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Avian proteomics: advances, challenges and new technologies

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Abstract. Proteomics is defined as an analysis of the full complement of proteins of a cell or tissue under given conditions. Avian proteomics, or more specifically chicken proteomics, has focussed on the study of individual tissues and organs of interest to specific researchers. Researchers have looked at skeletal muscle and growth, and embryonic development and have performed initial studies in avian disease. Traditional proteomics involves identifying and cataloguing proteins in a cell and identifying relative changes in populations between two or more states, be that physiological or disease-induced states. Recent advances in proteomic technologies have included absolute quantification, proteome simplification and the ability to determine the turnover of individual proteins in a global context. This review discusses the current developments in this relatively new field, new technologies and how they may be applied to biological questions, and the challenges faced by researchers in this ever-expanding and exciting field.

The holy grail of proteomics is the description of identity, quantity and state of all proteins in a cell under a specific set of conditions. Proteomics complements and extends the study of genomes and transcript data, reflecting the true biochemical outcome of genetic information. Whilst genomic and transcriptomic data provide the 'blueprint' for the possibility of cell function, they do not always inform on the actual protein content and thus the structural and biochemical effectors of a cell. Although only about ten years old, proteomics has seen rapid developments from the early studies which could be classified as 'identification proteomics' to 'characterisation proteomics' (post-translational modifications (Gruhler et al., 2005; Pedrioli et al., 2006), protein-protein interactions (Gingras et al., 2005; Arifuzzaman et al., 2006) and proteome dynamics (Doherty et al., 2004, 2005; Hayter et al., 2005) and also, 'quantitative proteomics' in which the amounts of protein are defined either relative to a comparator system (relative quantification) or in absolute terms (pmol/cell, molecular/cell, absolute quantification). Thus, proteomics has already evolved from simple questions about which proteins are present to an advanced science that invokes demanding interrogation of the protein world, defining the quantities, post-translational variants, binding partners and intracellular stability. Such information is essential for the integration of proteomics into systems biology.

In this review, we will outline some of the recent advances in avian proteomics using case studies to emphasise the utility of proteomics in understanding the biology of Aves. Avian proteome studies have been limited, but include muscle development, egg production, craniofacial disorders and the chicken lens using proteomic technologies (Table 1). Advances made in avian genomics, particularly the publication of the chicken genome sequence (Groenen et al., 2000; Hillier et al., 2004; Masabanda et al., 2004; Burt, 2005) will facilitate enhanced interpretation of proteomic experiments, minimising the requirement for cross-species matching and de novo sequencing. This should improve confi-

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Table 1. Avian proteomic studies

Application	Separation method	Mass spectrometry	Reference
Muscle growth and development		MALDI-TOF	Beynon et al. 2005
	2DGE	MALDI-TOF	Doherty et al. 2004 a, b
	1DGE	MALDI-TOF	Doherty et al. 2005
	LC and 1DGE	ESI-MS	Hayter, et al. 2003
	2DGE	MALDI-TOF AND ESI-MS/MS	Hayter, et al. 2005
	1DGE and 2DGE	MALDI-TOF	McLean et al. 2004 a, b
Facial development	2DGE	MALDI-TOF/TOF	Mangum et al. 2005
Embryogenesis	2DGE	MALDI-TOF	Agudo et al. 2005
	2DGE		Almeida et al. 2005
	2DGE	MALDI-TOF	Han et al., 2005
	2DGE and LC	MALDI-TOF/TOF and ESI-MS	Parada et al. 2005
Ocular development	2DGE	MALDI-TOF	Lam et al. 2006
	2DGE and LC	ESI-MS/MS	Wilmarth et al. 2004
Modelling organ function	LC	ESI-MS/MS	McCarthy et al. 2006
Disease and immunology	LC	ESI-MS/MS	Burgess et al. 2004
	LC	ESI-MS/MS	Liu et al. 2006
	1DGE	ESI-MS/MS	Scott et al. 2005
	1DGE and 2DGE	MALDI-TOF	Stagsted et al. 2004
Laying propensity	2DGE and LC	ESI-MS/MS	Kuo et al. 2005
Plasma proteomics	2DGE	MALDI-TOF	Huang et al. 2006
	1DGE and LC	ESI-MS/MS	Corzo et al. 2004

dence in the protein identifications provided by a typical proteomics experiment and provide the basis for further exploration of the protein component of avian species.

Previous studies

The chick embryo is a well studied system in developmental biology, and unsurprisingly has been a focus of proteomic analysis, covering areas as diverse as differentiation (Parada et al., 2005; McCarthy et al., 2006) and facial development (Mangum et al., 2005). Agudo and colleagues (2005) used stage 29 (6.5 days) embryos to probe the proteome of the developing chick. This stage was used as it marks the beginning of the differentiation towards the morphology of the hatched chick. Whilst 2,100 spots were detected on 2-D gels (nine replicates from each of three extraction protocols), only 105 spots were analysed. From these spots, 63 unique proteins were identified. Proteins identified were implicated in formation of various organs, CNS development and specific embryonic development. The authors state that these proteins represent potential biomarkers for embryonic development, however, as with many proteomic studies, only a small proportion of the proteins that were visualised on a 2-D gel were identified.

A similar study (Han et al., 2005) analysed chicken gonadal primordial germ cells to probe the molecular and physiological mechanisms underlying avian germ cell development. Again 2-D gels were used to separate the proteins and 300 individual protein spots were visualised. Proteins were identified from 89 spots with 44 unique proteins identified. This was said to represent 23.3% of the visualised proteins. It was observed that vimentin, a tissue specific, developmentally regulated protein was highly expressed in the cells. Vimentin is a member of the intermediate filament protein family and is valuable in the diagnosis of undifferentiated neoplasms. The presence of albumin (and albumin related proteins) was attributed to a role in maintaining homeostasis of the cells.

In a recent study embryonic cerebrospinal fluid (E-CSF) from embryos at stage 24 was studied (Parada et al., 2005). E-CSF fills a cavity in brain vesicles, forming a physiologically sealed system. This fluid, from stage 18 to 30 has a high protein concentration, higher than that of adult CSF. E-CSF plays an important role in CNS development, in the expansion of cephalic cavities and in the survival, proliferation and differentiation of neuroectodermal stem cells, in collaboration with known organising centres. Stage 24 was chosen for analysis as, at this stage, embryos show the greatest rate of neural stem cell proliferation and it is when the process of neurogenesis begins. Twenty six unique proteins, resolved by 2-D gel electrophoresis (2-DGE) were identified, most of which formed charge 'trains' that were hypothesised to be due to phosphorylation (although this was not confirmed). One of the known functions of E-CSF is to generate an expansive force by exerting positive pressure against the neuroepithelial walls. Identified extracellular matrix proteins and those classified as proteins of osmotic pressure and metal carriers may be implicated in this function. The interdependent and simultaneous actions of several devel-

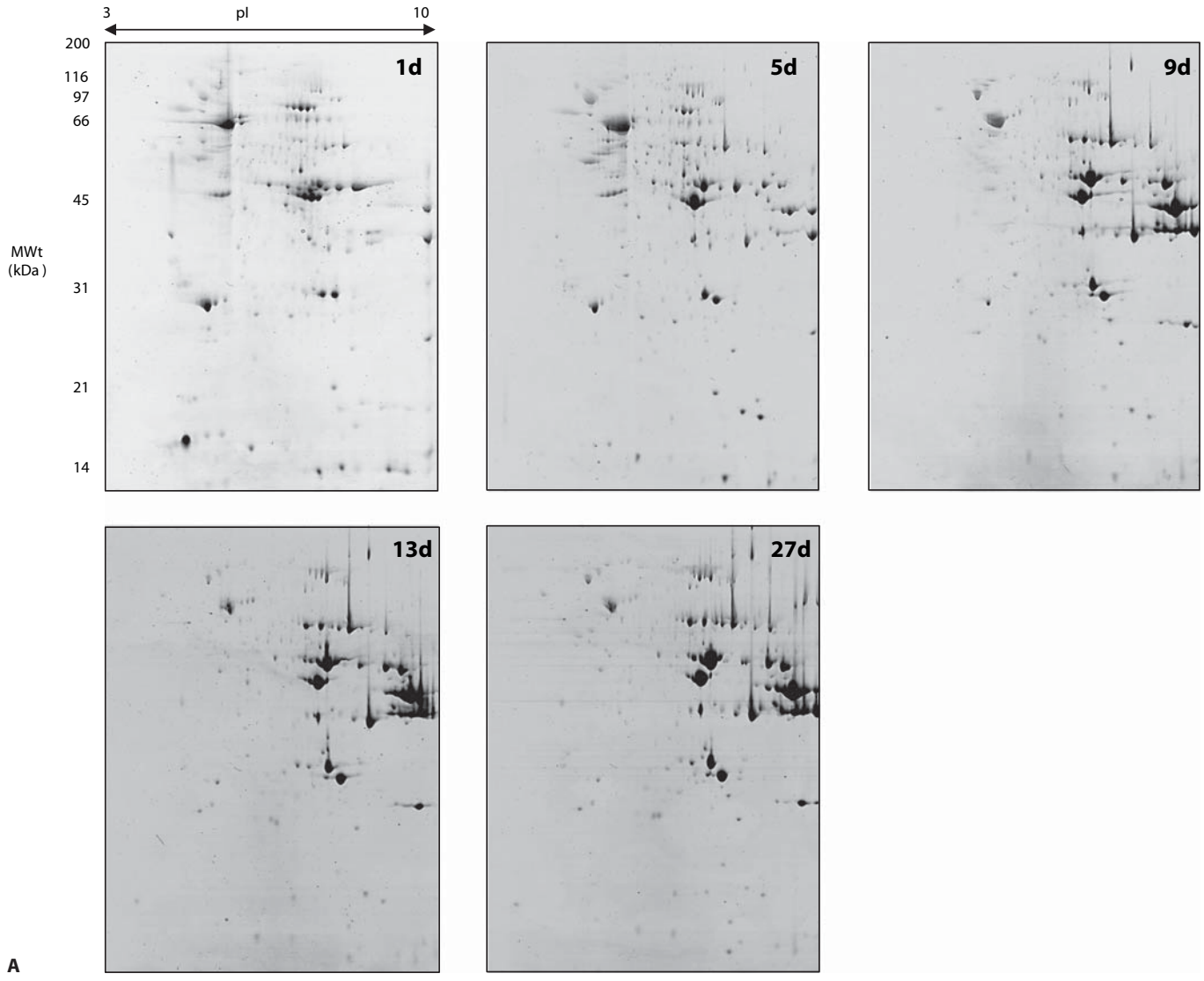
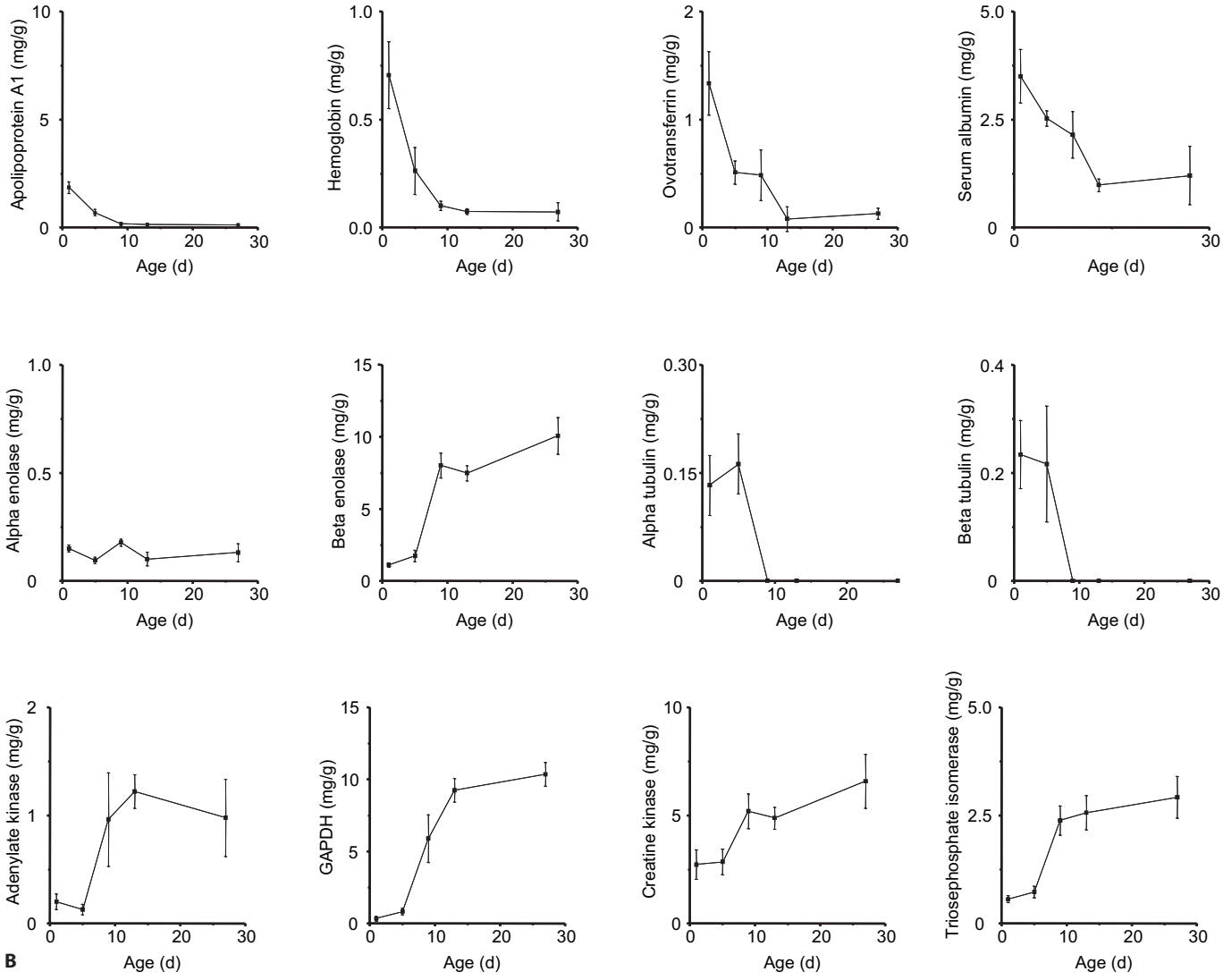


Fig. 1. Changes in protein expression in layer chicken skeletal muscle over 27 days post-hatching. (**A**) Chicken skeletal muscle from layer chickens at time-points from hatch to 27 days post-hatch were analysed by 2-DGE. Proteins were separated by charge (pI) in the first dimension and by molecular weight in the second dimension. Proteins were visualised by staining with colloidal Coomassie blue. Dramatic differences were observed in the protein profile over the time-course which represents a specialisation of the muscle as the bird matures. In particular, the older birds showed a predominance of glycolytic protein expression. (**B**) The concentration of a number of proteins was mapped over time using scanning densitometry (mean (n = 3) ± SE). The expansion of several proteins including creatine kinase, GAPDH and triose-phosphate isomerase was determined whereas other proteins such as apolipoprotein A1 and ovotransferrin decreased in concentration.

opmental mechanisms are required for CNS patterning and development. These processes are active in the stage studied. One of the functions of the E-CSF during this process is to contribute to neurogenesis via the exertion of a trophic role on the ectoderm. The E-CSF works with the isthmic organiser in the establishment of a proper pattern of protein expression. Proteins identified in this study such as apolipoproteins, retinol and vitamin D carriers and proteins related to quiescence and cell death may be important in this capacity. The authors, however, do note that the E-CSF proteome may contain other proteins, not identified by this approach and that, complementary methods will be further required before complete characterisation can be achieved.

Early craniofacial development in all vertebrate species progresses in a similar manner. Chicken embryos were used in a study by Mangum and colleagues (2005) due to their availability and the opportunity to isolate specific developmental stages. First branchial arch (BA1) tissues undergo major expansion between the E2 and E5 stages. Cellular profiles of growth and remodelling of BA1 are established but little is



known about events at the molecular level. Comparative proteomic analyses by 2-DGE of tissues from E2 and E5 showed differences in 40 spots (8% of the visible proteome) despite a 6-fold increase in tissue mass. 25 spots were analysed leading to the identification of 21 proteins, 14 of which were differentially expressed. These 14 proteins were ascribed to one of three groupings; molecular chaperones involved in protein production in either the cytosol or ER; cytoskeletal proteins involved in cell structure and mobility or plasma proteins. Additionally, some proteins were classified as involved in mitochondrial ATP production or chromosome structure.

McCarthy and colleagues (2006) used differential detergent fractionation (DDF) to study the avian bursa of Fabricius, the site of B cell development. The bursa contains both bursal B cells and a supporting stroma which is complex in nature. Little is known about the composition and interactions of these tissues. DDF was used to separate proteins prior to analysis by LC-ESI-MS/MS using a MudPIT protocol. In total 5,198 proteins were identified, 1,753 of which were B cell specific and 1,972 which were stroma specific. Only 28.3% of the total proteins identified were common to both tissues and included membrane proteins which are known to have general cellular function. A number of transcription factors were also identified, over 50% of which had not previously been identified in immune tissue from other species. In addition to the large number of protein identifications, the authors used this information to model specific biochemical pathways using human and mouse pathways as scaffolds. Specifically, the biological processes for programmed cell death, differentiation and proliferation in the chicken were annotated and receptor ligands likely to be involved in bursal signalling were identified. This paper is the most comprehensive study of an avian proteome to date, and represents the potential for proteomics to probe complex biological systems.

Our own studies have focussed on changes in the most abundant proteins in the low-salt, water soluble component of chicken skeletal (pectoralis) muscle (Doherty et al., 2004). This preparation excludes the myofibrillar apparatus which

contains such abundant proteins that they tend to overwhelm the muscle protein profile. Even when resolved by 1-D gels, the soluble proteins show a marked bias in abundance, such that very few proteins are visible. Layer chicks were obtained immediately post-hatching and reared to 27 days. At intermediate time-points, birds were culled, skeletal muscle excised and a soluble protein preparation prepared. 2-DGE of the protein extract was carried out at five time-points and the protein profile (Fig. 1) revealed that dramatic changes were occurring in protein expression from hatching to maturity. At one day post-hatch, protein spots were observed at all molecular weights and PI. Approximately 90% of the protein content of the sample was accounted for by 60 individual protein spots. Fifty three individual proteins were identified (not including post-translational variants) including structural proteins (actin, myosin light chain), proteins involved in the synthesis and modification of new proteins (elongation factor 2) and glycolytic proteins (enolase, triose-phosphate isomerase). At nine days post-hatching (and all subsequent time-points), the protein profile became simpler and markedly more specialised. The gels showed pronounced expansion of glycolytic enzymes. Relative quantification by scanning densitometry allowed the varying concentrations of individual proteins to be determined. Greatest expansion was observed for GAPDH, with other proteins such as haemoglobin and ovotransferrin decreased to trace levels.

Some indication of the complexity of interactions between transcript and protein levels was provided by the developmental switch in enolase isoforms. Alpha-enolase mRNA is dominant in the embryonic chick but expression of beta-enolase mRNA is almost exclusive in mature muscle (Tanaka et al., 1995). Although we saw dramatically enhanced expression of beta-enolase protein between 1 and 27 days post-hatch, the amount of alpha-enolase protein remained constant over the 27 day experiment. It has previously been shown that low levels of alpha-enolase activity could be detected in birds up to 40 days post-hatch but that activity of beta-enolase, in relation to total protein concentration, dramatically increased (Tanaka et al., 1985). Whilst transcript levels can indicate the potential for a protein to be synthesised, they can not specify whether a protein will be expressed, modified (post-translational modification, PTM) or what the half-life of that protein will be in vivo. In the case of alpha enolase, it is likely that there is still some low level transcription occurring after the chick has hatched but this is swamped by the substantial expression of the beta isoform. Additionally the alpha enolase may undergo minimal degradation i.e. has a long half-life, so that while little new alpha enolase is synthesised by the chick, neither is it degraded and so protein concentration levels remain relatively unchanged even when factoring expansion of the protein pool due to growth. In broiler chickens, the change in global protein expression occurs earlier than in layers (unpublished data) indicating that muscle maturation occurs more rapidly in the developing broiler chicks. This can be rationalised as birds which grow more quickly will require large energy supplies to facilitate growth.

During this study, we observed that in chicks immediately post-hatch there was a sub-cutaneous gel-like fluid coating and permeating the pectoralis muscle. Hatching requires an optimal level of water loss from eggs (McLean et al., 2004) and we conjecture that this gel either reflects an attempt to partition water within the body of the embryo, or might act as a water reserve in the first few hours ex ovo. The gel comprised of a small number of proteins in high concentrations in a profile that was similar to that of chicken plasma and resembling neither yolk nor white. However, on 2-D gels it was apparent that the subcutaneous gel, although having similarities to plasma, lacked fibrinogen. Activation of the final stages of the coagulation process might account for the enhanced viscosity of the subcutaneous gel, rendering it immobile in the subcutaneous space. The redirection of plasma in this way may be an evolutionary response to the constraints of development in a closed system that is subject to the irregularities of the environment. Chicken serum has also been analysed by 2-DGE and mass spectrometry (Huang et al., 2006) as has chicken plasma (Corzo et al., 2004; McLean et al., 2004).

Taiwanese domestic strains (B and L2) have been bred for 20 generations, from a common base population, with selection for egg production (Kuo et al., 2005). While genetically similar, egg production was found to be significantly enhanced in L2 hens over B hens and so they were chosen for a comparative proteomics study. The number of eggs laid by a hen is determined by the number of follicles destined for ovulation and the capacity of the oviduct to transform the ova into a hard-shelled egg. This process is under the strict control of hormones and other factors including energy metabolism and apoptosis of the oocyte and follicle-associated cells (Etches et al., 1984, 1990). As reproductive activity is controlled by the hypothalamus-pituitary-gonad axis, small changes in hypothalamic function may affect laying in hens. The hypothalami from hens prior to the start of egg-laying (18 weeks) and during egg-laying (60 weeks) were analysed by 2-DGE. Differences were found in 25 protein spots out of a total of 430 visualised between the L2 and B chicks at 18 weeks whereas at 60 weeks 60 differentially stained spots were found. Eight spots were found to differ at both time points. These included heterogenous nuclear ribonuclearprotein H3 (HNRPH3) and transcription factor E2F1 which are known to participate in the regulation of gene transcription and mRNA stability. Proteins which play a role in signal transduction (serine/threonine protein phosphatase 2A-ββ and wnt-11) were also altered as were proteins involved in lipid metabolism and homeostasis (VLDL receptor and apolipoprotein A1). It was postulated that HNRPH3 may act as a predictor of egg production rate with lipid metabolism and distribution in the hypothalamus determining the rate of egg yield.

Proteomic studies have also been undertaken on the avian eye (Wilmarth et al., 2004; Lam et al., 2006). The ocular lens in vertebrates is composed of highly concentrated soluble proteins (crystallins) that constitute more than 90% of the total lens protein. There are seven different β-crystallins known in chicken, which is similar to other vertebrate spe-

cies and also δ-crystallin, closely related to arginosuccinate lyase, the major crystalline in chicken ocular lens. 2-DGE was performed on water-soluble lens protein from ten week old chicken (Wilmarth et al., 2004). At this age, the abundance of δ-crystallin has decreased to levels similar to those of the major β-crystallins. The gel images showed approximately 70 spots, of which 29 were digested. 40 spots were thought to be β-crystallins (from mass alone) and 22 were positively identified as such. Splice variants and major truncation products were observed. Truncations were attributed to N-terminal extension removal although this was not confirmed.

Proteomic investigations have the potential to inform our understanding of biological systems and the few studies to date concerning chicken display the breadth of research that can be undertaken. Any biological system can be studied with limitations defined by sample availability and environment. Samples containing high concentrations of salts or those from specialised systems such as membranes may prove problematic, particularly when analysing by 2-DGE, but are not impossible to analyse. Tremendous effort is being channelled into overcoming technical difficulties so that more of the proteome can be accessed and in turn probed to answer specific biological questions.

Proteome dynamics

The studies outlined above use proteomics to compare protein profiles from samples under different physiological conditions and then identify those proteins that exhibit differential expression. This however represents a 'snapshot' of the proteome and does not define the mechanisms required to shift from one state to another. Recent work in our laboratory has established methods to determine rates of protein synthesis and degradation (protein turnover) on a proteome wide scale in intact animals, specifically chickens (Doherty et al., 2005). This ability to define the flux of proteins represents a missing link in systems biology analyses and is essential for the linking of mRNA and protein abundance data. A common misconception is that knowing the mRNA concentration of a particular gene product allows accurate prediction of the resulting protein concentration. However, this precludes any contribution from protein degradation.

Layer chickens were grown from five days on a semi-synthetic diet wherein 50% of the valine had been replaced with a stable isotope labelled variant of valine – [$^{2}H_{8}$]valine ('heavy' valine). Assuming that the dietary amino acid equilibrates rapidly with the endogenous body pool, there would be 50% probability of incorporating [$^{2}H_{8}$]valine in place of unlabelled valine ('light' valine) at any position in the protein sequence (Fig. 2). Thus, a protein that is being synthesised rapidly will incorporate heavy valine faster than a low turnover protein. However, the rate and extent of incorporation can only be ascertained if the true intracellular isotope abundance of the precursor pool is known. This will be less than 50% because of dilution by endogenous sources. In this regard, stable isotope labelling has a real advantage over radiolabelling, inasmuch as it permits mass isotopomer distribution analysis (MIDA). MIDA was developed by Hellerstein and colleagues (Hellerstein and Neese, 1992, 1999; Papageorgopoulos et al., 1999, 2002). Mass spectra of a monovaline peptide containing both heavy and light valine will contain two peak envelopes; one for the light variant and one for the heavy variant, separated by the m/z offset of the label. For di- and tri-valine peptides, three and four peak envelopes respectively will be observed, again with each envelope separated by the m/z of the label. MIDA uses deconvolution of the peak envelopes arising from multi-valine containing peptides to permit calculation of the relative isotope abundance of the heavy amino acid in the precursor pool. In our study this was calculated to be 0.35, significantly less than the value of 0.5 in the dietary input material due to incorporation of light amino acid available in the precursor pool from degradation of pre-existing proteins in the tissue of interest and from other tissues.

Once the RIA is calculated, it is possible to use this value to deconvolute the mono-valine peak envelopes to determine the percentage of newly synthesised protein. A precursor RIA of 0.35 means that for every valine residue to be incorporated there is a 35% probability that it will be the heavy variant. We are currently applying these approaches to the study of muscle proteome dynamics in relation to growth rate (Doherty et al., 2005). In a separate study, we purified the 20S core of the proteasome and after 2-D gel electrophoresis, we were able to identify all of the expected α and β proteins (Hayter et al., 2005). The rate of protein replacement was determined for each of the individual subunits using the approach outlined above. Using MIDA and mass spectrometry of tryptic peptides derived from the resolved spots, we were able to show that different subunits are synthesised at different rates (from 52 to 81% replacement over 120 h).

Challenges

Avian proteomics is a relatively new field with only a few researchers probing the global protein expression profile. With the advent of a near-fully annotated genome sequence, this will undoubtedly change. The following section shall detail some of the challenges both in terms of proteomic techniques and biological questions that will be faced in the coming years.

Infection and disease

Avian disease is a key issue for all poultry researchers. Much has been made of the emergence of avian flu and its impact on human health but chickens suffer from other pathogenic diseases such as necrotic enteritis, caused by *Clostridium perfringens* (Long et al., 1974; Wise and Siragusa, 2005), chicken anaemia virus (Todd et al., 1992) and mycoplasma type infections (Levisohn and Kleven, 2000;

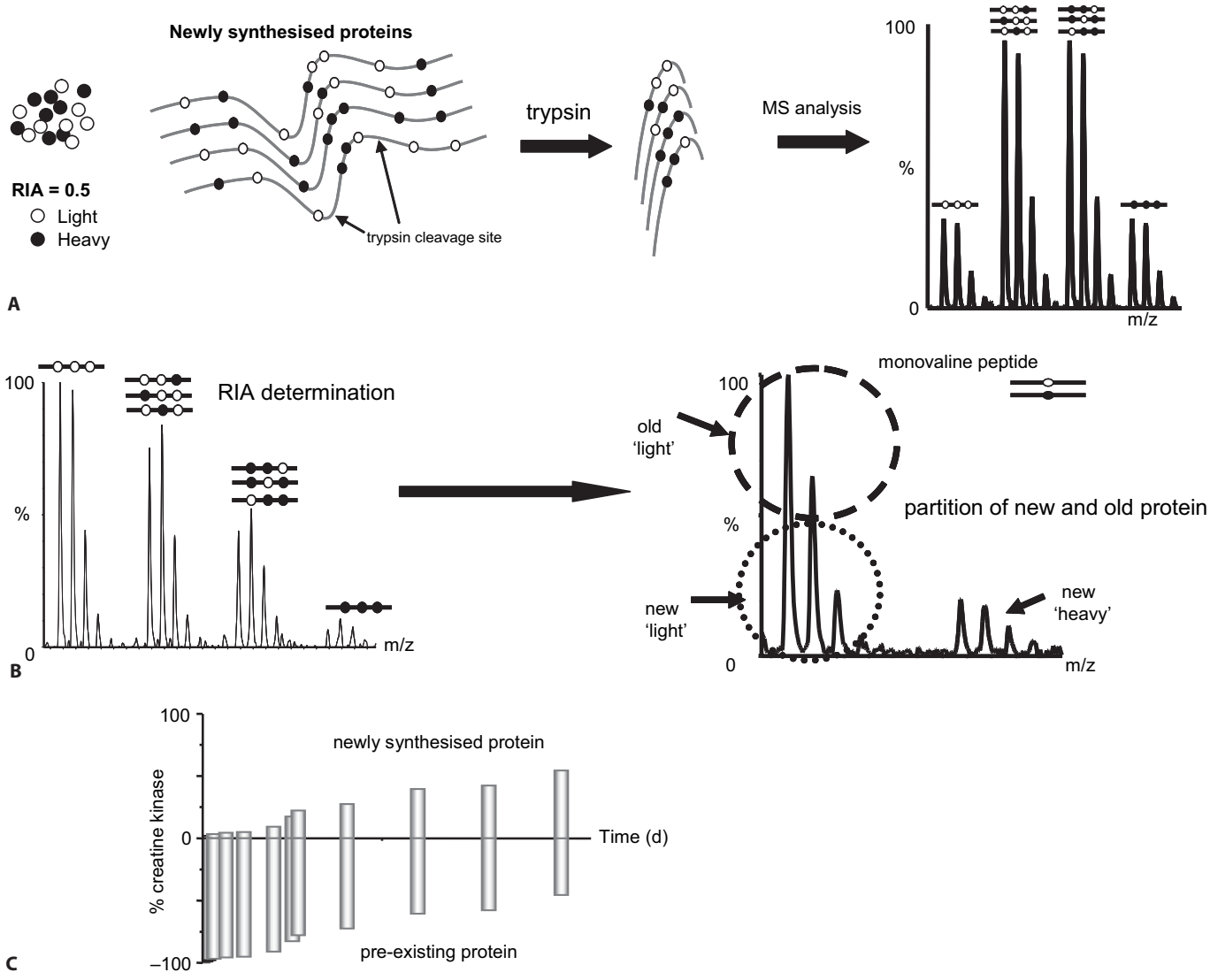


Fig. 2. Stable isotope labelling approaches to determine protein turnover in chicken skeletal muscle. In a typical proteomics experiment, limit peptides are derived from the proteolytic digest of a sample of interest. These are subjected to mass spectrometric analysis and the protein identified either by searching databases with peptide mass fingerprint or sequence information. In proteome turnover, the mass spectrum is manually inspected for peak envelopes attributed to isotopomers of the same peptide. (**A**) Chickens were fed a diet which contained 50% of the available valine as [$^2H_8$]valine. If the relative isotope abundance (RIA) of the precursor pool remained 0.5, i.e. no dilution of the precursor pool from external sources of valine, a tri-valine containing peptide would show a typical binomial (1:3:3:1) distribution of the ions derived from labeled peptides. (**B**) In reality, the stable-isotope labeled amino acid is diluted by unlabeled amino acid from the recycling of proteins in the tissue of interest and other tissues in the bird. In the example shown, a peptide containing three valine residues has been identified from GAPDH. The ratio of the peak intensities can be used to determine the true RIA of the precursor pool directly from the protein of interest. This can be used to deconstruct the peak pairs derived from a monovaline peptide to allow calculation of the amount of newly synthesised material and the amount of residual material. (**C**) This is carried out for several time points allowing replacement plots to be determined and ultimately synthesis and degradation rates. The replacement plot for creatine kinase is shown.

Ramirez et al., 2006) to name but a few. Defining the proteomics of these diseases requires not only a proteomic knowledge of the chicken and the infected organs but requires, perhaps more importantly, a proteomic understanding of the pathogen and the interplay between them. In addition to diseases inherent to avian species, the chicken is also utilised as a model for non-avian disease (Brown et al., 2003; Stern, 2005). This will increase its importance in proteomic research as it has in genome research. Broilers and layers are exposed to different life conditions (age, housing, food) and thus suffer from different diseases (Hocking et al., 1996; Anderson-MacKenzie et al., 1998; Currie, 1999). Immunoproteomics, the term used to define the proteomic analysis of immune responses to infectious pathogen is still in its infancy (Burgess, 2004). As the need to vaccinate chickens, in place of dosing birds with in-feed antibiotics,

increases, so will the necessity to define pathogen-bird protein interactions. We predict that the interaction between host and pathogen will be a growth area in proteomic and avian research. An early study has sought to combine proteomics with conventional immunology to probe the oxidative stability of proteins in chicken skeletal muscle (Stagsted et al., 2004). Soluble proteins were prepared from muscle (pectoralis and thigh) from chicks from four different feeding regimes to probe the effect of diet on oxidative stability. Gels were probed for proteins containing carbonyls or 3-nitrotyrosine. It was found that in both muscle types, enolase was extensively modified. Such modifications of enolase have previously been observed in bacteria (Cabiscol et al., 2000), yeast (Tamarit et al., 1998) and in human brain affected by Alzheimer's disease (Castegna et al., 2002). It was postulated that enolase may be a good indicator of the antioxidative state of the muscle.

Meat quality

An area of obvious interest to breeders of avian species is that of meat quality (Bendixen, 2005). The chicken remains a significant food animal and the ability to predict high quality meat traits is of immense value to the poultry industry. Protein biomarkers for muscle growth and meat quality would aid breeding programmes and ultimately provide benefits for consumers. The application of proteomic technologies to meat quality science, not only for avian species, is still in its infancy but the potential is substantial. Skeletal muscles are heterogeneous populations of fibres and are modified during their life-time relative to age, sex and breed. Whilst much is known about the genetics underlying muscle traits, little is known about the effects of the complex post-mortem mechanisms such as proteolysis, intracellular pH, ion transport and protein-protein interactions, on meat quality. There is a clear role for proteomic technologies in addressing these areas which will enhance the knowledge obtained from genetic studies.

New technologies

Even though the field of proteomics is advancing rapidly, there remain technological challenges that must be addressed. In particular, there is a shift away from gel-based technologies to high-throughput LC-MS/MS methodologies. Whilst this does not allow the rapid visualisation afforded by 2-DGE, or the ability to discriminate between protein variants, it allows higher sample throughput and analysis of proteins not possible by gel technologies (membrane proteins, extremes of pI and mass, insolubility in gel buffers). However problems still exist with issues of dynamic range. In plasma and mature muscle, protein profiles are dominated by a subset of highly abundant proteins that obscure the less abundant proteins. It is likely that the main effectors of biological or pathological differences between tissues are a result of expression changes or modifications to lower abundance proteins, and there is an urgent need to devise strategies to reach deeper into the proteome and analyse low abundance proteins.

Proteome simplification

Many researchers are seeking methods to address the issue of dynamic range with novel technologies and applications being explored. Immunodepletion of proteins such as albumin and immunoglobulin which swamp plasma samples is a common method (Granger et al., 2005; Brand et al., 2006) although antibodies are generally species specific and may have limited success across species boundaries. Other procedures rely on chemistry of specific amino acids. For example, in ICAT experiments, cysteine residues are tagged and selectively extracted resulting in a simplified representation of the proteome (Borisov et al., 2002). However, this may result in a key protein being missed if it does not contain a cysteine-containing peptide in the appropriate m/z range. A recent development in our laboratory (McDonald et al., 2005; McDonald and Beynon, 2006) has facilitated selective recovery of N-terminal peptides which not only simplifies the analytes but gives added confidence in the assignment of protein identifications. Briefly, the proteins from cell or tissue of interest are acetylated, blocking the N-termini of the protein and any accessible lysine residues. The proteins are then subjected to a proteolytic digest with an enzyme such as (but not exclusively) trypsin which results in the generation of limit peptide fragments which have exposed N-termini. The peptide mixture is reacted with a biotinylation reagent which reacts with all 'free' N-termini i.e. those from the newly formed internal peptides. The original N-termini of the proteins are blocked with an acetyl group and can therefore not react with the biotinylation reagent. The analyte mixture is subsequently passed through a streptavidin column which binds all peptides which have been biotinylated and allows the selective recovery of the N-terminal peptides alone. As the position of each peptide is known in the protein, it is possible to minimise the bioinformatic search space, facilitating a more confident protein assignment from a single peptide per protein. Large numbers of proteins can therefore be identified from a single LC-MS/MS analysis which is a substantial gain in proteomic power. The technique is obviously limited by observed m/z of the peptide and the dataset will be reduced by some peptides falling outside the analytical boundaries. In theory it is possible to overcome this technical difficulty with strategic experimental design.

Other laboratories have also addressed this problem of proteome simplification with alternate but equally elegant strategies. Combined fractional diagonal chromatography (COFRADIC, Gevaert et al., 2005, 2006) uses diagonal electrophoresis and diagonal chromatography to separate peptides in analytical space prior to mass spectrometry. This method has been used to isolate N-terminal peptides, further reducing the complexity of the analyte (Gevaert et al., 2003). Proteins are subjected to alkylation (to block cysteine

residues) followed by acetylation of all free amino groups, including the N-termini of the proteins. Tryptic digestion exposes the N-termini of internal peptides as in the previous method (McDonald et al., 2005). The peptide mixture is separated by reverse phase HPLC (RP-HPLC) and fractions collected. 2,4,6-trinitrobenzenesulfonic acid (TNBS) is added to each peptide fraction. Internal peptides react with TNBS to form hydrophobic trinitrophenyl-peptides (TNP-peptides). The true N-terminal peptides are unable to react with the TNBS due to prior modification. Each fraction is separated by RP-HPLC using the same conditions as for the initial separation. Those peptides modified with TNBS, that is, the internal peptides, experience a shift in retention time and are discarded. The N-terminal peptides are unmodified and thus will elute at exactly the same retention time, providing a purified N-terminal fraction which can be interrogated by mass spectrometry and the proteins identified by a single peptide.

Whilst the preceding methods address the need for simplification of proteomic experiments, they do not fully answer the problem of dynamic range. In analytes such as plasma and skeletal muscle, there is a bias towards the identification of highly abundant 'house-keeping' proteins. For instance, plasma is predominantly composed of nine abundant proteins (albumin, IgG, haptoglobin, transferrin, transthyretin, α_1-antitrypsin, α_1-glycoprotein, hemopexin and α_2-macroglobulin). Protein concentrations in plasma cover almost ten orders of magnitude and it is likely that any important biomarkers or functional proteins are at the lower end of that scale! Although immunodepletion kits can successfully remove the majority of these abundant proteins, they do nothing to increase the concentration of the lower abundance proteins. Righetti and colleagues (2006) have developed a technique based on solid-phase combinatorial chemistry which aims to 'equalise' the protein pool. 'Protein Equaliser Technology' uses a diverse library of combinatorial ligands (hexapeptides) which have been coupled to spherical porous beads. Each bead has millions of copies of a unique ligand and, as the beads are synthesised using combinatorial chemistry techniques, it is probable that each bead has a different ligand from every other bead. It is estimated that the library will contain approximately 64 million different ligands, providing potential bait for almost every protein in a complex proteome. Abundant proteins will quickly saturate its corresponding bead thus limiting the amount of those proteins to be analysed downstream. The remainder of the abundant proteins will be kept in solution and will not be involved in any further analysis. Therefore, all proteins, providing they almost saturate the binding capacity of their respective bead, will be present in the final analyte in equal concentrations. Once the proteins have bound to the beads they are subjected to various elution protocols which, depending on the strength of the interaction between the protein and the bead, should allow for subfractionation of the equalised proteome. Conventional proteomic techniques have previously detected and identified a maximum of 500 unique gene products in a single experiment. This new technique identified 3,200 unique gene products with 1,500 of these previously undetected using any other technique. Although this methodology is in the early stages of development and validation, it is a hugely promising avenue that should have the potential to exploit the previously hidden proteome.

Quantification

The ability to accurately quantify the absolute concentrations of individual proteins in a complex mixture, as encountered in a proteomics experiment is vital for biomarker applications and to validate inter-laboratory experiments. The majority of proteomic experiments to date have used scanning densitometry of proteins separated by gel electrophoresis to obtain relative levels of protein expression i.e. comparison of one state with another defined as percentage or 'fold' changes in expression. This procedure has a number of key limitations. 2-DGE in particular has been shown to introduce error with 20–54% loss of protein reported for a single step in the 2D procedure (Zhou et al., 2005).

Recent developments in our laboratory have allowed, for the first time, rapid, absolute quantification of multiple proteins in a complex mixture (Beynon et al., 2005; Pratt et al., 2006). This technique, known as QconCAT, is a significant advance on AQUA (Gerber et al., 2003; Kirkpatrick et al., 2005), a method which involves the chemical synthesis of heavy isotope labelled signature peptides. These labelled peptides act as internal standards for the analyte peptide and thus protein to be quantified. A drawback of AQUA is that for each peptide to be quantified, an internal standard must be chemically synthesised which is both costly and time-consuming (although a number of companies have now made these commercially available) and independently quantified. QconCAT technology is based on the concatenation of a number of signature peptides into a synthetic gene which can be expressed in a host strain such as *E. coli*. The bacteria are grown in a medium which is supplemented with the heavy isotope of choice (either a labelled amino acid or $[^{15}N]H_4Cl$). The resulting protein (the QconCAT) contains all of the labelled signature peptides (Q-peptides) for the proteins of interest. We have successfully applied this technology to the absolute quantification of the major soluble proteins from chicken skeletal muscle. Signature peptides from 20 of the most abundant chicken skeletal muscle proteins were selected using prior knowledge of the ionisation, and thus detectability, of these peptides in MALDI-MS. Peptides selected did not contain histidine, cysteine or methionine so as to minimise down-stream effects which could hamper purification (histidine) or quantification (cysteine and methionine) or which could complicate protein expression by the formation of disulfide bridges. The peptide sequences were placed in a random, linear order (concatenated) in silico and the resultant amino acid sequence back-translated following addition of required flanking amino acid sequence. The flanking amino acids were required for a number of reasons:

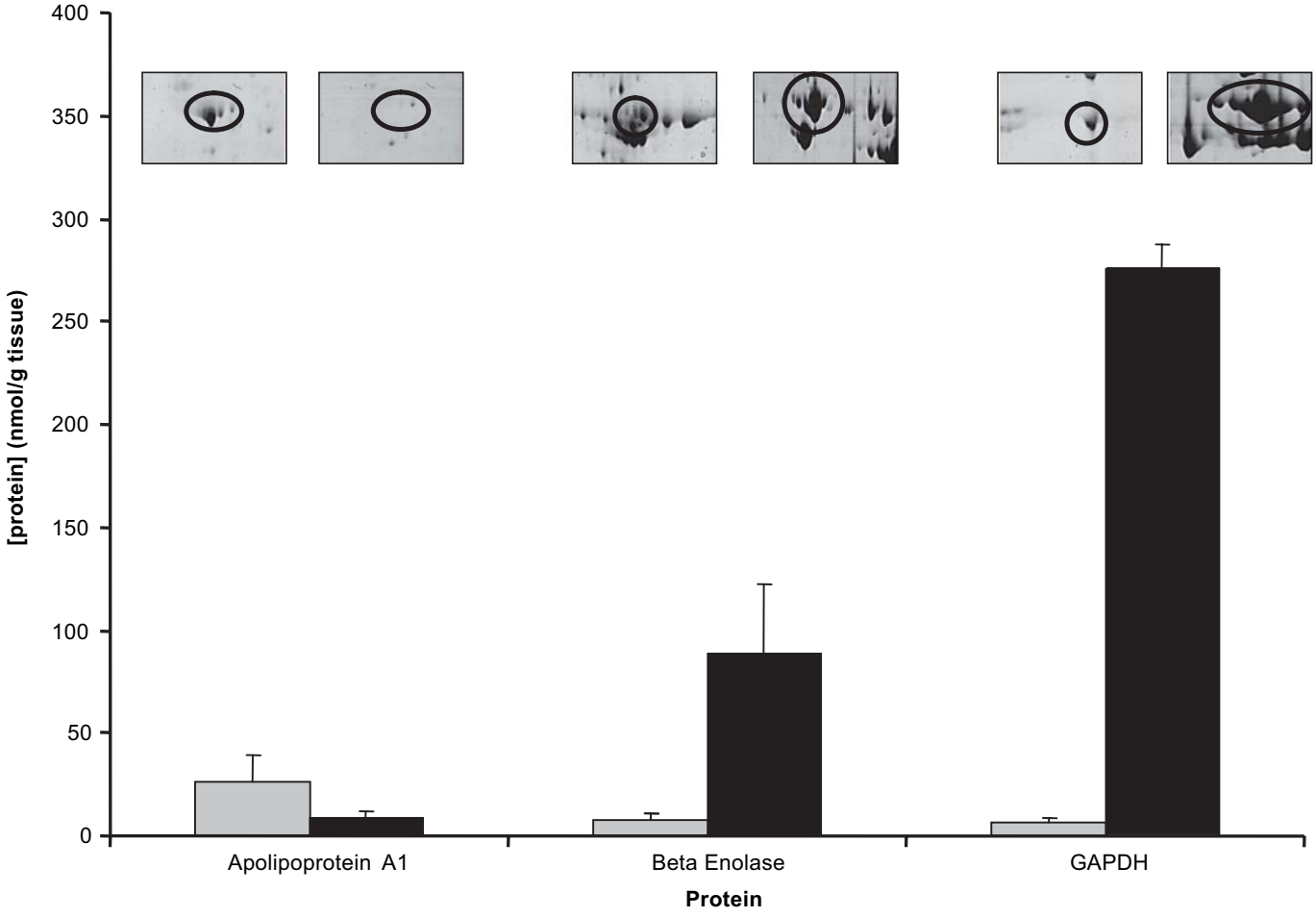


Fig. 3. Absolute quantification of proteins in chicken skeletal muscle by QconCAT. The absolute concentration of selected skeletal muscle proteins from chicken was determined using the QconCAT technology. Birds from 1 and 27 day post-hatch were used and the change in concentration with age was determined (mean (n = 3) ± SE). The changes in the concentrations of three proteins are shown: Apolipoprotein A1 which shows a marked decrease in concentration; beta enolase which increases in concentration and GAPDH which undergoes the greatest increase in concentration. Insets show the 2D gel images with each of the proteins quantified circled.

- The insertion of a C-terminal or N-terminal His-tag to facilitate rapid purification of the expressed protein
- An optional single cysteine containing motif for additional quantification using colorimetric assay
- An initiator methionine
- Sacrificial peptides flanking the true Q-peptides to maintain Q-peptide integrity at the N- and C-termini.

Once the gene is optimised it is checked for any putative secondary RNA structure that might impede translation and the peptides re-ordered if necessary. The gene is then synthesised by ligation of overlapping oligonucleotides and amplified using PCR. The gene is excised from plasmid DNA using specific restriction enzymes and ligated into a pET expression vector containing the His-tag motif as a fusion to the vector. The QconCAT vector is used to transform *E. coli*. The cells containing QconCAT are then grown in a minimal medium where access to amino acids and other nutrients can be controlled. The ability to add and subtract specific nutrients allows the experimenter to supplement the medium with stable-isotope labelled amino acids or, for example ^{15}N-ammonium chloride thus ensuring that all of the proscribed amino acids will be labeled. The choice of label can be critical. This has been extensively reviewed elsewhere (Beynon and Pratt, 2005 and 2006) but criteria include:

- If using an amino acid, the presence of that amino acid in all signature peptides
- The use of an essential amino acid i.e. the bacterium should not be able to synthesise the amino acid itself and thus dilute the chosen label with unlabelled amino acid
- A sufficient mass offset between unlabelled and labelled amino acids such that they can be readily distinguished in mass spectrometric space.

QconCAT protein expression is induced by addition of IPTG and the authenticity of the protein can readily be assessed by 1DGE of the cell lysate followed by excision, tryp-

tic digestion and MALDI-ToF MS of the proposed QconCAT containing band. Once the protein is identified and verified as the QconCAT, it is purified rapidly using nickel affinity chromatography (as the protein contains a His-Tag). Additionally, because the QconCAT protein is a completely synthetic construct, it is unlikely to have any higher order structure and thus tends to form as insoluble aggregates in the cell. This immediately confers a further degree of purity as it is easily separated from the abundant intracellular soluble proteins by centrifugation. Once validation studies have been performed (Beynon et al., 2005) the QconCAT can be added directly to the analyte that is to be quantified, in our case, the soluble fraction of chicken pectoralis muscle. In our pilot study of this new and exciting technology, the QconCAT protein was added immediately after the muscle was homogenised and centrifuged to isolate the soluble muscle proteins. However, if one was interested in the analysis of myofibrillar or membrane proteins, it could be added directly to the homogenised tissue. This means that in all subsequent processing steps the QconCAT protein and analyte proteins are present. Therefore, any processing that affects the analyte will also affect the QconCAT protein, minimising any errors in quantification. We have shown this method to be exquisitely sensitive with low errors obtained. Indeed, biological variation was found to be far greater than analytical variation (Fig. 3). The change in absolute concentration of several abundant proteins between chicks immediately post-hatch and those that were grown to 27 days post-hatch was determined. Our previous 2-DGE-based studies had shown that several proteins showed great expansion whereas others showed decreased expression. Using QconCAT technology we were able to express such changes in absolute terms, not simply percentage differences. For example GAPDH, a glycolytic enzyme involved in catalysis of the NAD(H) dependent conversion of D-glyceraldehyde 3-phosphate to 3-phospho-D-glyceroyl phosphate was shown to increase from 6.7 ± 1.7 nmol/g of tissue immediately post-hatch to 275.3 ± 11.9 nmol/g of tissue at 27 days post-hatch (mean ± SE). Apolipoprotein A1, however, fell in concentration from 26 ± 13 nmol/g of tissue to 9 ± 2nmol/g of tissue. Apolipoprotein A1 is involved in cholesterol transfer from tissues to liver and acts as a cofactor for lecithin cholesterol acyltransferase. Unlike mammalian apolipoproteins which are expressed predominantly in the liver and intestine, avian apolipoproteins are expressed in numerous other tissues including skeletal muscle. Elevated synthesis of apolipoprotein A1 in the skeletal muscle of newborn chick is hypothesised as a role for the protein as a local lipid transporter in early post-hatching development. It is feasible to surmise that the depletion with age is a real biological event.

QconCAT quantification is clearly a powerful technique that will have implications in many areas of avian biology. One might imagine a situation where a group of biomarkers for either accelerated growth or a specific disease are discovered. Instead of looking for a fold-change in expression or determining the concentration of individual biomarkers independently, it is possible to design a QconCAT that will determine absolute concentrations of each target protein in a single experiment. Additionally, biomarkers tend to exist in the cell or tissue at relatively low concentrations. As the analyst will only be looking for one or two signature peptides for each protein it is easy to envisage a simple experimental design incorporating LC separation with selective ion monitoring mass spectrometry to direct your mass spectrometer to specifically detect your ions of interest and thus minimise any effects of dynamic range.

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