consuming anabolic activities such as carbohydrate, lipid, and protein synthesis (Hardie et al., 2006). In fact, it has been suggested that the AMPK pathway arose very early in eukaryotic evolution as a protective mechanism to allow organisms to adapt quickly and survive periods of energy deprivation (Kahn et al., 2005; Hardie et al., 2006).

Although initial investigations of AMPK were focused on its role as an energy sensor involved in controlling cellular energy balance, increasing evidence derived from a growing list of AMPK downstream target proteins now indicates a broader multifunctional role for AMPK in regulating global aspects of cellular and whole-organism homeostasis, such as the control of food intake, BW gain, gene transcription, mitochondrial biogenesis and function, cell cycle progression, apoptosis, cellular proliferation, cellular polarity, and cellular structure (Kahn et al., 2005; Hue and Rider, 2007). Considerable insight into the physiological functions of AMPK has come from animal models involving the genetic modification or knockout of AMPK α catalytic subunit genes (Viollet et al., 2003). Recently, the first animal models completely lacking all AMPK activity (AMPK-null mutants) have been reported for *Drosophila* through chemically induced mutagenesis (Mirouse et al., 2007) or via genetic manipulation of the single α AMPK subunit gene contained in this species (Lee et al., 2007). The AMPK-null mutants, all of which died during embryonic development, even in the presence of sufficient nutrients, exhibited energy-dependent defects in genomic integrity (i.e., defective mitotic cells) and abnormal epithelial cell structure and polarity (Lee et al., 2007; Mirouse et al., 2007). Similar findings have been observed for mice in which both LKB1 alleles had been knocked out. These LKB1-null mutants fail to activate AMPK and exhibit multiple abnormalities, culminating in embryonic lethality by midgestation (Alessi et al., 2006). Results obtained from these models conclusively established that AMPK activity is indispensable for embryogenesis (Forcet and Billaud, 2007). In fact, it has been suggested that embryonic cells may be more susceptible to AMPK dysfunction, such as that caused by mutation, because of a greater demand for a limited nutrient supply during development (Forcet and Billaud, 2007). Thus, it is likely that AMPK also functions as an energy-dependent regulator of embryonic growth and development.

There have been numerous reports of LKB1 and AMPK expression and activity in cells and tissues from a variety of species (Hardie, 2005, 2007; Kahn et al., 2005). However, very few of these studies have involved embryos or embryonic cells at different stages of development. Although there have been several recent reports of the expression of LKB1 and AMPK in chicken tissues and cultured cells (for references, see Proszkowiec-Weglarz and Richards, 2007), there have been no reports concerning the occurrence or functioning of this important energy-responsive regulatory pathway in the developing avian embryo. Therefore, the objective of this study was to characterize the expression of the AMPK pathway in selected tissues from chicken embryos during the latter half of incubation and at hatch.

MATERIALS AND METHODS

Animals and Experiments

All animal studies were conducted according to research protocols approved by the Beltsville Animal Care and Use Committee (USDA, Agricultural Research Service). Fertile chicken eggs (SPF eggs, Charles River Laboratories Inc., Wilmington, MA) were incubated in a standard commercial incubator. Samples of liver, brain, heart, and breast muscle were collected from 10 to 20 embryos on d 12, 14, 16, 18, and 20 of incubation (**e12**, **e14**, **e16**, **e18**, and **e20**, respectively), and at hatch. All tissue samples were snap-frozen in liquid nitrogen and stored at −80°C before RNA or protein isolation.

Reverse Transcription-PCR

Total RNA was isolated from tissue samples by using the TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription (**RT**) reactions (20 μL) consisted of 1.0 μg of total RNA, 50 U of Superscript III reverse transcriptase (Invitrogen), 40 U of an RNase inhibitor (Invitrogen), 0.5 m*M* deoxynucleotide triphosphates, and 100 ng of random hexamer primers. Polymerase chain reaction was performed in 25 μL reactions containing 20 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 1.0 unit

of Platinum Taq DNA polymerase (Invitrogen), 0.2 m*M* deoxynucleotide triphosphates, 2.0 m*M* Mg^{2+}, 10 pmol of each gene-specific primer (for primer sequence, see Proszkowiec-Weglarz et al., 2006a), 5 pmol each of an appropriate mixture of primers and competimers specific for 18S rRNA (QuantumRNA Universal 18S Standards kit, Ambion Inc., Austin, TX), and 1 μL of the RT reaction. Thermal cycling parameters were 1 cycle at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 8 min. Appropriate negative controls were run to ensure PCR accuracy and specificity.

Capillary Electrophoresis with Laser-Induced Fluorescence Detection

Relative quantification of PCR products was accomplished by using capillary electrophoresis with laser-induced fluorescence detection as described previously (Richards and Poch, 2002). Briefly, aliquots (2 μL) of RT-PCR samples were first diluted 1:100 with deionized water. A P/ACE MDQ instrument (Beckman Coulter Inc., Fullerton, CA) equipped with an argon ion laser-induced fluorescence detector was used. Capillaries were 75 μm i.d. × 32 cm μSIL-DNA (Agilent Technologies, Palo Alto, CA). EnhanCE dye (Beckman Coulter Inc.) was added to the DNA separation buffer (0.5%, wt/vol, hydroxypropylmethylcellulose in Tris-borate-EDTA buffer) to a final concentration of 0.5 μg/mL. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity at 8.1 kV for 3.5 min. The P/ACE MDQ software (Beckman Coulter Inc.) was used to calculate the integrated peak area for each PCR amplicon.

Quantification of Gene Expression

The levels of gene expression were determined as the ratio of integrated peak area for each PCR product relative to that of the coamplified 18S rRNA internal standard. Values are presented as the mean ± SEM of individual expression ratio determinations (n = 6).

Tissue Protein Extraction

Samples of liver, brain, heart, and breast muscle tissue (n = 4) collected on e12, e16, e20, and at hatch were homogenized in lysis buffer (50 m*M* Tris-HCl, pH 7.5; 250 m*M* sucrose, 10 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride, 1 m*M* dithiothreitol, 2 m*M* phenylmethylsulfonyl fluoride, 0.1 m*M* benzamidine, 1 m*M* EDTA, 1 m*M* ethylene glycol tetracetic acid, 2 m*M* sodium orthovanadate, 5 μg/mL of soybean trypsin inhibitor, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and T-PER tissue protein extraction reagent (Pierce, Rockford, IL). Homogenates were then centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatants were collected and snap-frozen in liquid nitrogen. Protein concentration in tissue extracts was estimated by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analyses

Tissue protein extracts (50 μg) were subjected to SDS-PAGE under reducing conditions on 10% gels according to the method of Laemmli (1970) Separated proteins were then transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA) by using a semidry electroblotting system (Bio-Rad Laboratories) for 1.5 h at 16 to 25 V in Tris-glycine buffer containing 20% methanol. Membranes were blocked for 2 h in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (Sigma) or for 1 h in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE), and incubated overnight with one of the following primary antibodies: 1) phospho-AMPK α (Thr172) antibody (1:1,000, Cell Signaling Technology Inc., Beverly, MA), 2) AMPK α antibody (1:1,000, Cell Signaling Technology Inc.), 3) AMPK α-1 antibody (1:1,000, Upstate Biotechnology Inc., Lake Placid, NY), 4) AMPK β antibody (1:1,000, Upstate Biotechnology Inc.), 5) β-actin antibody (1:1,000, Cell Signaling Technology Inc.), 6) phospho-LKB1 (Thr189) antibody (1:500, Cell Signaling Technology Inc.), 7) phospho-LKB1 (Ser428) antibody (1:500, Cell Signaling Technology Inc.), 8) LKB1 (27D10) rabbit monoclonal antibody (1:1,000, Cell Signaling Technology Inc.), 9) phospho-acetyl-CoA carboxylase (Ser79) antibody (1:5,000, Cell Signaling Technology Inc.) or 10) acetyl-coenzyme A carboxylase antibody (1:2,000, Cell Signaling Technology). β-Actin was intended to serve as a loading (internal) control, but its variability among samples precluded that function. Data are included to demonstrate such variation. Table 1 presents a summary of the antibody reagents used in this study, including the homology between the antigens used to produce the antibodies listed above and the corresponding chicken target protein amino acid sequences. Detection of protein bands was performed by using donkey anti-rabbit horseradish peroxidase conjugated secondary antibody (1:10,000, Amersham Biosciences, Piscataway, NY) with ECL Plus Western blotting detection reagents (Amersham Biosciences) or infrared dye-labeled goat polyclonal anti-rabbit IgG (1:20,000, Li-Cor Biosciences). The membranes were then exposed to Kodak BioMax MR films (Kodak, Rochester, NY), developed, scanned, and the band intensities were quantified by densitometry with Imagequant software (Molecular Dynamics Inc., Sunnyvale, CA). When using an infrared dye-labeled secondary antibody, the membranes were scanned directly after incubation and the band intensities were quantified with the Odyssey infrared imaging system (Li-Cor Biosciences). Immunodetection of AMPK α-1, AMPK β, and β-actin proteins was performed after stripping the polyvinylidene fluoride membranes in

stripping buffer (100 m*M* 2-mercaptoethanol, 2% SDS, and 62.5 m*M* Tris-HCl, pH 6.7). Dilutions and incubations with antibodies were performed in Tris-buffered saline containing 0.1% Tween-20 and 1% nonfat dry milk for the ECL Plus system or in Odyssey blocking buffer supplemented with 0.1% Tween-20 (primary antibody) or 0.1% Tween-20 and 0.01% SDS (Sigma; secondary antibody) for the infrared dye system. For determination of AMPK phosphorylation level, 40 mU of an AMPK standard (**ST**; Upstate Biotechnology Inc.) was loaded on the gels with chicken samples. The ST consisted of partially purified rat liver AMPK, and 1 U of ST activity was defined as the amount of ST protein required to incorporate 1 nmol of phosphate into 100 μ*M* SAMS peptide substrate per minute at 30°C, with a final ATP concentration of 100 μ*M*.

Statistical Analysis

Gene and protein expression data were subjected to ANOVA by using the GLM procedure (The SAS System for Windows, v. 9.1; SAS Institute, Cary, NC). Duncan's multiple range test option of the GLM procedure was used to determine the significance of mean differences. Significance was set at $P < 0.05$.

RESULTS

mRNA Expression

Messenger RNA expression data for LKB1, its co-activating proteins, and AMPK subunits in chicken liver during embryonic development and at hatch are shown

Table 1. Characteristics of antibodies for 5′-adenosine monophosphate-activated protein kinase (AMPK) pathway proteins used in the analysis of chicken tissue and cell extracts

Protein	Antibody	Source antigen	Commercial supplier[1]	Homology[2] (%)	References for chicken analyses[3]
LKB1	1. LKB1 (27D10) rabbit monoclonal antibody (catalog no. 3050)	Human	Cell Signaling Technology Inc.	90	1
	2. Rabbit polyclonal phospho-LKB1 (Ser428) antibody (catalog no. 3051)	Human	Cell Signaling Technology Inc.	75	1, 7
	3. Rabbit polyclonal phospho-LKB1 (Thr189) antibody (catalog no. 3054)	Human	Cell Signaling Technology Inc.	92	8
AMPK α	1. Rabbit polyclonal anti-AMPK α-pan antibody (catalog no. 07-181)[4]	Human	Upstate Biotechnology Inc.	84/89[5]	3
	2. Rabbit polyclonal anti-AMPK α-1 antibody (catalog no. 07-350)	Human	Upstate Biotechnology Inc.	94	1, 4, 6, 7
	3. Rabbit polyclonal anti-AMPK α-2 antibody (catalog no. 07-363)	Human	Upstate Biotechnology Inc.	80	1, 8
	4. Rabbit polyclonal AMPK α antibody (catalog no. 2532)[4]	Human	Cell Signaling Technology Inc.	81/88[5]	1, 5, 6, 7
	5. Rabbit polyclonal phospho-AMPK α (Thr172) antibody (catalog no. 2531)[4]	Human	Cell Signaling Technology Inc.	100	1–7
AMPK β	Rabbit polyclonal anti-AMPK β antibody (catalog no. 07-670)	Human	Upstate Biotechnology Inc.	100/100[6]	3, 6, 7
AMPK γ[7]	—	—	—	—	—
ACC[8]	1. Rabbit polyclonal acetyl-coenzyme A carboxylase antibody (catalog no. 3662)	Human	Cell Signaling Technology Inc.	100	3, 6, 7
	2. Rabbit polyclonal phosphoacetyl-coenzyme A carboxylase (Ser 79) antibody (catalog no. 3661)	Rat	Cell Signaling Technology Inc.	92	1, 2, 7
	3. Rabbit anti-phosphoacetyl-coenzyme A carboxylase (Ser 79) antibody	Rat	Upstate Biotechnology Inc.	92	3, 5

[1]Cell Signaling Technology Inc. (Beverly, MA); Upstate Biotechnology Inc. (Lake Placid, NY).

[2]Percentage homology between peptide (antigen) used to produce the antibody and the corresponding chicken sequence; data obtained with the BLASTP program (http://ncbi.nlm.nih.gov) or from the commercial supplier.

[3]References: 1 = Blättler et al. (2007); 2 = Proszkowiec-Weglarz et al. (2006a); 3 = Tosca et al. (2006); 4 = Tosca et al. (2008); 5 = Chabrolle et al. (2007); 6 = Proszkowiec-Weglarz and Richards (2007); 7 = present study; 8 = unpublished data of the authors.

[4]Antibody detects both isoforms of mammalian AMPK α subunits (α-1 and α-2).

[5]First number indicates homology with chicken AMPK α-1 predicted protein and the second number indicates homology with chicken AMPK α-2 predicted protein.

[6]First number indicates homology with chicken AMPK β-1 predicted protein and the second number indicates homology with chicken AMPK β-2 predicted protein.

[7]All commercially available AMPK γ antibodies show low (<80%) homology with chicken predicted γ protein sequences and thus are expected to demonstrate low or no cross-reactivity with avian proteins.

[8]ACC = acetyl-coenzyme A carboxylase.

in Figure 1. There were no significant changes in LKB1 mRNA expression from e12 to hatch (Figure 1a). Expression of mRNA for the β isoform of STRAD increased significantly ($P < 0.05$) during embryonic development in the liver. We were unable to detect mRNA for the STRAD α isoform expressed in this tissue (Figure 1b). No significant changes in mRNA expression for either MO25 isoform occurred during embryonic development (Figure 1c). However, mRNA expression for the MO25 α isoform was significantly ($P < 0.05$) greater than that for MO25 β throughout this period of development.

The expression of AMPK α-1 subunit mRNA decreased significantly ($P < 0.05$) from e12 to hatch, whereas the α-2 subunit mRNA showed no significant ($P > 0.05$) changes during this time in the liver (Figure 1d). In addition, α-1 was the predominant AMPK α subunit mRNA expressed in the liver throughout development. Both AMPK β subunit genes were expressed in similar amounts on e12, e14, e16, and at hatch (Figure 1e). On e18, the β-2 subunit mRNA was more highly expressed, whereas on e20 β-1 was the predominant AMPK β subunit mRNA expressed in the liver. Hepatic AMPK γ-1 and γ-2 subunits were highly expressed, whereas γ-3 mRNA was not detected in the liver during the latter half of incubation or at hatch (Figure 1f). Expression of γ-1 mRNA increased significantly ($P < 0.05$), whereas γ-2 mRNA expression decreased as development proceeded. At hatch, AMPK γ-1 was the γ subunit mRNA predominantly expressed in the liver (Figure 1f).

Figure 2 summarizes mRNA expression patterns for the upstream LKB1 complex and AMPK subunits in the brain during embryonic development and at hatch. A small but significant ($P < 0.05$) increase in LKB1 mRNA expression was observed on e18 (Figure 2a). Although both STRAD isoform mRNA were expressed in the brain throughout development, the α isoform was always the predominant mRNA expressed. Furthermore, expression of the STRAD α isoform mRNA increased significantly ($P < 0.05$) toward hatch (Figure 2b). The expression of MO25 α mRNA decreased significantly ($P < 0.05$) at hatch, whereas MO25 β mRNA expression showed a small but significant ($P < 0.05$) increase from e12 to hatch (Figure 2c).

Expression of the AMPK α-1 subunit mRNA was greater in comparison with the α-2 subunit in the brain (Figure 2d). Expression of both subunit mRNA was significantly ($P < 0.05$) less at hatch compared with e12. A similar decrease in mRNA expression at hatch was observed for both AMPK β subunits (Figure 2e). The AMPK γ-1 and γ-2 subunits exhibited similar expression from e12 to e18 in the brain, followed by a small but significant ($P < 0.05$) decline in γ-2 mRNA expression at hatch (Figure 2f). Expression of AMPK γ-3 mRNA was not detected in the brain during the latter half of incubation or at hatch.

Figure 3 depicts the mRNA expression profiles for the LKB1 complex and AMPK subunits in breast muscle during development. The greatest mRNA expression for LKB1 was observed on e20 (Figure 3a). Expression of STRAD β mRNA was significantly ($P < 0.05$) greater than that for the α isoform on e12. However, expression of β subunit mRNA increased, whereas α subunit mRNA declined significantly ($P < 0.05$) during incubation such that at hatch, STRAD α was the predominant isoform mRNA expressed in breast muscle (Figure 3b). Expression of MO25 α mRNA decreased significantly ($P < 0.05$) between e12 and e20, whereas no changes in MO25 β mRNA expression were observed during this time (Figure 3c).

After an initial decline between e12 and e16, AMPK α-1 mRNA expression in breast muscle remained less than that of α-2 through hatch. Expression of AMPK α-2 subunit mRNA remained unchanged through e20, whereas it was significantly ($P < 0.05$) elevated at hatch (Figure 3d). Moreover, from e14 forward through hatching, α-2 mRNA was the predominant AMPK α subunit mRNA expressed in breast muscle (Figure 3d). Similarly, AMPK β-2 mRNA was expressed predominantly in breast muscle. Although β-1 subunit mRNA expression decreased, β-2 mRNA expression increased significantly ($P < 0.05$) from e12 to hatch (Figure 3e). All 3 mRNA for AMPK γ subunits were expressed in breast muscle during embryonic development. The γ-1 and γ-2 subunit mRNA were expressed similarly from e12 to hatch, whereas the expression of γ-3 mRNA increased and declined during this time. In general, the level of γ-3 mRNA expressed in breast muscle remained significantly ($P < 0.05$) less in comparison with that for the γ-1 and γ-2 subunits from e12 through hatch (Figure 3f).

Figure 4 presents the changes in LKB1, STRAD, MO25, and AMPK subunit mRNA expression patterns in the heart from e12 through hatch. The expression of LKB1 mRNA was significantly ($P < 0.05$) greater on e14, e18, and e20 compared with e12 or at hatch (Figure 4a). There were no significant differences in expression of the 2 STRAD isoform mRNA between e12 and e18. However, on e20 and at hatch, mRNA expression for STRAD α was significantly ($P < 0.05$) greater than expression of the β isoform (Figure 4b). Expression of MO25 α mRNA increased from e12 to hatch, whereas no significant ($P > 0.05$) changes were observed in the β isoform mRNA (Figure 4c). Like all the other tissues studied, the α isoform was the predominantly expressed MO25 mRNA in the heart throughout development.

From e12 to hatch, the expression of AMPK α-2 subunit mRNA increased significantly ($P < 0.05$) in the heart, whereas expression of α-1 mRNA declined. During this time, α-2 was the predominant AMPK α subunit mRNA expressed in the heart (Figure 4d). Only minor changes occurred in mRNA expression for both AMPK β subunits (Figure 4e). Throughout the period of study, β-2 was the AMPK β subunit mRNA predominantly expressed in the heart. Like breast muscle, mRNA for all 3 AMPK γ subunits were expressed in the heart, with mRNA for the γ-1 subunit being predominantly expressed. A small increase in AMPK γ-1

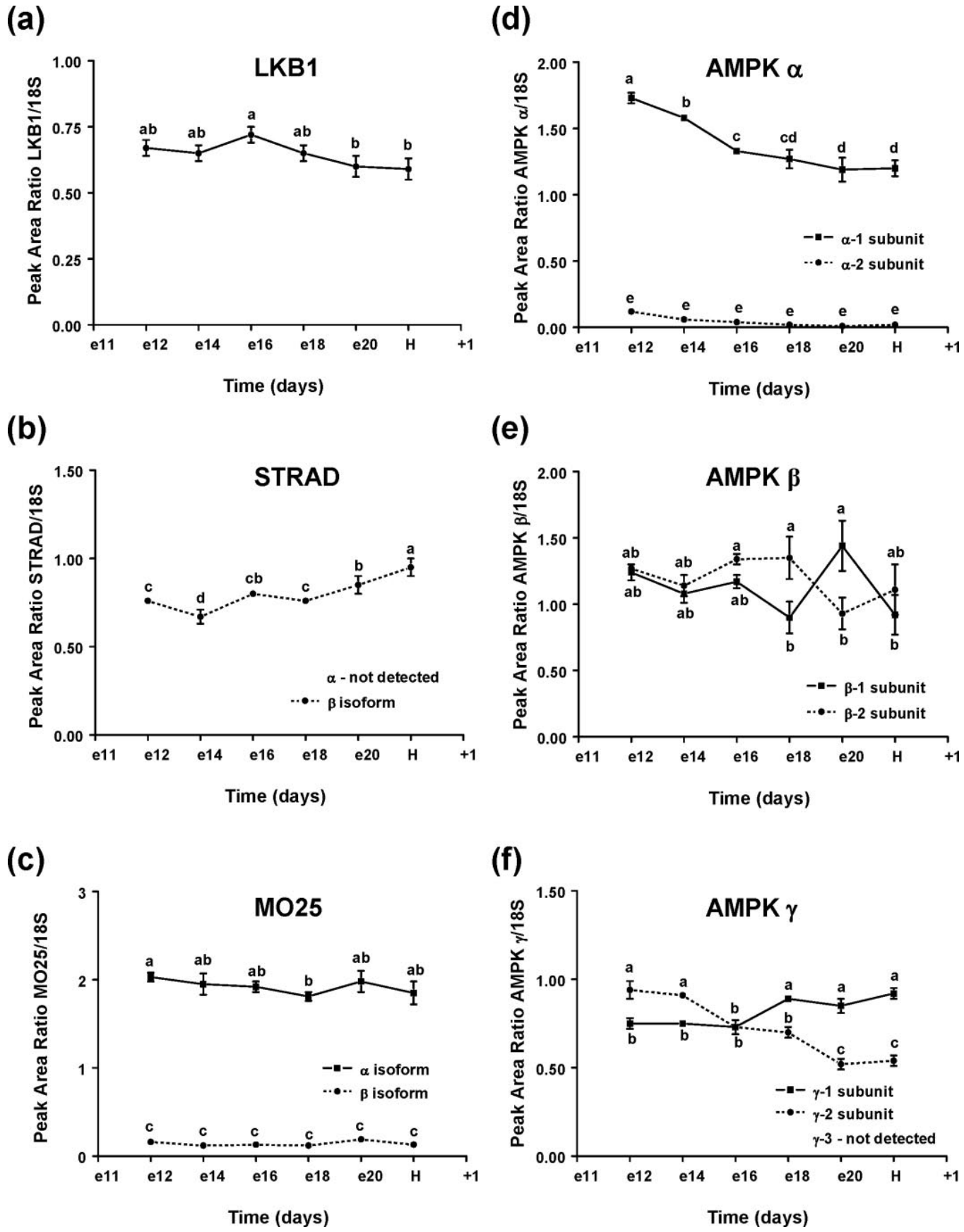

Figure 1. Expression of LKB1 (a), STE20-related adaptor (STRAD; b), mouse protein 25 (MO25; c) mRNA, and 5′-adenosine monophosphate-activated protein kinase (AMPK) α (d), β (e), and γ (f) subunit mRNA in chicken embryonic liver at d 12 (e12), 14 (e14), 16 (e16), 18 (e18), and 20 (e20) of incubation and at hatch (H). Reverse transcription-PCR and capillary electrophoresis with laser-induced fluorescence detection were used to quantify the amount of gene expression relative to an 18S rRNA internal standard. Values represent the mean ± SEM of 6 determinations. [a–d]Different letters denote statistically significant ($P < 0.05$) differences for mean comparisons.

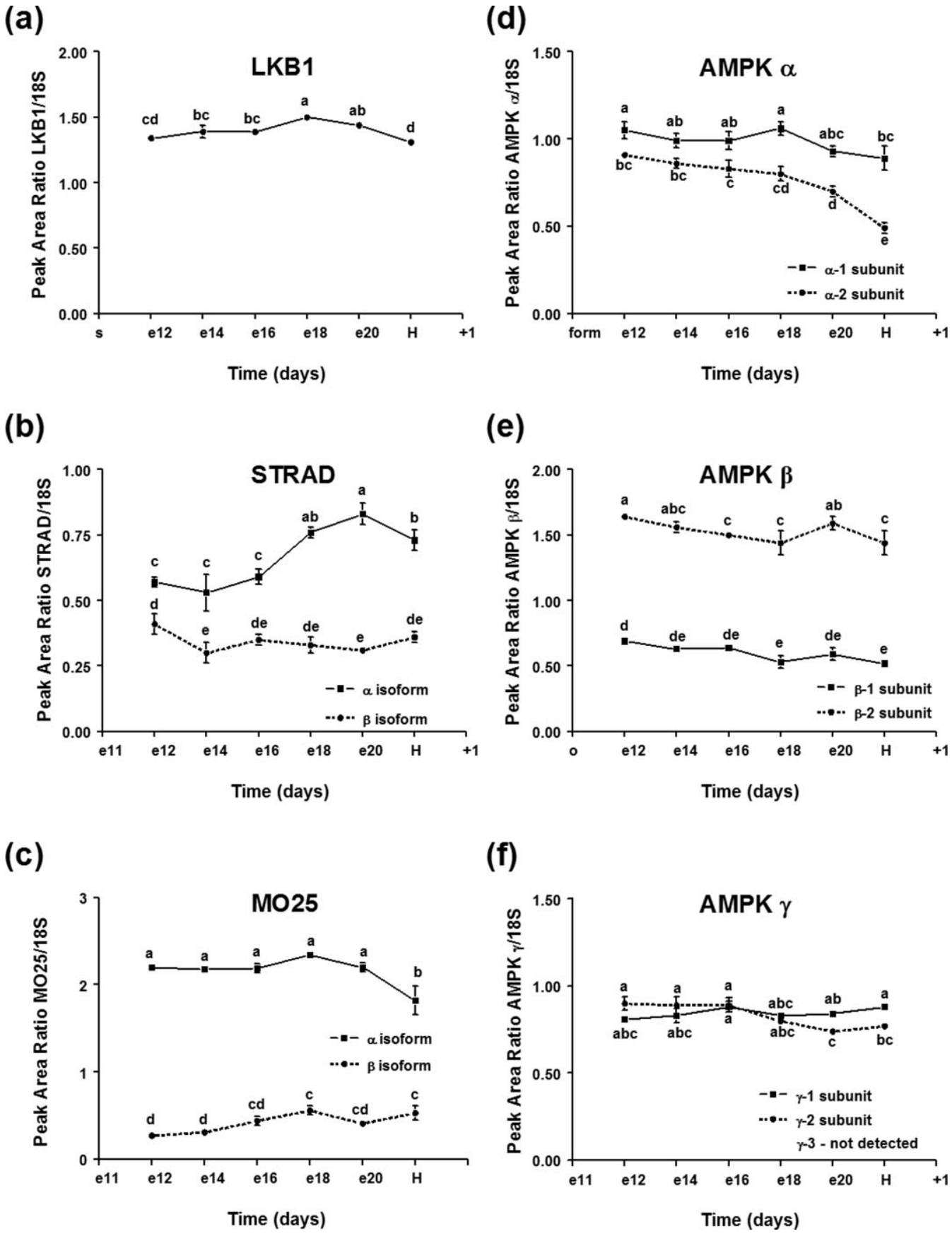

Figure 2. Expression of LKB1 (a), STE20-related adaptor (STRAD; b), mouse protein 25 (MO25) (c) mRNA, and 5′-adenosine monophosphate-activated protein kinase (AMPK) α (d), β (e), and γ (f) subunit mRNA in chicken embryonic brain at d 12 (e12), 14 (e14), 16 (e16), 18 (e18), and 20 (e20) of incubation and at hatch (H). Reverse transcription-PCR and capillary electrophoresis with laser-induced fluorescence detection were used to quantify the amount of gene expression relative to an 18S rRNA internal standard. Values represent the mean ± SEM of 6 determinations. [a–e]Different letters denote statistically significant ($P < 0.05$) differences for mean comparisons.

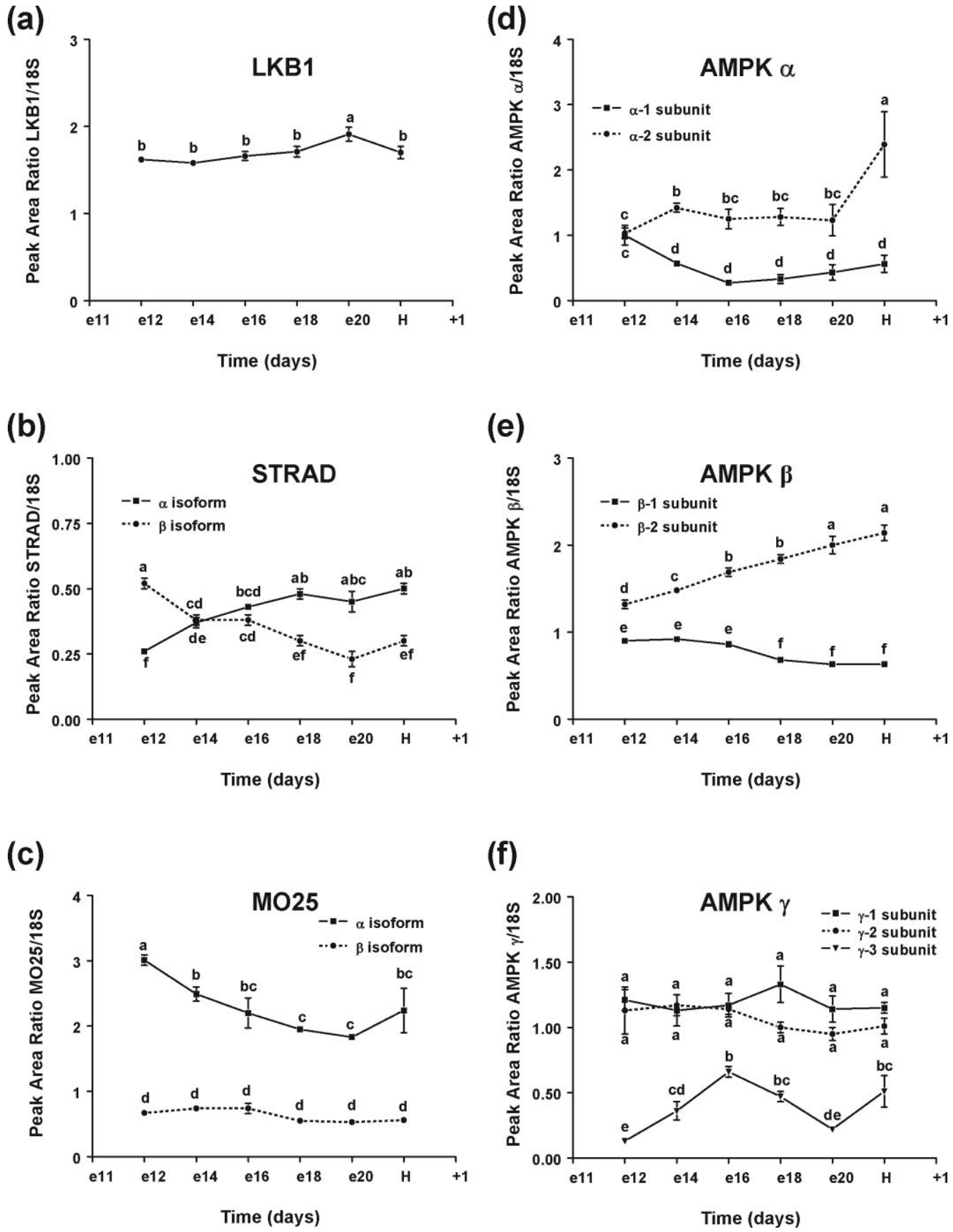

Figure 3. Expression of LKB1 (a), STE20-related adaptor (STRAD; b), mouse protein 25 (MO25; c) mRNA, and 5′-adenosine monophosphate-activated protein kinase (AMPK) α (d), β (e) and γ (f) subunit mRNA in chicken embryonic breast muscle at d 12 (e12), 14 (e14), 16 (e16), 18 (e18), and 20 (e20) of incubation and at hatch (H). Reverse transcription-PCR and capillary electrophoresis with laser-induced fluorescence detection were used to quantify the amount of gene expression relative to an 18S rRNA internal standard. Values represent the mean ± SEM of 6 determinations. [a–f]Different letters denote statistically significant ($P < 0.05$) differences for mean comparisons.

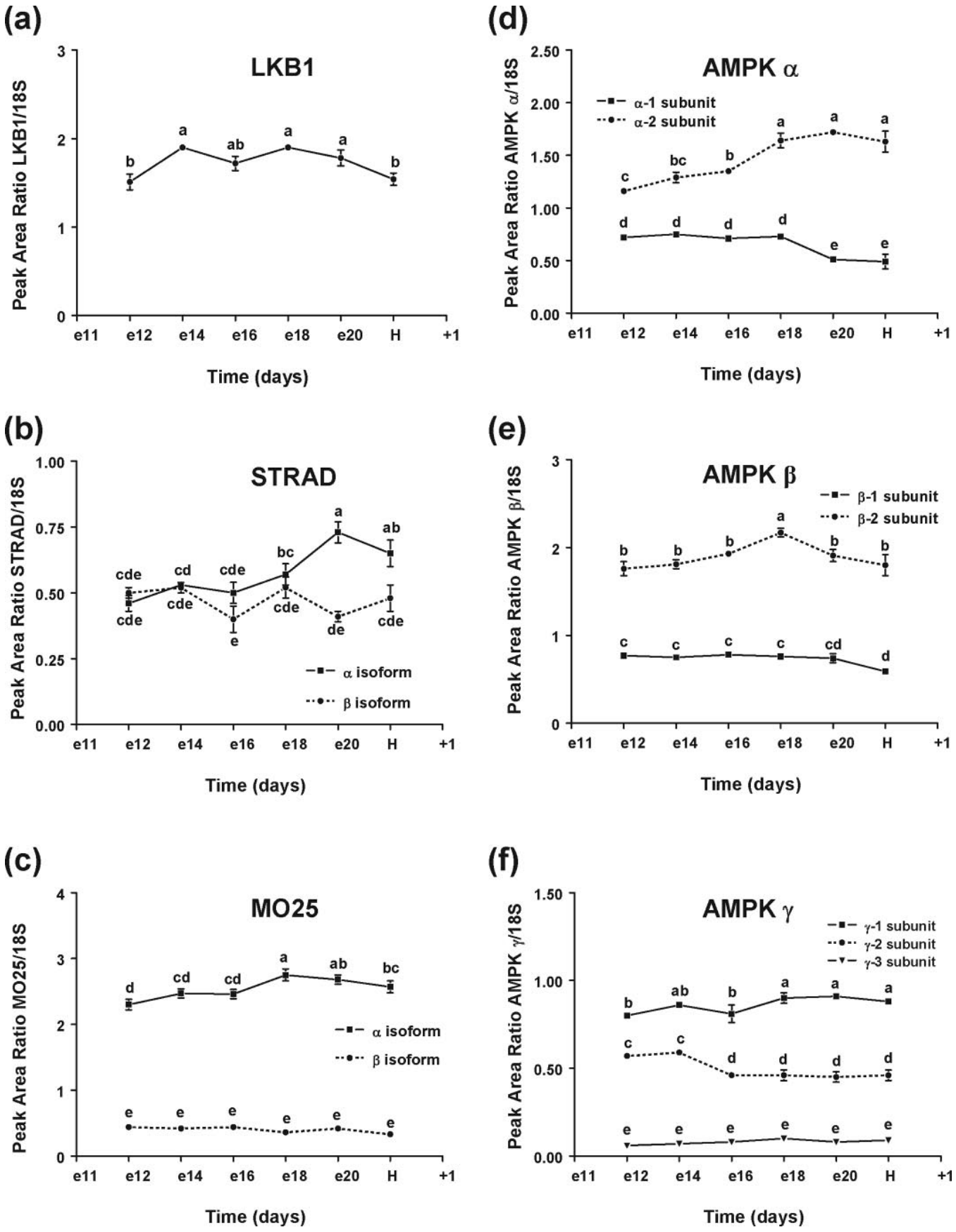

Figure 4. Expression of LKB1 (a), STE20-related adaptor (STRAD; b), mouse protein 25 (MO25; c) mRNA and 5′-adenosine monophosphate-activated protein kinase (AMPK) α (d), β (e), and γ (f) subunit mRNA in chicken embryonic heart at d 12 (e12), 14 (e14), 16 (e16), 18 (e18), and 20 (e20) of incubation and at hatch (H). Reverse transcription-PCR and capillary electrophoresis with laser-induced fluorescence detection were used to quantify the amount of gene expression relative to an 18S rRNA internal standard. Values represent the mean ± SEM of 6 determinations. [a–e]Different letters denote statistically significant ($P < 0.05$) differences for mean comparisons.

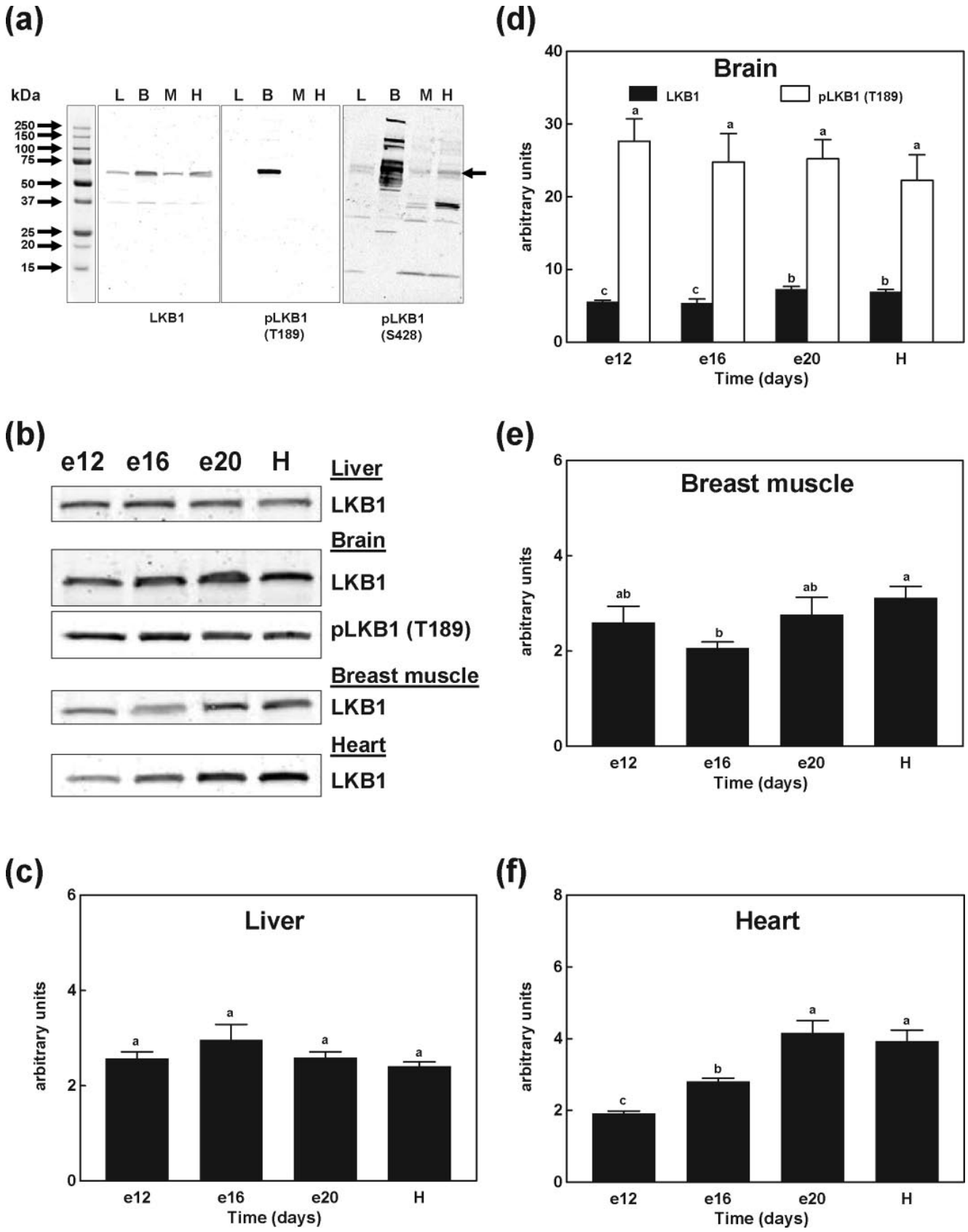

Figure 5. Western blot analysis of LKB1 and phospho-LKB1 (pLKB1, T189, and S428) in liver (L), brain (B), breast muscle (M), and heart (H) from chicken embryos at d 12 (e12), 16 (e16), and 20 (e20) of incubation and at hatch. (a) Representative immunoblots for tissues collected at hatch (H) and stained for LKB1 and pLKB1 (T189 and S428) protein along with a series of molecular weight markers (left). The arrow (right) indicates the LKB1 band. (b) Representative immunoblots stained for LKB1 and pLKB1 (T189, brain only) protein for tissues collected at e12, e16, e20, and at hatch (H). Densitometry was used to quantify band intensities for LKB1 and pLKB1 (T189, brain only) in the (c) liver, (d) brain, (e) breast muscle, and (f) heart. Data are expressed as arbitrary units, and values represent the mean ± SEM of 4 determinations. [a–c]Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

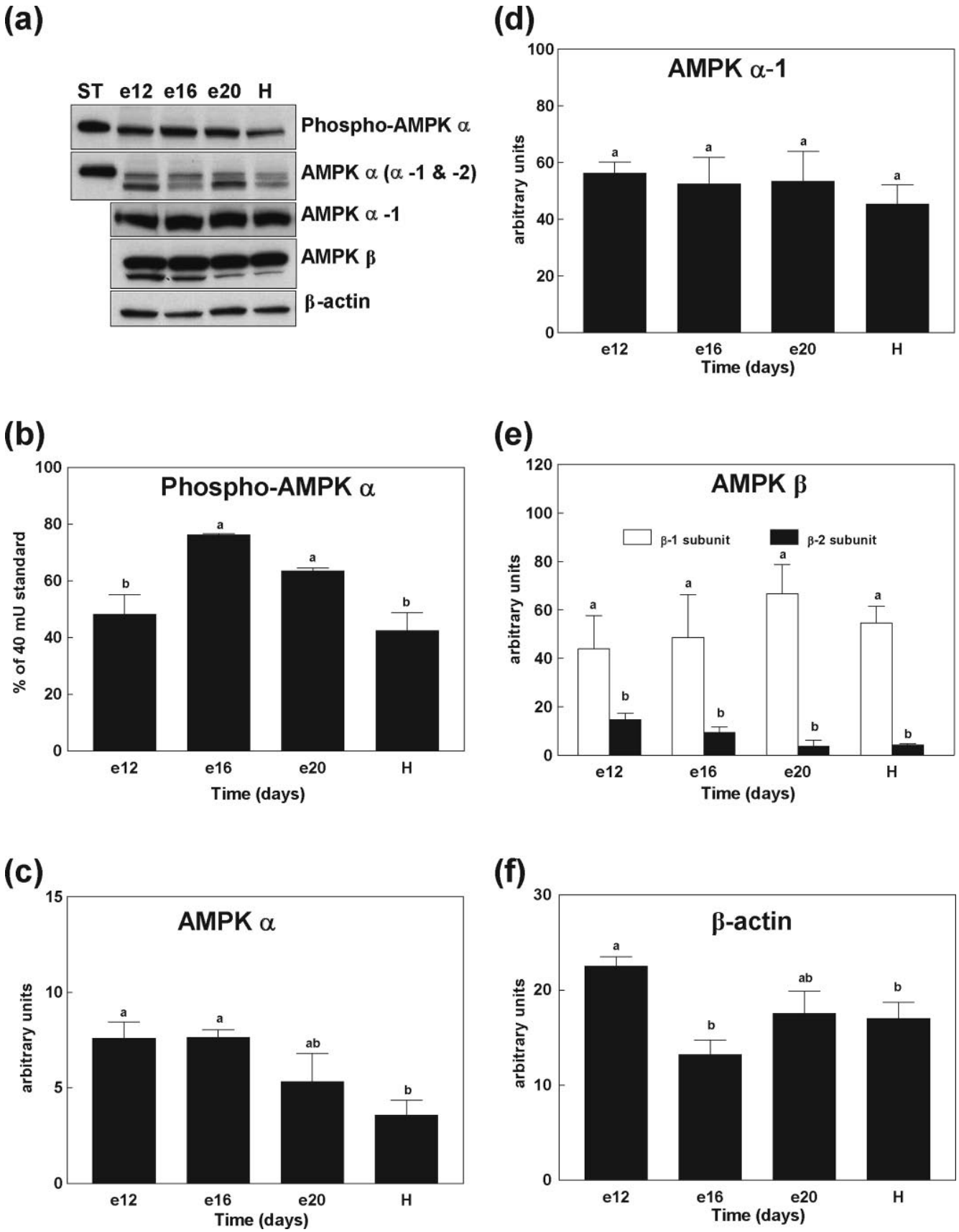

Figure 6. Western blot analysis of phospho-5′-adenosine monophosphate-activated protein kinase (phospho-AMPK) α, AMPK α, AMPK α-1, AMPK β, and β-actin protein in chicken embryonic liver at d 12 (e12), 16 (e16), and 20 (e20) of incubation and at hatch (H). (a) Representative immunoblots stained for phospho-AMPK α, AMPK α, AMPK α-1, AMPK β, and β-actin protein. Densitometry was used to quantify band intensities for phospho-AMPK α (b), AMPK α (c), AMPK α-1 (d), AMPK β (e), and β-actin (f). Data are expressed as percentage of 40 mU of AMPK standard (ST) or arbitrary units, and values represent the mean ± SEM of 4 determinations. [a,b]Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

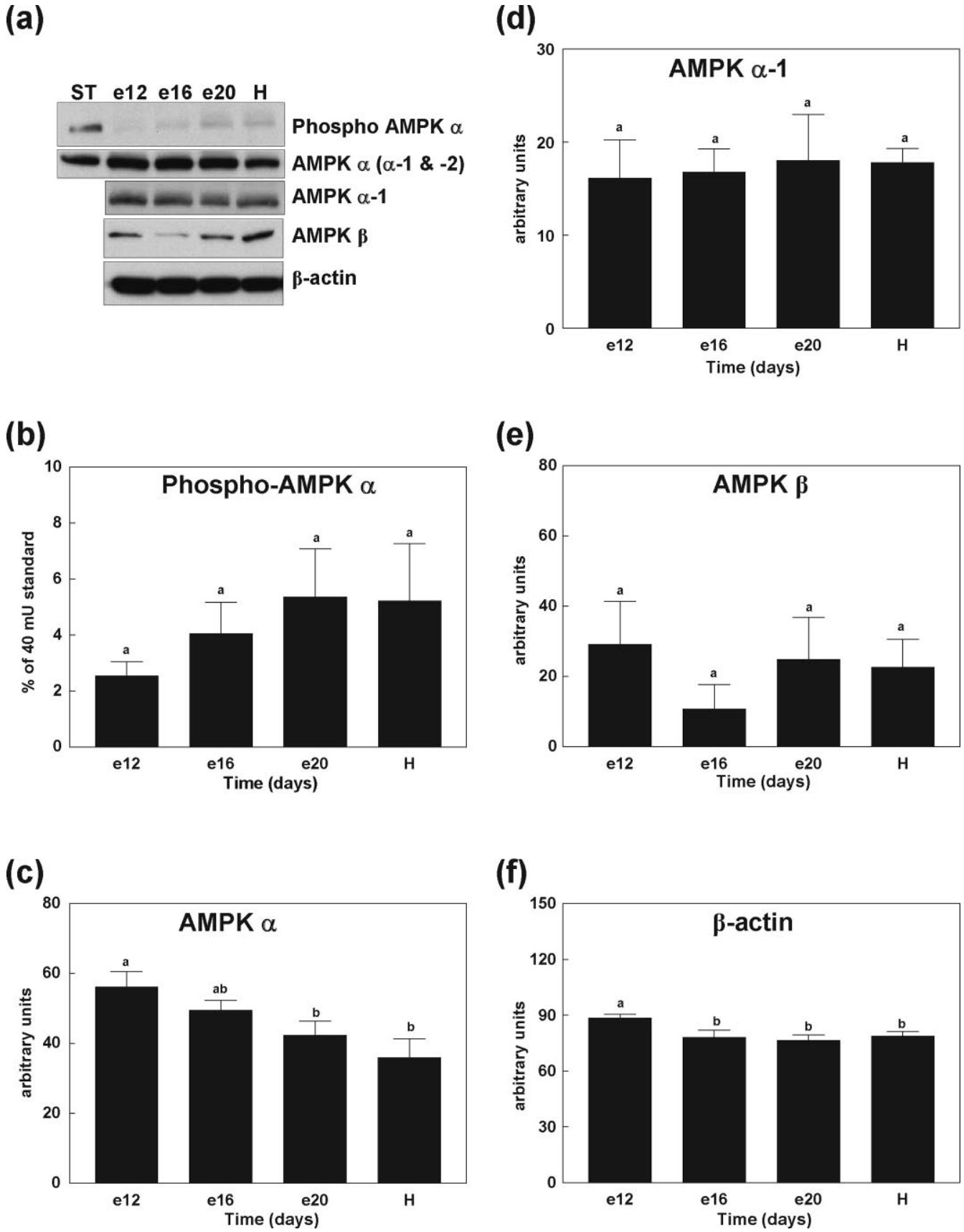

Figure 7. Western blot analysis of phospho-5′-adenosine monophosphate-activated protein kinase (phospho-AMPK) α, AMPK α, AMPK α-1, AMPK β, and β-actin protein in chicken embryonic brain at d 12 (e12), 16 (e16), and 20 (e20) of incubation and at hatch (H). (a) Representative immunoblots stained for phospho-AMPK α, AMPK α, AMPK α-1, AMPK β, and β-actin protein. Densitometry was used to quantify band intensities for phospho-AMPK α (b), AMPK α (c), AMPK α-1 (d), AMPK β (e), and β-actin (f). Data are expressed as percentage of 40 mU of AMPK standard (ST) or arbitrary units, and values represent the mean ± SEM of 4 determinations. [a,b]Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

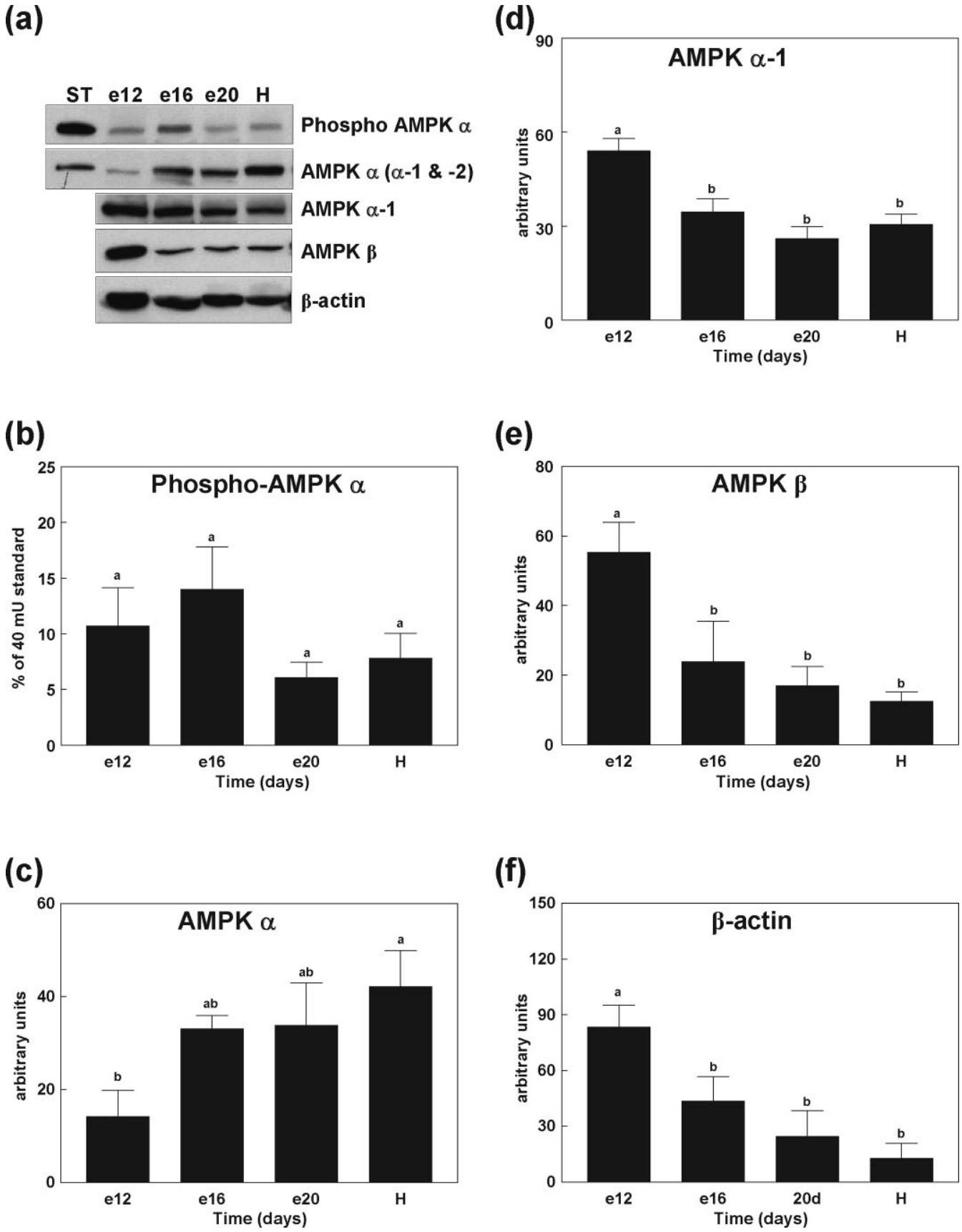

Figure 8. Western blot analysis of phospho-5′-adenosine monophosphate-activated protein kinase (phospho-AMPK) α, AMPK α, AMPK α-1, AMPK β, and β-actin protein in chicken embryonic breast muscle at d 12 (e12), 16 (e16), and 20 (e20) of incubation and at hatch (H). (a) Representative immunoblots stained for phospho-AMPK α, AMPK α, AMPK α-1, AMPK β, and β-actin protein. Densitometry was used to quantify band intensities for phospho-AMPK α (b), AMPK α (c), AMPK α-1 (d), AMPK β (e), and β-actin (f). Data are expressed as percentage of 40 mU of AMPK standard (ST) or arbitrary units, and values represent the mean ± SEM of 4 determinations. [a,b]Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

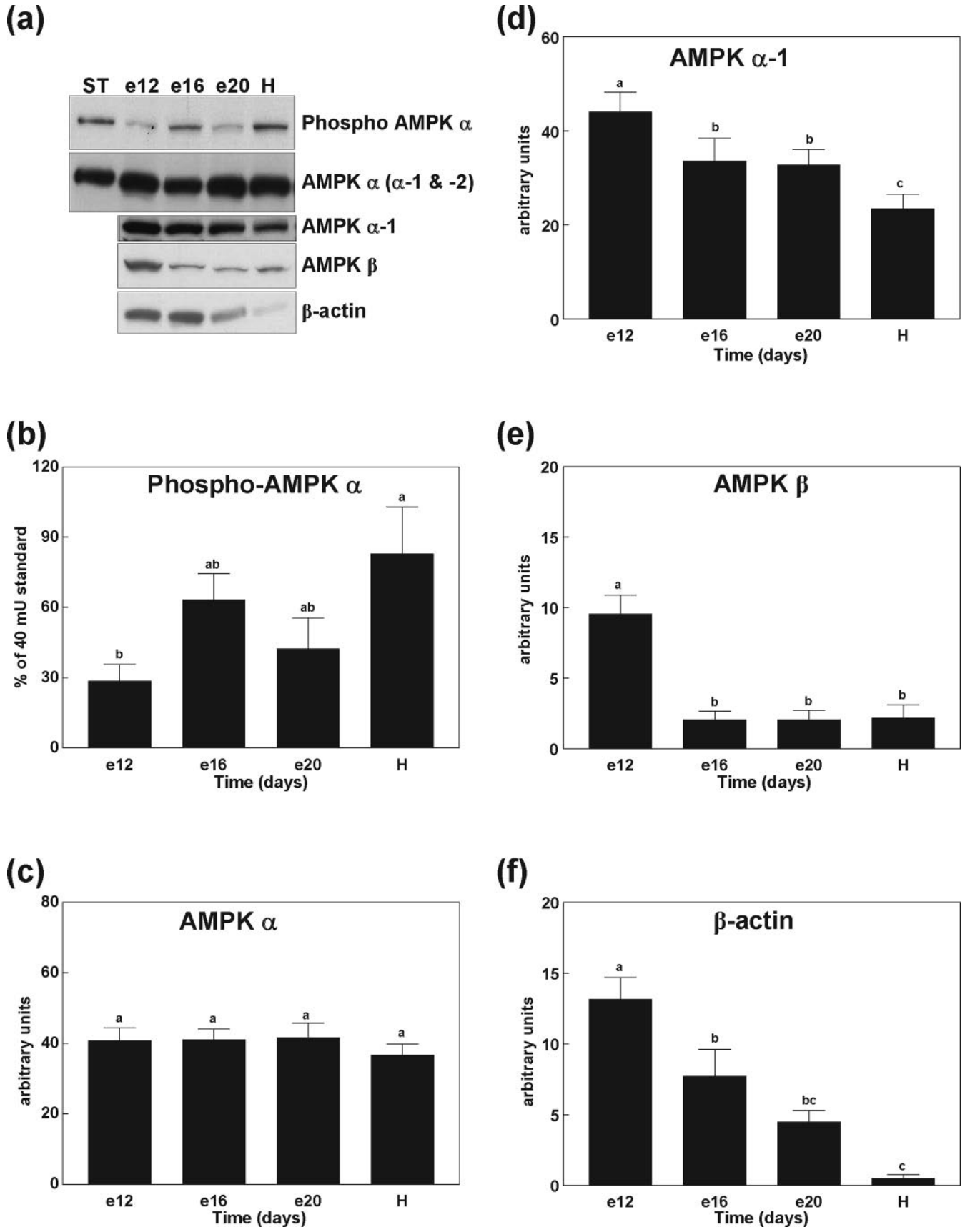

Figure 9. Western blot analysis of phospho-5′-adenosine monophosphate-activated protein kinase (phospho-AMPK) α, AMPK α, AMPK α-1, AMPK β, and β-actin protein in chicken embryonic heart at d 12 (e12), 16 (e16), and 20 (e20) of incubation and at hatch (H). (a) Representative immunoblots stained for phospho-AMPK α, AMPK α, AMPK α-1, AMPK β, and β-actin protein. Densitometry was used to quantify band intensities for phospho-AMPK α (b), AMPK α (c), AMPK α-1 (d), AMPK β (e), and β-actin (f). Data are expressed as percentage of 40 mU of AMPK standard (ST) or arbitrary units, and values represent the mean ± SEM of 4 determinations. [a–c]Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

mRNA expression and a small decrease in γ-2 mRNA expression were observed from e12 to hatch, whereas no significant ($P > 0.05$) changes occurred in γ-3 mRNA expression during this time (Figure 4f).

Protein Expression

The expression of total and phosphorylated LKB1 (**pLKB1**) protein in the liver, brain, breast muscle, and heart tissues collected on e12, e16, e20, and at hatch was determined by Western blot analysis (Figure 5). At hatch, LKB1 protein expression was greatest in the brain, followed by the heart, breast muscle, and liver (Figure 5a). Using specific antibodies that recognize 2 different sites of phosphorylation (T189 and S428; numbers refer to the human sequence) on LKB1 (Table 1), we found that only the brain demonstrated phosphorylation at T189, whereas all tissues contained LKB1 phosphorylated at S428 (corresponding to S435 in the chicken sequence; GenBank accession no. ABI23430). The brain displayed the greatest amount of pLKB1 (Ser 428) at hatch (Figure 5a). Although the expression of pLKB1 (T189) tended to decline during development, no significant differences were observed for this modification from e12 to hatch (Figure 5b and 5d). In both the brain and heart, total LKB1 protein increased significantly ($P < 0.05$) between e12 and hatch (Figure 5b, 5d, and 5f).

Figure 6 summarizes the expression of phosphorylated AMPK α [**pAMPK α** (T172)]and AMPK α and β subunit proteins in the liver on e12, e16, e20, and at hatch. A significant ($P < 0.05$) increase in pAMPK α (T172) was observed on e16 and e20 compared with e12 and at hatch (Figure 6b). Although expression of the total AMPK α subunit protein (α-1 and α-2) had declined significantly ($P < 0.05$) by hatch, no changes in α-1 subunit protein expression were observed throughout the experimental sampling period (Figure 6c-d). The predicted molecular mass of both AMPK β subunit proteins (β-1 and β-2) is approximately 30 kDa (Proszkowiec-Weglarz et al., 2006a). However, chicken liver AMPK β subunit proteins were found to migrate anomalously on SDS-PAGE, showing 2 bands (Figure 6a). Based on earlier reports involving mammalian AMPK β subunit proteins (Woods et al., 1996; Thornton et al., 1998), the higher molecular weight band (40 kDa, top band in Figure 6a) was identified as the β-1 protein, whereas the lower molecular weight band (34 kDa) was identified as the β-2 protein. This anomaly was also reported by Tosca et al. (2006) for the SDS-PAGE separation of AMPK β subunit proteins derived from the chicken ovary. No significant ($P > 0.05$) changes were observed in the expression of either β subunit protein in the liver from e12 to hatch (Figure 6e). The expression of β-actin protein changed significantly ($P < 0.05$) during the experimental period and, for this reason, was not used for data normalization (Figure 6f). Because equivalent amounts of extract protein were applied to each well, this approach was not required.

Changes in the expression of pAMPK α (T172) and AMPK α and β subunit proteins in brain tissue collected during the latter half of embryonic development and at hatch are depicted in Figure 7. Although there was an increasing trend, no significant ($P > 0.05$) changes were observed in the amount of AMPK α subunit phosphorylation (Figure 7b). Total AMPK α subunit protein expression declined significantly ($P < 0.05$), whereas no changes were detected in AMPK α-1 protein expression from e12 to hatch (Figure 7c and 7d). A single band was observed for AMPK β subunit protein in the brain (Figure 7a), and no significant ($P > 0.05$) changes were found during the latter half of incubation or at hatch (Figure 7e). Similar to what was observed in the liver, a significant ($P < 0.05$) decrease in β-actin protein expression in the brain occurred from e16 to hatch (Figure 7f).

Figure 8 presents the expression of pAMPK α (T172) and AMPK α and β subunit proteins in breast muscle between e12 and hatch. No significant ($P > 0.05$) changes in AMPK α phosphorylation were determined during the period of study (Figure 8b). The level of total AMPK α protein expression increased significantly ($P < 0.05$) between e12 and hatch (Figure 8c). In contrast, the level of AMPK α-1 protein expression decreased significantly ($P < 0.05$) during this period (Figure 8d). Expression of AMPK β protein was significantly ($P < 0.05$) less from e16 to hatch compared with the level observed on e12 (Figure 8e). Moreover, only a single band was detected for AMPK β protein by Western blotting (Figure 8a). Expression of β-actin protein declined significantly ($P < 0.05$) in the breast muscle from e12 to hatch (Figure 7f).

The expression of pAMPK α (T172) and AMPK α and β subunit proteins in the heart from e12 to hatch is shown in Figure 9. A significant ($P < 0.05$) increase in AMPK α phosphorylation was observed in the heart between e12 and hatch (Figure 9b). No significant ($P > 0.05$) changes occurred in the expression of total AMPK α protein, whereas the expression of AMPK α-1 subunit protein decreased significantly ($P < 0.05$) from e12 through hatch (Figure 9c-d). Like the brain and breast muscle, only a single band was detected for the AMPK β subunit protein by Western analysis (Figure 9a), and its expression was significantly ($P < 0.05$) less from e16 to hatch compared with that observed on e12 (Figure 9e). A dramatic decline in heart β-actin protein expression similar to that seen for breast muscle (Figure 8f) was observed from e12 to hatch (Figure 9f).

Figure 10 depicts Western blots for total acetyl-coenzyme A carboxylase (**ACC**) and the phosphorylated form of ACC [**pACC (S79)**], a major downstream target for AMPK in the liver, brain, breast muscle, and heart collected at different times during development. Although the expression of ACC protein was very low in all the tissues, we were able to detect to-

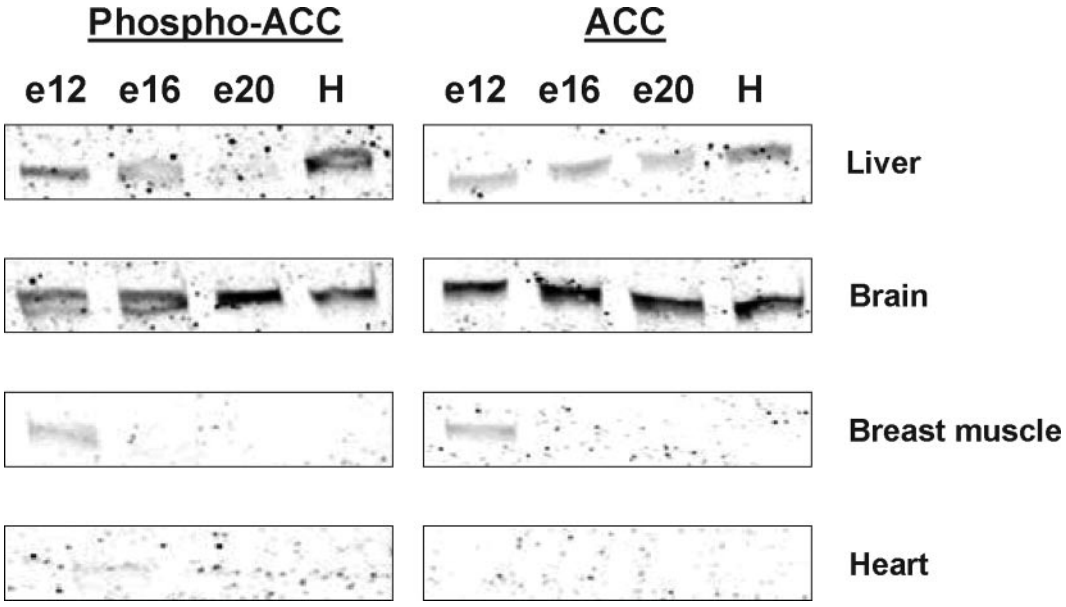

Figure 10. Representative Western blots for phospho-acetyl-coenzyme A (phospho-ACC) and total acetyl-coenzyme A carboxylase (ACC) protein in liver, brain, breast muscle, and heart collected on d 12 (e12), 16 (e16), and 20 (e20) of incubation and at hatch (H).

tal and pACC (S79) expression in the liver and brain throughout the period of study. Moreover, the greatest expression of ACC and pACC (S79) was observed in the brain. In the breast muscle, both ACC and pACC (S79) were detected only on e12 and only in extremely small amounts. We were unable to detect expression of ACC in the heart at any time during the study period. In the brain, the expression of pACC (S79) appeared to increase to a peak at e20, whereas in the liver, the expression of pACC (S79) was greatest at hatch. Total ACC protein expression in the brain appeared to be constant during development, in contrast to the liver, which exhibited increasing total ACC protein expression from e12 to hatch.

DISCUSSION

This study is the first to identify and characterize the AMPK pathway in chicken embryos and newly hatched chicks. We demonstrated that this pathway is active in different tissues during the latter half of incubation and at hatch. This could indicate an important developmental role for the AMPK pathway in the chicken, as has been suggested for other species (Forcet and Billaud, 2007). The AMPK pathway is known to be an important regulator of cellular energy dynamics in peripheral tissues such as the liver and skeletal muscle by controlling glucose and lipid metabolism (Imai et al., 2006). Therefore, it is reasonable to assume that this pathway plays a crucial role in maintaining energy balance during perinatal development of the chicken as the embryo transitions from a lipid-rich nutrition source (yolk) in ovo to a high-carbohydrate diet immediately posthatch.

The LKB1 gene was expressed in all tissues collected from embryos and hatched chicks. This is consistent with previous observations in mammals showing that LKB1 is expressed in varying amounts in all fetal tissues (Alessi et al., 2006). Similar to our previous report for 3-wk-old broilers (Proszkowiec-Weglarz et al., 2006a), LKB1 mRNA expression was greatest in the breast muscle, heart, and brain and decreased in the liver during embryonic development and at hatch. Our results from Western blot analysis showed a protein expression profile for LKB1 among tissues similar to that observed for mRNA expression. That is, LKB1 protein expression was greater in the brain, breast muscle, and heart than in the liver. However, differences were also observed between mRNA and protein expression for LKB1. The increase in LKB1 protein expression in the brain and heart toward hatch was not reflected in the changes in mRNA expression. It is not known whether such differences in total protein levels correspond to changes in the catalytic activity of LKB1 present in each tissue. Instead, the level of activity for LKB1 in these tissues is more likely influenced by the degree of phosphorylation at 8 specific residues within the protein (Alessi et al., 2006). The LKB1 protein is known to possess 4 sites (i.e., amino acid residues T185, T189, T336, and S404; numbers refer to the mouse sequence) that are autophosphorylated by LKB1 itself (Alessi et al., 2006). The purpose of this autophosphorylation is currently unknown because mutation of any of these sites apparently does not affect the catalytic activity of LKB1, despite the fact that T185 and T189 are located within the kinase domain of the protein (Alessi et al., 2006). However, our observation that the T189 residue of LKB1 is strongly and specifically phosphorylated in the brain throughout development suggests a potentially unique function for this particular posttranslational modification in chickens. It is possible that this modification in the brain relates to some aspect of its matura-

tion, because LKB1 has been reported to play an important role in neural differentiation and development through its regulatory effects on neuronal polarization in the embryonic cortex (Asada et al., 2007; Barnes et al., 2007). The polarizing activity of LKB1 in the brain is specifically enhanced by protein kinase A and p90 ribosomal S6 kinase, both of which phosphorylate the S428 residue (Barnes et al., 2007). Although the downstream targets of pLKB1 (S428) have been shown to be AMPK-related brain-specific kinases (Bright et al., 2008), it has been suggested that AMPK or other downstream substrates of LKB1 might also regulate the function of adult neurons (Barnes et al., 2007). In this study, we found that LKB1 was also phosphorylated on S435 (equivalent to S428 in the human sequence or S431 in the mouse sequence) and that the expression of pLKB1 (S435) was greatest in the brain compared with the other tissues collected from embryos and hatched chicks (Figure 5a). Furthermore, the greater levels of LKB1 expression in the brain compared with other tissues of the developing chick embryo could also reflect the fact that the brain has an extremely high metabolic rate and high lipid content (Turnley et al., 1999). Thus, our findings could indicate a role for LKB1 in neural development in chickens.

In this study, we also determined the mRNA expression patterns for 2 LKB1-associated proteins, STRAD and MO25, which are required to activate LKB1 through complex formation. It is clear that mRNA encoding LKB1 and its associated proteins are expressed throughout development in chickens. As with LKB1 mRNA expression, the profiles observed for the STRAD and MO25 isoforms were similar to those in corresponding tissues obtained from 3-wk-old broilers (Proszkowiec-Weglarz et al., 2006a). However, we did observe tissue-specific mRNA expression patterns that could potentially influence the type of LKB1 complex formed and thus its ability to activate downstream targets such as AMPK. For example, the fact that the liver expresses mRNA only for the STRAD β isoform suggests that the LKB1 complex in this tissue would likely differ from the ones formed in the brain, breast muscle, and heart, which express both STRAD isoforms. In contrast, all tissues preferentially expressed the α isoform mRNA for MO25. It has been reported that LKB1 complexes with different isoform combinations for STRAD and MO25 display different activities, with the LKB1:STRADα:MO25α complex exhibiting the greatest level of activity for activating different kinases in the AMPK-related subfamily (Hawley et al., 2003; Lizcano et al., 2004). Thus, mRNA expression profiles for the LKB1-associated proteins STRAD and MO25 provide additional insight into the potential for activation of AMPK by LKB1 in different tissues during the development of chickens.

All 7 AMPK subunit genes were expressed in varying amounts in the liver, brain, breast muscle, and heart during the latter half of incubation and at hatch in chickens. In most cases, the mRNA expression profiles were similar to those observed previously in 3-wk-old broilers (Proszkowiec-Weglarz et al., 2006a). However, there was evidence for tissue-specific AMPK subunit mRNA expression. The liver and brain preferentially expressed the α-1 AMPK subunit isoform mRNA, whereas the breast muscle and heart preferentially expressed mRNA for the α-2 subunit. Expression of the α-1 protein was greatest in the liver, which exhibited similar levels on e12, e16, e20, and at hatch, whereas the levels of this particular AMPK subunit isoform protein declined toward hatch in the breast muscle and heart. Differential expression of AMPK α subunit isoform genes may indicate potential differences in AMPK activation and function. For example, it has been reported that AMPK complexes containing the α-2 subunit have a greater tendency to localize to the nucleus, and therefore may be more likely to be involved in gene regulation events (Salt et al., 1998; Winder and Thompson, 2007). In contrast, AMPK complexes containing the α-1 subunit have been reported to localize to the cytoplasm or cell membrane, thus targeting AMPK activity to discrete cellular locations (Hue and Rider, 2007). This suggests that the α isoforms are not completely interchangeable for AMPK complex formation and that they may not necessarily compensate for each other in determining overall AMPK activity. Thus, in chickens there may be differences in the activation and function of AMPK in muscle (heart and skeletal muscle) compared with other tissues such as the liver and brain because of differential expression of the α subunit isoforms.

We have previously reported that the AMPK γ-3 subunit is expressed only in the skeletal muscle and heart in chickens (Proszkowiec-Weglarz et al., 2006a,b). This also appears to be the case for the developing embryo and hatched chick. Moreover, in 3-wk-old broilers we observed that in skeletal muscle, mRNA for the γ-3 subunit was more highly expressed than for either of the other 2 γ subunits. However, in this study, significantly reduced amounts of AMPK γ-3 mRNA compared with AMPK γ-1 and γ-2 were observed in the skeletal muscle and heart during the latter half of incubation and at hatch (Figures 8 and 9). This indicates a developmental pattern for expression of the AMPK γ-3 subunit. In fact, we observed that the mRNA expression of γ-3 increased by 48 h posthatch, and from this point forward, it became the predominant AMPK γ subunit expressed by the breast muscle in chickens (Proszkowiec-Weglarz and Richards, 2007). However, in the heart, no dramatic increase in γ-3 subunit expression occurred posthatch as observed in the breast muscle. In fact, at 3 wk of age, a similar mRNA expression profile was observed for all 3 AMPK γ subunits (i.e., γ-1 > γ-2 > γ-3) in broilers as was found in the embryonic heart. Therefore, this expression pattern for the AMPK γ-3 subunit suggests that it may play an important role in posthatch skeletal muscle development and function. Deshmukh et al. (2008) have suggested that a temporal inverse relationship in the ac-

tivities of the protein kinase B (Akt/PKB) and AMPK pathways may be central to regulation of target of rapamycin signaling and its subsequent effect on protein synthesis and muscle mass accretion. Specifically, they found that the α-2 and γ-3 subunits were required to form a functional AMPK heterotrimeric complex that would inhibit insulin-induced target of rapamycin pathway signaling in skeletal muscle. Birk and Wojtaszewski (2006) also reported on the differential regulation of AMPK heterotrimers in human skeletal muscle during exercise-induced activation of the AMPK pathway. They found that the α-2/β-2/γ-3 heterotrimer was the predominant functional AMPK complex found in skeletal muscle. Developmental changes in the expression of AMPK γ-3 subunit mRNA in skeletal (breast) muscle (Figure 8) could indicate the relationship of AMPK pathway activity to posthatch control of protein synthesis and muscle mass accretion in broiler chickens.

Niesler et al. (2007) demonstrated a changing expression profile of AMPK subunit isoforms in differentiating C2C12 cells, a myoblast cell line, with the α-1, β-1, β-2, and γ-3 subunits being expressed in low amounts in undifferentiated myoblasts but increasing dramatically with subsequent differentiation to myotubes. They concluded that differentiated C2C12 skeletal myotubes are better able to deal with in vitro cellular stresses to inhibit apoptosis compared with the undifferentiated C2C12 skeletal myoblast cells because they express more functional AMPK heterotrimers owing to the availability of increased subunit protein as differentiation proceeds. Our results indicated that the levels of AMPK α-1 and β subunit proteins actually declined in breast muscle during incubation (Figure 8d and 8e), which is contrary to the findings of Niesler et al. (2007). However, the level of total AMPK α protein (i.e., α-1 and α-2 combined) did increase during this time (Figure 8c) along with an increasing trend in activated (phosphorylated) AMPK (Figure 8b). This emphasizes the importance of the AMPK subunit expression profile in determining the amount of functional AMPK in a specific tissue during development. Moreover, these observations point to a potentially important role for the AMPK pathway in regulating satellite stem cell differentiation and skeletal muscle development. This remains to be demonstrated in avian muscle.

Unfortunately, it is currently not possible to study the expression of each of the AMPK γ subunits at the protein level in chickens because of a lack of the specific antibodies needed to detect them in Western blot analysis (Table 1). Because all the commercially available antibodies have been developed against the mammalian sequence (rat or human) contained in the N-terminal region of the subunit protein and because that region is the most highly variable among species, this makes them unsuitable for detection of avian AMPK γ subunit proteins. Thus, it will be necessary to develop unique avian-specific antibodies that recognize a homologous γ subunit sequence to enable the study of AMPK γ subunit protein expression in avian species.

Activation of AMPK involves the phosphorylation of the T172 residue located within the activation loop of the kinase domain present in the α subunits (Hawley et al., 1996). Using Western blot analysis, we determined that pAMPK (T172) was present in the liver, brain, breast muscle, and heart during the latter half of incubation and at hatch. Moreover, the liver and heart showed the highest levels of activated (phosphorylated) AMPK, with lower levels present in the skeletal muscle and brain. These are all organs in which changes in AMPK activation have been shown to influence metabolic activity by the actions of AMPK on numerous downstream protein targets (Kahn et al., 2005). Acetyl-coenzyme A carboxylase, both the cytoplasmic (ACC-α, involved in lipogenesis) and the mitochondrial-associated (ACC-β, involved in fatty acid oxidation) isoforms, are important downstream targets for AMPK and are frequently used to gauge the activity of AMPK in different tissues. The fact that we were able to detect ACC only in the liver and brain tissues probably reflects the fact that, in these tissues, the predominant form of ACC expressed is ACC-α, whereas in the skeletal muscle and heart the predominantly expressed isoform is ACC-β (Ruderman et al., 1999). The antibodies used to detect ACC and pACC (Table 1) are reported to detect both forms of ACC; thus, these differences may reflect differences in the expression of ACC isoforms in tissues during development. Moreover, based on the levels of pACC detected, AMPK activity [as judged by pACC (S79) levels] is clearly greater in the brain than the liver during incubation, and this may reflect the fact that the developing brain has a greater metabolic rate and demand for lipid produced via lipogenesis (Turnley et al., 1999). However, this result is in contrast to the assessment of AMPK activity based on pAMPK (T172) expression, which indicated reduced AMPK activity in the brain as compared with the heart or liver. Therefore, it is also possible that the anti-ACC and anti-pACC antibodies used in this study do not detect both isoforms of chicken ACC (α and β) equally well, as is assumed for mammalian ACC. Thus, our ability to detect ACC and pACC only in the liver and brain might also indicate that the antibodies used recognized only (or preferentially) the ACC-α isoform, which would be the predominant form in lipogenic tissues such as the liver and brain.

Our findings emphasize the need to evaluate the use of different methods (i.e., direct vs. downstream target phosphorylation) carefully to assess AMPK activities, which do not always agree. Such discrepancies could arise from differences in the mode of upstream activation by AMPK kinases or in the nature of the downstream target proteins. It has been proposed that AMPK complexes containing the α-2 subunit could participate in more metabolically related actions activated by LKB1, whereas AMPK complexes with the α-1 subunit might function more in calcium-dependent actions controlled by the calcium-calmodulin-dependent protein kinase kinase (Hue and Rider, 2007). Analysis of pAMPK (T172)

gives an estimate of total activated AMPK, but does not account for differential activities of the 2 separate α-containing AMPK heterotrimeric complexes (α-1 vs. α-2). To perform that type of analysis (i.e., isoform-specific AMPK activity), prior immunoprecipitation of the samples to be analyzed for pAMPK (T172) with an α-specific antibody is required (Zhou et al., 2001; Birk and Wojtaszewski, 2006). This is not yet feasible for avian samples because of the poor performance of the AMPK α-2 antibodies that are currently commercially available (Table 1; unpublished data of the authors). In addition, many of the known AMPK downstream targets are multisite phosphorylated proteins containing one or more AMPK sites in addition to sites for other protein kinases, including those related to AMPK that are also activated by LKB1 (Lizcano et al., 2004). That creates the potential for shared or overlapping control of downstream actions attributable to cross-talk between AMPK and other cell-signaling pathways (Hue and Rider, 2007). Thus, it must be noted that the phosphorylation status of ACC or other recognized AMPK downstream targets, although important, does not necessarily constitute an exclusive measure of AMPK activity.

In conclusion, in this study we demonstrated the expression and activity of the AMPK pathway in the liver, brain, breast muscle, and heart from embryos during the latter half of incubation and from newly hatched chicks. Our findings included expression of a major upstream AMPK kinase, LKB1, and its associated proteins, different AMPK subunits, and an important downstream target of AMPK. Together, our results indicate the existence of a functioning AMPK pathway in the liver, brain, breast muscle, and heart, and they suggest a role for this pathway in the perinatal development of chickens. Future studies will continue to explore the important role(s) of AMPK in the metabolic adjustments required to achieve energy homeostasis during the transition from embryo to hatched chick.

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