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REGULAR ARTICLE

Proteome dynamics in complex organisms: Using stable isotopes to monitor individual protein turnover rates

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The complete definition of changes in a proteome requires information about dynamics and specifically the rate at which the individual proteins are turned over intracellularly. Whilst this can be achieved in single-cell culture using stable isotope precursors, it is more challenging to develop methods for intact animals. In this study, we show how dietary administration of stable isotope-labelled amino acids can obtain information on the relative rates of synthesis and degradation of individual proteins in a proteome. The pattern of stable isotope-labelling in tryptic peptides can be deconstructed to yield a highly reliable measure of the isotope abundance of the precursor pool, a parameter that is often difficult to acquire. We demonstrate this approach using chickens fed a semisynthetic diet containing [$^2H_8$]valine at a calculated relative isotope abundance (RIA) of 0.5. When the labelling pattern of gel-resolved muscle proteins was analyzed, the intracellular precursor isotope abundance was 0.35, consistent with dilution of the amino acid precursor pool with unlabelled amino acids derived from degradation of pre-existing proteins. However, the RIA was stable over an extended labelling window, and permitted calculation of the rates of synthesis and degradation of individual proteins isolated by gel electrophoresis. For the first time, it is feasible to contemplate the analysis of turnover of individual proteins in intact animals.

Keywords:
Isotopes / Protein turnover rate / Proteome dynamics

1 Introduction

Discrepancies between transcriptome and proteome data are well recognized, and the correlation between mRNA and cognate protein levels is imperfect [1–5]. One reason for these weak correlations is the lack of information on the flux or turnover of these molecules. When the intracellular concentration of any protein changes, this can be brought about by alterations in the rate of synthesis or the rate of degradation, or indeed, both processes may respond. The full definition of macromolecular expression must therefore include information on the intracellular stabilities of both mRNA and protein.

We have previously developed strategies to measure rates of protein turnover on a proteome-wide scale in continuous culture yeast cells [6]. Using stable isotope-labelled amino acids as precursors in protein synthesis it was possible to monitor the loss of labelled amino acids by MALDI-TOF-MS. Precise control over precursor labelling afforded by the continuous culture environment and the availability of auxotrophic mutants to prevent dilution of precursor pool by

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Abbreviations: CK, creative kinase; **RIA**, relative isotope abundance

endogenously synthesized amino acids meant that the labelling strategies, and hence, experiments were relatively straightforward. However, we have been interested in applying similar approaches in intact animals where the degree of experimental control is much more limited, and where different strategies are required. First, it would be impossible to obtain full labelling due to the pre-existence of unlabelled material in the animal. Secondly, the continuous reutilization of amino acids as a result of the normal synthesis and degradation cycle would dilute the precursor pool. Thirdly it would be difficult to administer label in such a way that proteins would become fully labelled. Appropriate experimental design and data analysis can overcome many of these difficulties, and we have developed an approach that can, for the first time, recover rates of turnover, on a protein-by-protein, proteomewide scale in a complex organism.

2 Materials and methods

2.1 Experimental animals and tissue preparation

Layer chickens (*Gallus gallus*, ISA Brown) were obtained 1 d post-hatching and reared to 6 d on a synthetic diet in which valine was at the level that was just sufficient for optimal growth. The diet comprised (g/kg): ground wheat: 750; soya oil: 40; cellulose: 30; dicalcium phosphate: 18; limestone: 15; vitamin and mineral mix: 5; salt: 3; choline chloride (50%): 1.5; arginine: 8.8; glycine: 6; histidine: 1.78; isoleucine: 5.15; leucine: 6.9; lysine: 8.75; methionine: 3.8; cystine: 2.2; phenylalanine: 3.75; tyrosine: 4; proline: 6; threonine: 5.75; tryptophan: 1.1; aspartic acid: 29; alanine: 20; glutamic acid: 20; valine (normal or deuterated): 5.55. The composition of the vitamin/mineral mix was designed to provide, per kg diet: vitamin A: 12 000 IU; vitamin D_3: 5000 IU; vitamin E: 50 mg; vitamin K: 3 mg; thiamine: 2 mg; riboflavin: 7 mg; vitamin B_{12}: 15 μg; nicotinic acid: 50 mg; pantothenic acid: 15 mg; biotin: 200 μg; folic acid: 1 mg; zinc: 80 mg; copper: 10 mg; iodine: 1 mg; iron: 80 mg; manganese: 100 mg; selenium: 200 μg; cobalt: 500 μg. At 6 d, birds were switched to the "heavy" diet, in which 50% of the valine in the diet (that proportion added as crystalline amino acid) was replaced with [$^2H_8$]valine (Cambridge Isotope Laboratories, Andover, MA, USA). The dietary changeover point was taken as $t = 0$. Three birds were culled at this point and pectoralis muscle was collected. Birds were fed the heavy diet for 120 h during which three birds were sampled at each of 14 times.

2.2 Gel electrophoresis

Pectoralis muscle (100 mg) was homogenized in 0.9 mL sodium phosphate buffer (20 mM, pH 7.0) containing protease inhibitors (Complete Protease Inhibitors, Roche, Lewes, UK). The homogenate was centrifuged at 15 000 $\times g$ for 45 min at 4°C and the supernatant fraction was used for analysis. The protein content of each sample was assayed using the Coomassie Plus Protein Assay (Perbio Science UK, Tattenhall, UK). Proteins were separated by electrophoresis through a 12.5% w/v SDS-PAGE gel and stained using Coomassie blue (Bio-Safe™, Bio-Rad, Hercules, CA, USA). All 42 samples (3 birds × 14 time points) were analyzed simultaneously. Gel imaging software (Phoretix 1D, NonLinear Dynamics, Newcastle, UK) was used to quantify each protein. Briefly, the band volume corresponding to each protein was determined as a fraction of the total integrated density of all bands on the gel. From this, as the total amount of protein loaded was known, the absolute amount of each protein could be calculated. This was subsequently corrected to account for mass of tissue per bird. Gel plugs containing proteins of interest were excised and the proteins were subjected to in-gel tryptic digestion and peptide extraction using a MassPrep™ digestion robot (Waters Micromass, Manchester, UK) using previously published protocols [7].

2.3 MALDI-TOF-MS

Peptides were analyzed using a MALDI-TOF mass spectrometer (M@LDI™; Micromass, Manchester, UK) over the range of 950–3500 Th. Proteins were identified by peptide mass fingerprinting by manual searching using our MASCOT [8] server (Ver. 1.9; MatrixScience, London, UK). The initial search parameters allowed for a single trypsin missed cleavage, carbamidomethyl modification of cysteine residues, oxidation of methionine, and an m/z error of ±250 ppm. An additional parameter, coded as a pseudo post-translational modification was included to search (automatically or manually) for peptides containing deuterated valine [9]. A mass difference of 7.03 Da is indicative of incorporation of a single labelled valine residue because of the loss of the α-carbon deuteron [6].

2.4 Data analysis

The relative isotope abundance (RIA) of the precursor pool was determined using multiple peptides containing either two or three valine residues. Once the precursor RIA was determined, this parameter permitted measurement of the rates of synthesis and degradation of each individual protein. For these calculations, peptides containing single valine residues were preferred, but where necessary, peptides containing multiple valine residues were used.

3 Results and discussion

3.1 Experimental design

There are compelling reasons for the use of a labelled amino acid as a precursor for protein synthesis. Other carbon precursors, such as glucose [10] label different amino acids to varying extents, and it is difficult to determine absolute rates of labelling of protein when the dynamics of labelling of the

different amino acid pools could vary. Most animals are incapable of synthesis of a subset of amino acids, and use of one of these amino acids as labelling precursor would eliminate dilution by synthesis *de novo*. However, the precursor pool is still diluted by amino acids released through ongoing degradation of pre-existing tissue proteins.

We chose to develop our approach in the chicken because the differences in growth rate between birds bred for meat production and egg production is one of the most dramatic model systems for tissue growth [11–14]. [$^2H_8$]valine was selected as the precursor, as this is an essential amino acid, and is the second most abundant amino acid in the chicken proteome [9]. Further, the mass difference between labelled and unlabelled peptides would be large enough to be isolated in *m/z* space from the [^{13}C] natural isotope envelope of each peptide. Under long light periods, the chicken will eat almost continually, which together with the retention of food in the crop means that the precursor is delivered to the intestine at an almost steady rate. Therefore, the precursor RIA should rise quickly to a plateau and then remain essentially constant throughout the labelling period. If the pre-plateau phase makes a minor contribution to the total time integral of the labelling window, it can safely be ignored [15].

A diet in which all the valine was labelled would have to be totally synthetic, comprising only crystalline amino acids, and would be sufficiently unpalatable to the birds that they would not receive adequate nutrition to attain their growth potential. Therefore, we designed a cereal based diet in which 50% of the total valine was introduced as crystalline [$^2H_8$]L-valine. Further, the dietary amount of valine was formulated to be at the limit for maximal growth ensuring optimal utilization of this precursor for protein synthesis.

Unlike cells in culture, where starting biomass can be small, the bird provides a substantial pre-existing source of amino acids that enter the precursor pool. Thus, the precursor pool dynamic is complex. The deuterated valine is diluted by unlabeled amino acid from two sources; the unlabelled proteins in the diet and the unlabelled proteins in the body of the animal. For this reason, the RIA of the precursor pool must be determined experimentally. There is some debate over the most appropriate tissue pool that should be analyzed to recover the RIA, for example, the amino acyl tRNA or the free amino acid pool [16]. However, we show here that peptides containing multiple valine residues allow direct calculation of the precursor RIA, an approach that is convenient and indeed superior, because the peptides report directly on the precursor RIA.

Consider a precursor valine pool that has a true RIA of 0.5. Whilst a protein is being synthesized on the ribosome, there is a 50:50 chance that the next valine residue incorporated into the polypeptide chain will be either heavy (H) or light (L) (Fig. 1). After the labelling phase, the protein is purified, or for example, recovered from a 2-D gel, and digested to limit peptides with a protease such as trypsin. Of these peptides, some will contain 0, 1, 2, 3 or more valine residues. Obviously, peptides lacking valine residues cannot inform on

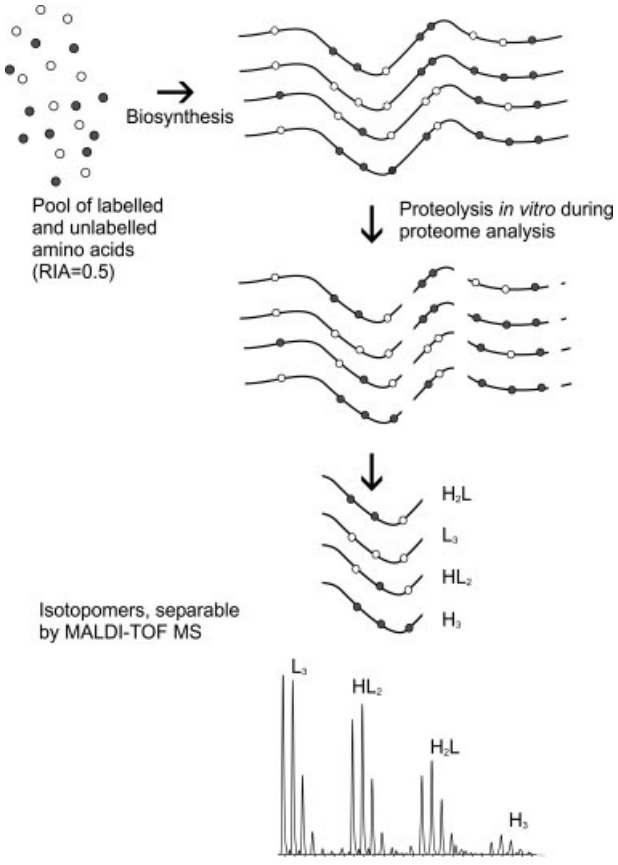

Figure 1. Stable isotope labelling of proteins *in vivo* using amino acid as precursor. Proteins labelled *in vivo* with a stable isotope-labelled amino acid incorporate the labelled amino acid according to the relative abundance of that amino acid in the precursor pool. Subsequent proteolysis yields peptides, amenable to analysis by MS in which the relative intensities of unlabelled and labelled peptide ions can be assessed. In this illustration, a peptide containing three instances of the labelled amino acid is used to indicate the complexity of the labelling pattern.

the rate of synthesis of the protein. Peptides containing a single valine residue are complex, as the 'light' species comprises an unknown mixture of peptides derived from pre-existing protein ('old') and from newly synthesised ('new') protein molecules where an unlabelled valine residue was incorporated. Without knowledge of the RIA of the precursor pool, it is impossible to disaggregate the intensity of the 'light' peptide into pre-existing and newly synthesised compartments. If the precursor pool can be set at 1.0, this issue does not arise, but this situation only pertains for stable isotope labelling in culture [6].

By contrast, peptides containing two or more valine residues can yield a direct measurement of the precursor RIA. Consider a peptide containing three valine residues. After incorporation of valine from the partially labelled precursor pool (RIA = 0.5) four variants of this peptide have been synthesized: L_3, L_2H, LH_2, and H_3 in the abundance ratio of 1:3:3:1, reflecting the fact that there are three ways to syn-

thesize the L_2H (positionally: -L-L-H-, -L-H-L- and -H-L-L-) and LH_2 (positionally, -L-H-H-, -H-L-H- and -H-H-L-) variants. However, this abundance ratio (of 1:3:3:1) would only be sustained if the precursor RIA was 0.5 and if there was no pre-existing pool of protein. Pre-existing (L_3) peptide, and any shift in precursor RIA from 0.5 leads to a distortion from this 1:3:3:1 abundance ratio. Only the L_3 peak contains pre-existing material; the LH_2, L_2H, and H_3 peaks can only have arisen from newly synthesized protein.

For simplicity, we define the precursor RIA as *r*. It follows that the relative abundance of the unlabelled precursor is (1 – *r*). The ratio of the intensities (*I*) of the LH_2 and L_2H peaks can therefore be expressed as:

$$\frac{I_{LH_2}}{I_{L_2H}} = \frac{3\cdot(1-r)\cdot r^2}{3\cdot(1-r)^2\cdot r} \text{ or } \frac{I_{LH_2}}{I_{L_2H}} = \frac{r}{(1-r)} \quad (1)$$

Thus, the RIA can be readily calculated.

$$r = \frac{I_{LH_2}}{I_{L_2H}} \Big/ \left(1 + \frac{I_{LH_2}}{I_{L_2H}}\right) \quad (2)$$

Similar arguments apply to a peptide containing two valine residues, although in this instance, the relationship is slightly different:

$$r = 2\frac{I_{H_2}}{I_{HL}} \Big/ \left(1 + 2\frac{I_{H_2}}{I_{HL}}\right) \quad (3)$$

Knowledge of the intensities of the stable isotope-labelled peptides containing multiple valine residues can therefore yield the precursor RIA. This is a real advantage of mass isotopomer analysis [17] – the ability to separate the differently labelled variants by mass means that it is possible to calculate, from multiple peptides and from multiple proteins, the true precursor isotope abundance of the pool of amino acids. Whether this is the intracellular free pool, the charged tRNA pool, or even a subcompartment of the charged tRNA pool is irrelevant, as the RIA of the precursor pool is calculated from the products.

Once the RIA value has been calculated, it is valid for all future calculations using that tissue (although it may be different in another tissue). Focus can then shift back to single valine peptides, where there is only a 'heavy' and a 'light' peak. The intensity of the light peak is a composite value, reflecting pre-existing unlabelled protein and newly synthesised protein that has incorporated unlabelled amino acid. However, the heavy peak can only reflect newly synthesised material, and, given the knowledge of precursor RIA, the intensity of this heavy peak can then be used to partition the light peak into the 'new' and 'old' components as shown below (Eqs. 5 and 6) where F_N is the fraction of newly synthesised protein. T_N, the total amount of newly synthesised protein has two components; that defined by the heavy peak, I_H, and that defined by part of the light peak, $I_{L,NEW}$. The light peak contains both pre-existing, old material, $I_{L,OLD}$, and newly synthesised peptides where an unlabelled valine residue was incorporated ($I_{L,NEW}$). Thus,

$$T_N = I_H + I_{L,NEW}$$

I_H, can also be represented by:

$$I_H = r\cdot T_N$$

where r is the RIA. Thus, $I_{L,NEW}$, is described by:

$$I_{L,NEW} = (1-r)\cdot T_N$$

From these two equations defining I_H and $I_{L,NEW}$, it follows that:

$$I_{L,NEW} = \frac{(1-r)\cdot I_H}{r} \quad (4)$$

As the fraction of the newly synthesised protein, F_N is defined by I_H plus $I_{L,NEW}$, divided by the summed ion intensities, it follows that:

$$F_N = \frac{I_{L,NEW} + I_H}{(I_L + I_H)}$$

If we then substitute for $I_{L,NEW}$ (Eq. 4) in this equation, we obtain:

$$F_N = \frac{\frac{I_H\cdot(1-r)}{r} + I_H}{I_L + I_H} \quad (5)$$

This can be further simplified to:

$$F_N = \frac{I_H}{(I_L + I_H)\cdot r} \quad (6)$$

which is the conventional equation used to describe fractional synthesis. Over the time of a labelling experiment, it should be possible to observe the increase in newly synthesized protein, and at the same time monitor the degradation or dilution (under conditions of growth) of pre-existing unlabelled protein.

3.2 Application to chicken muscle protein turnover

We administered [$^2H_8$]valine in the diet of layer chickens for a total of 120 h. There were several reasons for the choice of valine. As an essential amino acid, there would be no possibility of *de novo* synthesis complicating the labelling of the precursor pool – with our previous studies in *Saccharomyces cerevisiae*, we used a leucine auxotroph for the same reasons [6]. Secondly, the availability of [$^2H_8$]valine meant that there is clear baseline separation in the mass spectrum between the unlabelled and labelled peptides, simplifying the analysis [17]. Finally, valine is the second most abundant essential amino acid in the chick proteome [9]. Leucine is the most abundant amino acid, as it is in most proteomes, but there are complications in the use of this amino acid because of the isobaric isoleucine.

During this period of dietary administration of labelled valine, the birds grew at normal rates, and thus, the total body pool of protein was expanding. The proteins we have analyzed were those derived from the pectoralis muscle, which shows a modest increase in relative mass during growth [19]. Over the 120 h labelling period, we have assumed a linear rate of expansion of the pectoralis muscle, the same rate as of growth (0.32 ± 0.025%/h; mean ± SE, $n = 50$, $r^2 = 0.71$). At time points distributed over the 120 h labelling period, soluble pectoralis muscle proteins were resolved by one-dimensional gel electrophoresis (Fig. 2). We have previously shown that the soluble fraction of chicken skeletal muscle is relatively simple, dominated by about 20 highly abundant proteins [19] and the identities of these proteins were confirmed in this study by MALDI-TOF peptide mass fingerprinting (data not shown). The assignment of the major protein in each band was unambiguous, and once that identification was achieved, we restricted our analysis to those peptides that were derived from a theoretical tryptic digest of the same protein and which were isolated from other ions in the mass spectrum. It is highly unlikely that we have mis-incorporated ion intensity data from other trace peptides. Further, the one-dimensional analysis allows multiplexed parallel analysis of multiple tissue samples – for the present study we used three birds at each of 14 time points, a total of 42 different biological samples yielding a high level of statistical confidence in the turnover data. To illustrate the progressive change in the mass spectra, typical data for glyceraldehyde 3-phosphate dehydrogenase or triose phosphate isomerase mono-, di-, and trivaline peptides are presented in Fig. 3. We consistently observe that in skeletal muscle the offset mass between the light and heavy peptides is equal to $7.03n$ Da, where n is the number of valine residues. This reflects loss of the α-carbon deuteron through transamination, and is a useful indicator of the metabolic equilibration of the exogenous label with the endogenous amino acid pool.

The monovaline peptide (LVSWYDNEFGYSNR, $[M+H^+]$ m/z 1749.79 Th) is associated with a gradual appearance of the heavy variant, at 1756.82 Th. The appearance is slow, and for this protein is not readily apparent before 30 h. The pattern of labelling is substantially more complex for di- and trivaline peptides. The trivaline peptide from glyceraldehyde 3-phosphate dehydrogenase reveals all heavy variants, but the intensity of the H_3 peptide does not attain readily measurable labelling, even at 120 h. At a precursor RIA of 0.35, the proportion of the labelled material in H_3 could only reach $0.35^3 = 0.043$, less than 5% of the total. By contrast, for newly synthesized proteins, the L_2H peptide has a theoretical relative intensity of $3 \times 0.65^2 \times 0.35 = 0.44$, almost half of the total incorporated ion intensity. For low levels of incorporation (low turnover proteins) there may be some advantage in analyzing the single heavy isotopomer of a multivaline peptide, such as L_2H of a trivaline peptide, as this is the peptide that will contain most of the incorporated stable isotope.

The label distribution of di- and trivaline peptides was used to calculate a value for the precursor RIA as described in Section 3.1. Data from several proteins and from multiple peptides were combined over the labelling period (Fig. 4). Although individual data points (aggregated values derived from three birds and multiple peptide ions per time point)

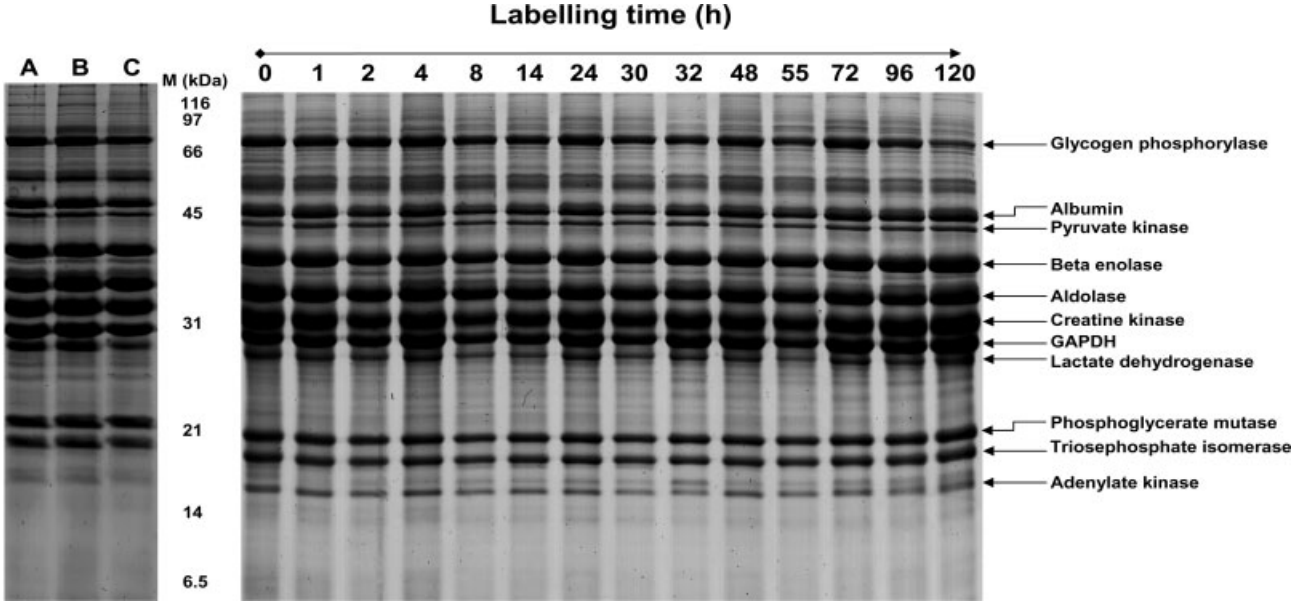

Figure 2. SDS-PAGE analysis of soluble proteins of chicken skeletal muscle. Layer chickens were fed a diet containing stable isotope-labelled valine from 6 d to 11 d of age. At times through this labelling period, triplicate animals were culled and used as a source of pectoralis skeletal muscle, from which a soluble protein preparation was analyzed by 1-D SDS-PAGE. The identities of most of the abundant proteins were confirmed by MALDI-TOF-MS. Three birds from a single time point ($t = 14$ h, birds A, B, and C) were compared to indicate the consistency of the electrophoretic pattern between individuals.

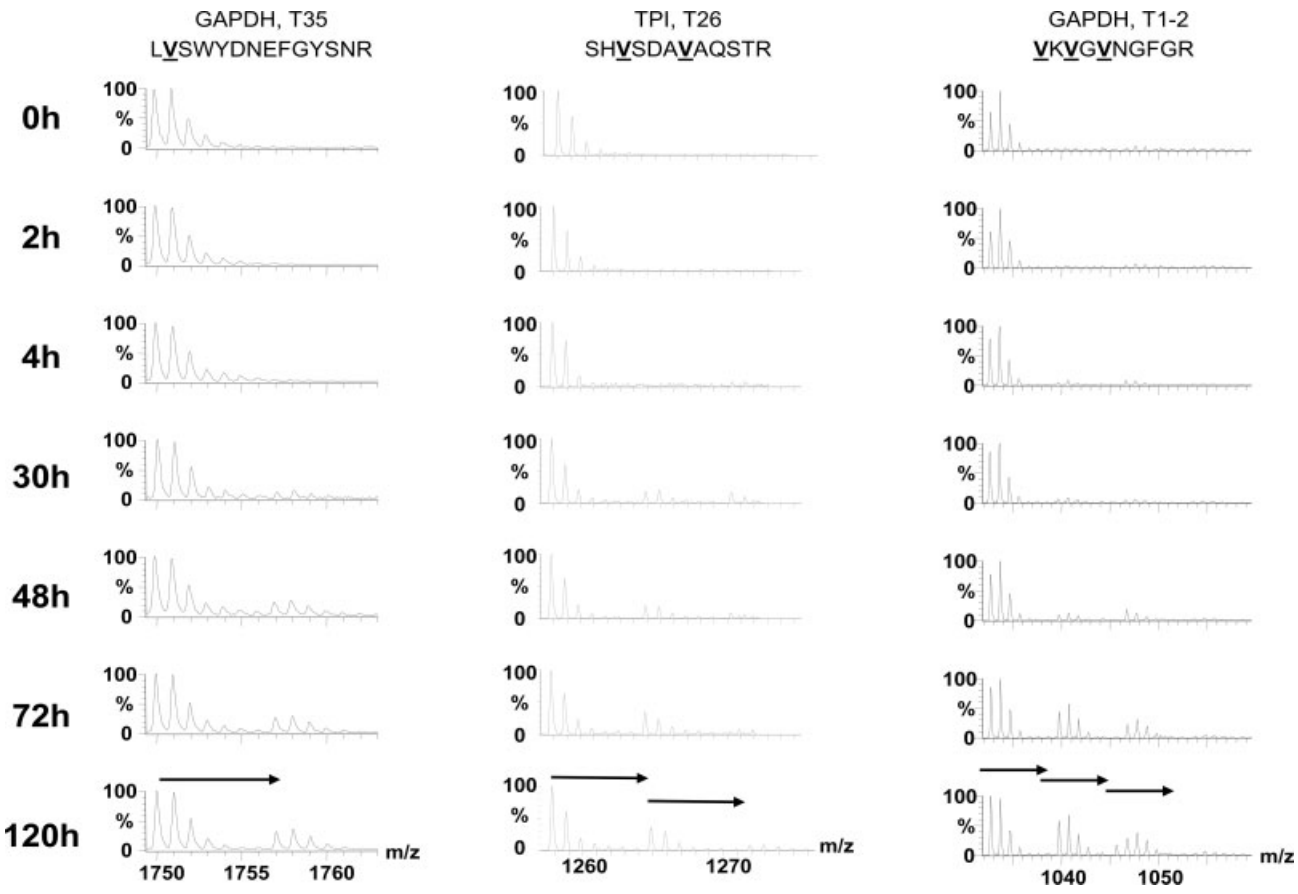

Figure 3. MALDI-TOF analysis of the incorporation of $[^2H_8]$L-valine into skeletal muscle protein. Chicken muscle proteins were labelled by oral administration of $[^2H_8]$L-valine for up to 120 h. At different times, soluble proteins were resolved by 1-D gel electrophoresis and protein bands corresponding to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI) were analyzed by in-gel tryptic digestion and MALDI-TOF-MS of the resultant peptides. Specific informative regions of the MALDI-TOF mass spectra are shown (monovaline: GAPDH, T35, L**V**SWYDNEFGYSNR; divaline; TPI, T26, SH**V**SDA**V**AQSTR; trivaline; GAPDH, T1-2, **V**K**V**G**V**NGFGR). The horizontal arrow defines a mass shift of 7.03 Da.

exhibited some variance, particularly at the start of the labelling curve when the extent of incorporation was slight, the collected data set were monotonic, and yielded an excellent fit to a monoexponential rise to plateau. The lower panel in Fig. 4 emphasizes the consistency between animals; the error attached to the RIA value for each animal reflects the variance within the value calculated using different peptides and proteins. The plateau value yielded a precursor RIA of 0.35 ± 0.01 ($n = 367$). The exponential constant for the rise to plateau was $2.4 \pm 1\,h^{-1}$, which although a less confident value, meant that the precursor pool reached the plateau value within a matter of hours (half time to attain plateau = 3.5 h) and the pre-plateau phase was an insignificant percentage of the total time integral for labelling of proteins (120 h). Moreover, the variation between different animals was relatively small (Fig. 4, lower panel), and although it would have been possible to calculate RIA for individual animals, the variance was sufficiently low that we deemed this unnecessary. Over a much longer labelling window, the RIA would eventually rise to 0.5, as all endogenous proteins were replaced by their stable isotope equivalents, but this would take much longer than the labelling window employed here. In the absence of intracellular protein degradation, an endogenous RIA of greater than 0.45 would be attained when the birds reached a body weight 10 times that at the start of the experiment – for the labelling experiment used here, the increase in body weight after 5 d of labelling was 1.6-fold. Intracellular proteolysis will diminish the time for the endogenous pool to acquire the same value as the exogenous dietary amino acid but this is clearly a slow process. However, the rate of increase was sufficiently low that we have assumed that the RIA was virtually constant over this labelling window.

Once the precursor RIA was obtained, the focus shifted to peptides containing a single valine residue, as the analysis was more straightforward. As expected, during the labelling period, the relative abundance of heavy peptides increased. As proteins are synthesized, they incorporate approximately two light valine residues for every heavy residue. Thus, an unlabelled peptide will comprise pre-existing ('old')

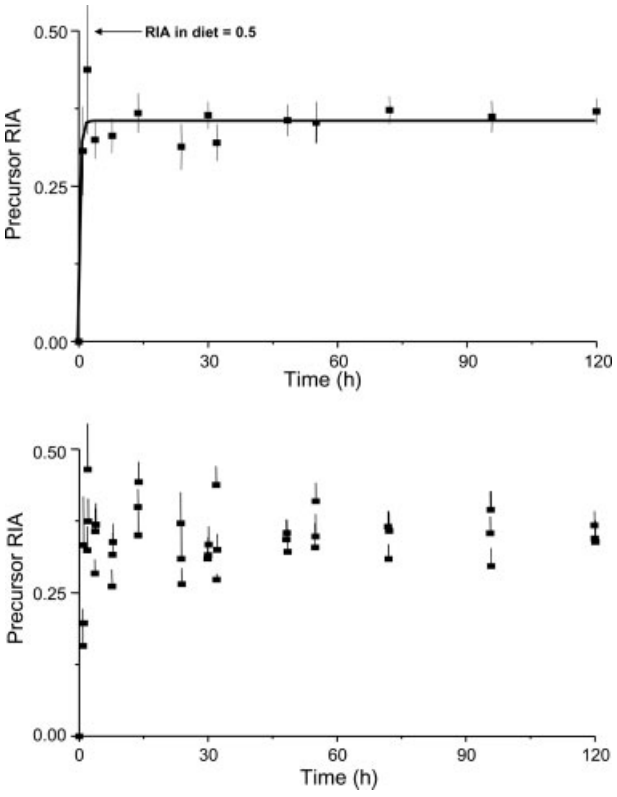

Figure 4. Precursor pool labelling kinetics. Chicks were fed a diet containing [$^2H_8$]L-valine at an RIA of 0.5 for up to 120 h. At different times, the precursor RIA was calculated for di- and trivaline peptides from creatine kinase, β-enolase, triosephosphate isomerase, and GAPDH. The top panel is the precursor labelling curve for all 42 animals, wherein each point reflects the aggregated data for multiple animals and multiple peptides at each time point (RIA = 0.354 ± 0.01, n = 367). The smooth curve is the best fit for a monoexponential rise to plateau. In the lower panel each data point shows the RIA calculated for an individual bird indicating that biological variation is minimal.

material as well as newly synthesized material ('new'), and it is essential to be able to disproportionate this peak into 'old' and 'new'. However, the labelled variant of the peptide can only have been synthesized *de novo*. Thus, the intensity of the heavy peak can be used to derive the disproportionation of the corresponding light peak. For the newly synthesized proteins, the unlabelled, light 'new' peak should have an intensity of (1-r)/r of the labelled peak; for an RIA of 0.35 this is 1.8 times. Any additional intensity of the light peak (*i.e.*, that intensity exceeding the value of 1.8 times the heavy peak intensity) reflected pre-existing 'old' material, and the rate of convergence of the light: heavy ratio to approximately 1.8 reflected turnover, that is, the rapidity with which this pre-existing material was degraded (Fig. 5). If the protein was increasing in abundance in the tissue (as a proportion of total protein) then this would need to be factored into the analysis, but for this study, we chose a time period (from 5 d to 10 d of age) where the muscle protein composition did not change dramatically (Fig. 2). We could therefore readily calculate changes in the pools of 'new' and 'old' protein over time.

The increase over time of the 'new' pool reflects *de novo* synthesis, the loss of the 'old' pool reflects protein breakdown, and because it is possible to acquire both fractions at each time, we therefore present the data as 'replacement plots' that track the shift from 100% 'old' material to predominantly new. This has been accomplished for eight major proteins in chicken skeletal muscle which represent the dominant bands in a 1-D gel electrophoresis analysis. There are two ways to express these data. First, it is possible to express the total of 'old' plus 'new' as 100%, reflecting the change in partitioning over time (Fig. 6). Then, from this calculated partition between new and old components, and factoring in the total pool size obtained from densitometry and assumptions about tissue expansion (see Section 2) it was possible to recalculate the data to reflect muscle growth (Fig. 7) and generate detailed time courses for the replacement of individual muscle proteins. All of the proteins showed substantial turnover during this time. The lowest rate of replacement was for creatine kinase and triosephosphate isomerase, such that 55 ± 4% (mean ± SE, n = 3) and 60 ± 2% of these protein pools, respectively, were replaced during the labelling period. The highest turnover was for β-enolase and phosphoglycerate mutase for which 81 ± 4% and 90 ± 9%, respectively, of the protein was replaced during the same period. Since all of the proteins were derived from the sarcoplasmic fraction of skeletal muscle, and thus, were presumably exposed to the same synthetic and degradative machinery, this labelling strategy provides a good estimate of relative rates of turnover of different proteins. With the exception of phosphoglycerate mutase, for which ion intensities were low, the variance in the data sets was remarkably small, and the replacement curves were accurately defined.

Interestingly, the trajectories were not defined well by single exponentials and two features were apparent. First, there was some evidence of periodic changes in the rate of isotope incorporation, which might reflect diurnal rhythms in protein synthesis. Previous studies have indicated that imprinting of the circadian system occurs in the prehatching period and is extended to the early posthatching period [20]. This may mean that although the birds have been maintained under a 23 h light period and will be eating almost continually, ensuring a constant infusion of isotope, protein synthesis may have been entrained by environmental factors and may show a diurnal variation even in the absence of extended light periods. Although the overall trends for protein replacement were clearly measurable, an appropriately finely grained labelling protocol could well reveal the finer detail of this pulsatile and diurnal variation in protein turnover. The precursor RIA did not show any evidence of the same type of variation, and we interpret this complexity in the labelling curve to changes in protein turnover rather than precursor pool labelling. Secondly, it was apparent that the rate of turnover of most of the proteins was declining

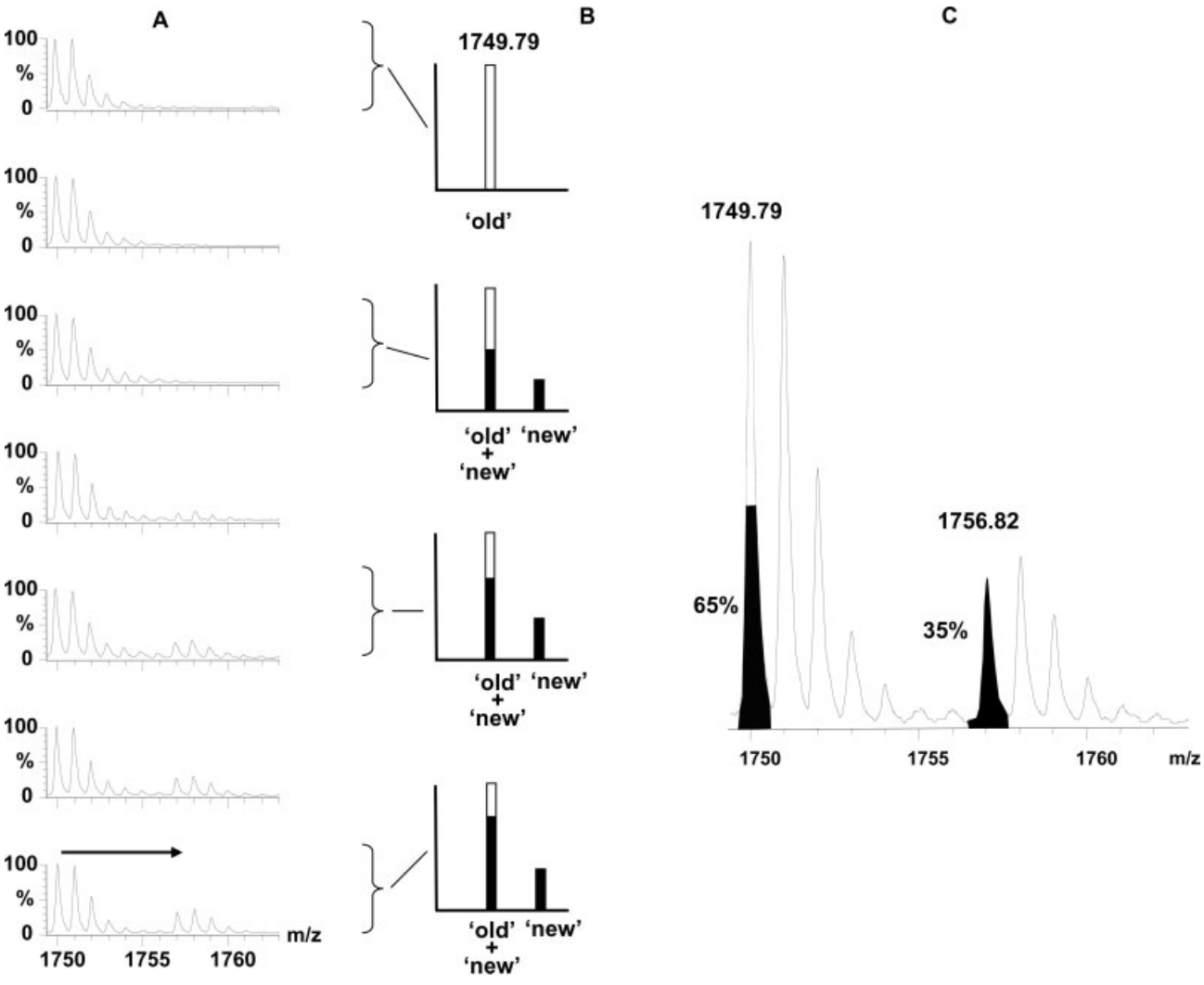

Figure 5. Calculation of turnover rate of proteins using monovaline-labeled peptides. In this example, a single peptide from one protein (GAPDH, T35, *m/z* 1749.79, L**V**SWYDNEFGYSNR) is monitored during 120 h of labelling (A). Provided that the RIA of the immediate precursor for protein synthesis is known, it is possible to deconstruct the labelling profile for peptides containing a single instance of the labelled amino acid. As the protein becomes progressively labelled, the stable isotope peptide becomes apparent in the mass spectrum as a secondary peptide ion, 7.03 Da heavier. Since the 'heavy' peptide can only be attributable to *de novo* synthesis, the intensity of this ion can be used to subsequently partition the 'light' ion intensity into that corresponding to pre-existing material ('old') and that attributable to newly synthesized protein that has incorporated an unlabelled valine residue in this position ('new'; B, indicated by shading; only the monoisotopic peaks are shaded for clarity, although the same arguments apply to the entire natural isotope distribution). At 120 h the unlabelled peak contains 65% of the newly synthesized protein; the remaining 35% is located in the 'heavy' peak (C). It is therefore possible to assess the rate of turnover of individual proteins, even from monolabelled peptides, once the precursor RIA has been calculated for that tissue.

throughout the labelling period. This would be expected in a rapidly growing tissue, and it is reassuring to observe the same behaviour here.

Some indication of the consistency of the analysis can be obtained from individual proteins for which there is more than one monovaline tryptic peptide. For creatine kinase (CK), peptide ions at 1217.62 Th and 1118.66 Th are both derived from monovaline peptides and can therefore be used to yield separate estimates of the partition between newly synthesized and pre-existing protein. When the percentage of newly synthesized CK is calculated for each peptide (and using the same value for RIA = 0.354) the correlation was excellent (r^2 = 0.95, n = 78, Fig. 8). Other proteins did not have useable monovaline peptides in the MALDI-TOF spectrum, either because they were too small or too large for the effective operational range of a typical MALDI-TOF instrument (in this case, 950–3500 Th), because they failed to ionize in the source, or because the isotope distribution overlapped the envelopes from other tryptic fragments or contaminant ions. It is possible to analyze both di- and triva-

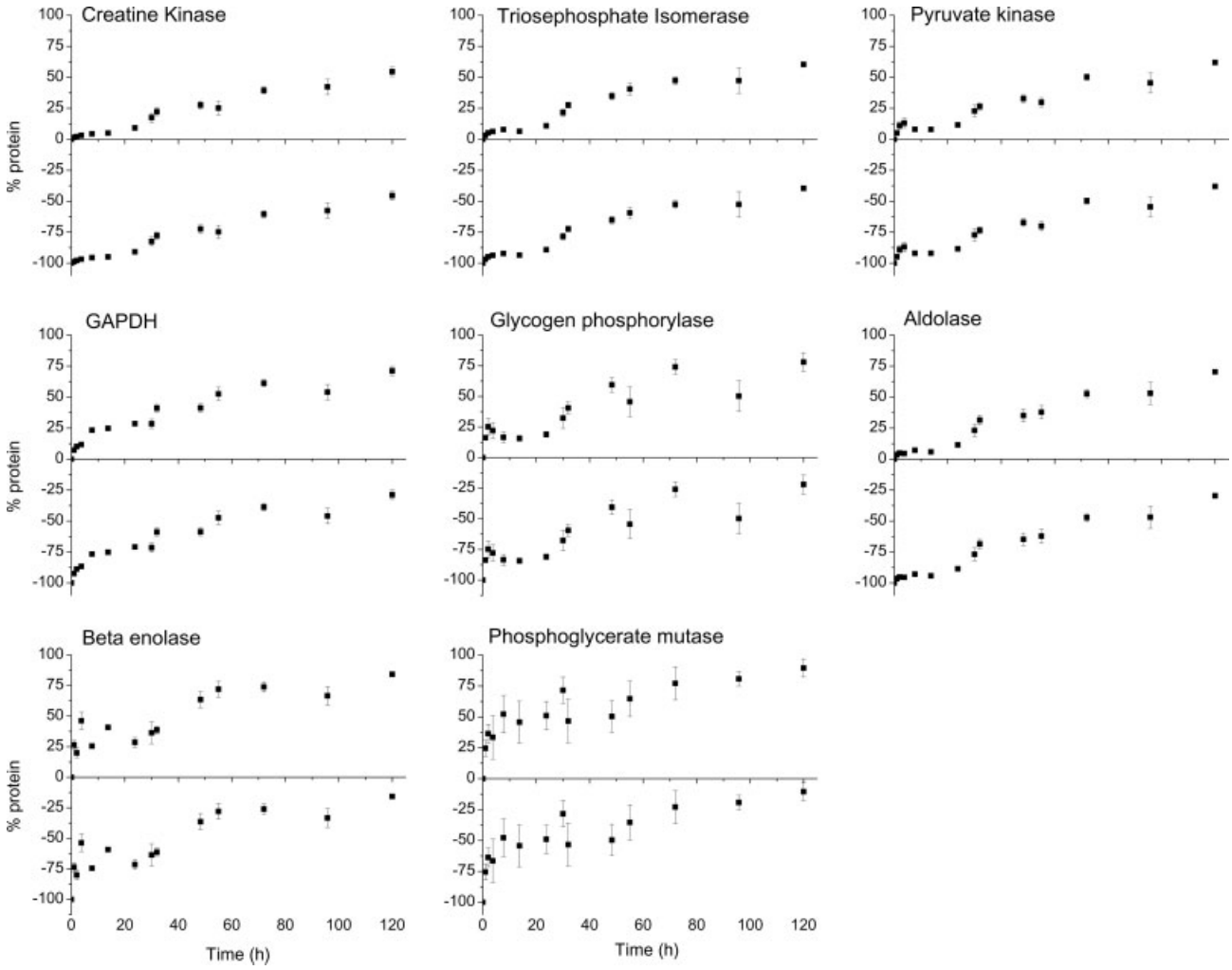

Figure 6. Turnover of individual soluble proteins in skeletal muscle measured by incorporation of stable isotope-labelled amino acids. Using the calculation defined in the text the transition to newly synthesised ("new", equal to $F_N \times 100$) from residual ("old", equal to $F_O \times 100$) protein was calculated over 120 h for eight abundant soluble proteins in chicken skeletal muscle. To aid in interpretation, the percentage of "old" protein has been plotted on a negative scale, such that the sum (new+(−old)) = 100 at all points, and thus reflects the partition between the new and old pools. Each time point is the aggregated data from three birds and multiple peptides per protein (mean ± SEM, n = 3–6).

line peaks in the same manner as the monovaline peaks and we compared turnover data from di- and trivaline peptides to those obtained from monovaline peptides using CK as exemplar. The correlation was very good (r^2 = 0.81 to 0.97, Fig. 9), and confirmed that multivaline peptides are just as valuable in the calculation of turnover rates. For some proteins in this study, such as adenylate kinase, it was not possible to obtain turnover rates. Although tryptic digests of the protein *in silico* indicated valine-containing peptides of suitable mass for MALDI-TOF analysis, they were not observed experimentally in either the light or heavy derivative. For such proteins, alternate matrices, ionization modes or mass spectrometric analysis might provide a solution. Additionally derivatization of peptides, for example of lysine-terminated peptides by guanidination, may improve detectability [21].

4 Concluding remarks

The ability to measure individual turnover rates in a complex, multicellular organism is a significant advance on our previous work with unicellular organisms. Although the isotope labelling becomes substantially more complex, the isotopomer patterns can eliminate the uncertainties in determination of the RIA of the precursor. Although we have used MALDI-TOF-MS for this study, there is no reason why a higher throughput LC-MS/MS approach could not be used. A minor complication might be that [^{2}H] amino acids exhibit differences in peptide elution times on reversed-phase separations. A [^{13}C] amino acid might be preferred for such studies, although as each bird maintained to 120 h was supplied with approximately 300 mg labelled amino acid, result-

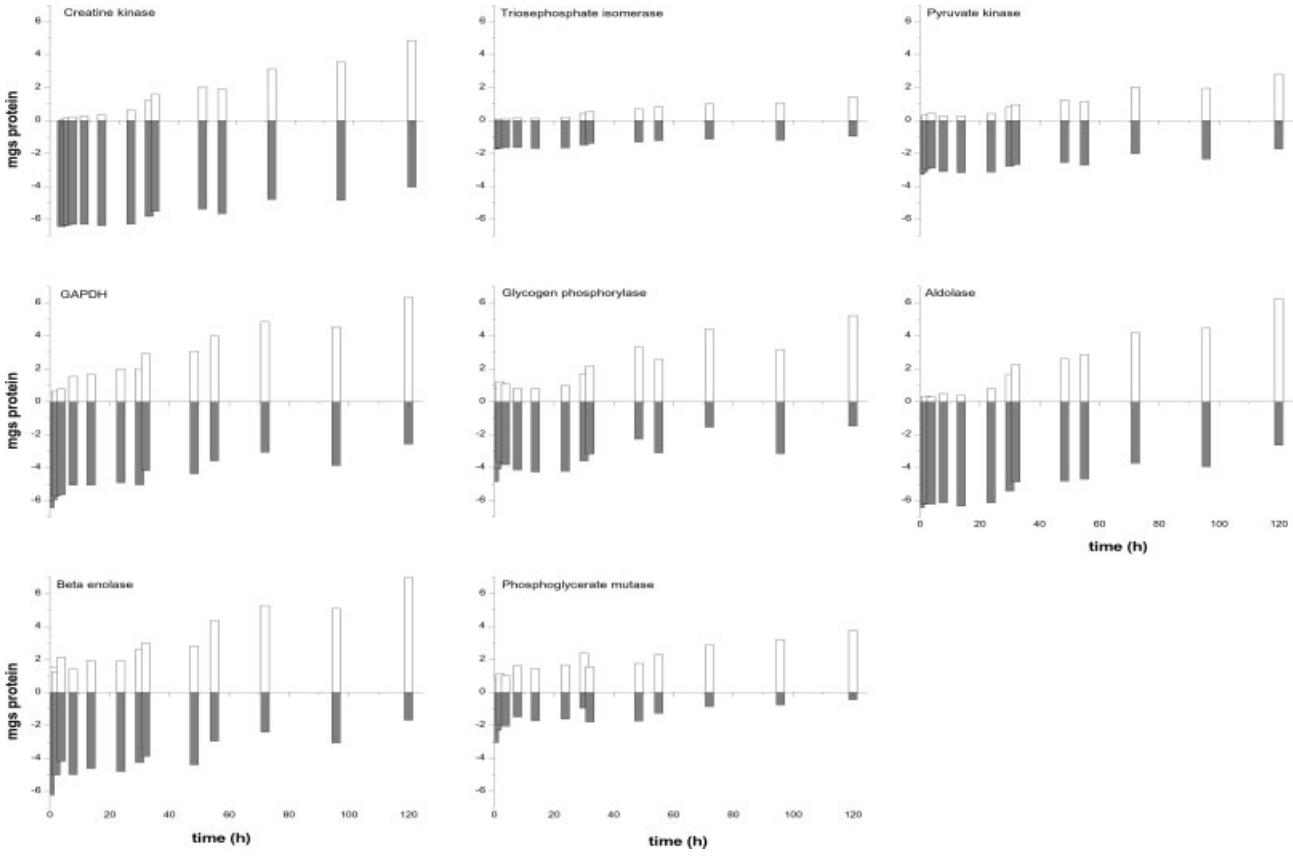

Figure 7. Protein turnover and growth. The partition between pre-existing and newly synthesized protein pools has been calculated for the eight abundant soluble proteins in chicken skeletal muscle between 0 and 120 h to taking into account the amount of each protein (determined by quantitative densitometry of the 1-D gel electrophoresis) and the expansion of the protein pool during growth. Protein is expressed as mg protein/g tissue at $t = 0$. Each time point is the aggregated data from three birds and multiple peptides per protein (mean ± SEM, $n = 3$–6).

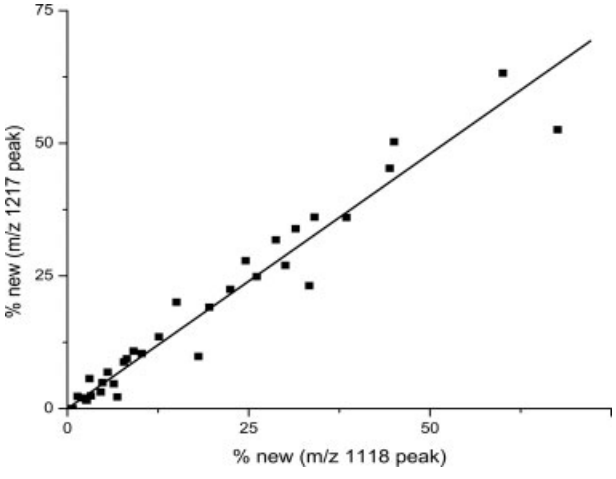

Figure 8. Correlation analysis of calculated turnover rates from monovaline peptides. Two monovaline tryptic peptides from CK (T11, DLFDPVIQDR, *m/z* 1217.62 and T6-7, VLTPELYKR *m/z* 1117.66) were used to calculate the proportion of newly synthesized protein. The calculation was repeated for each individual bird, and expressed as a scatter plot to indicate the agreement between two different monovaline peptides from the same protein.

ing costs will not be insignificant. At a dietary RIA of 0.5, dilution of the label by dietary and intracellular unlabelled amino acids leads to a tissue precursor RIA of 0.35. At most therefore, a protein that is completely replaced during the labelling window expresses unlabelled (light) peptide ions that are twice as abundant as the labelled (heavy) variant. The current generation of MALDI-TOF-mass spectrometers show exquisite sensitivity and high resolution, but are not particularly suited to determination of the relative abundances of stable isotope and unlabelled elements. We therefore consider that an incorporation of between 5% and 10% of stable isotope label is the minimum that could be measured with confidence. These limitations aside, the method outlined here has redundancy, a tolerance to individual variation and the ability to define turnover rates on single proteins isolated by gel electrophoresis, or indeed, by a predigested mixture of proteins resolved by LC-MS/MS.

Although it is now feasible to include proteome dynamics in functional genomics studies of intact animals, it is worth noting that the chicken was particularly suitable for these 'proof of concept' studies. Under appropriate lighting regimens, the birds can be persuaded to adopt a feeding pat-

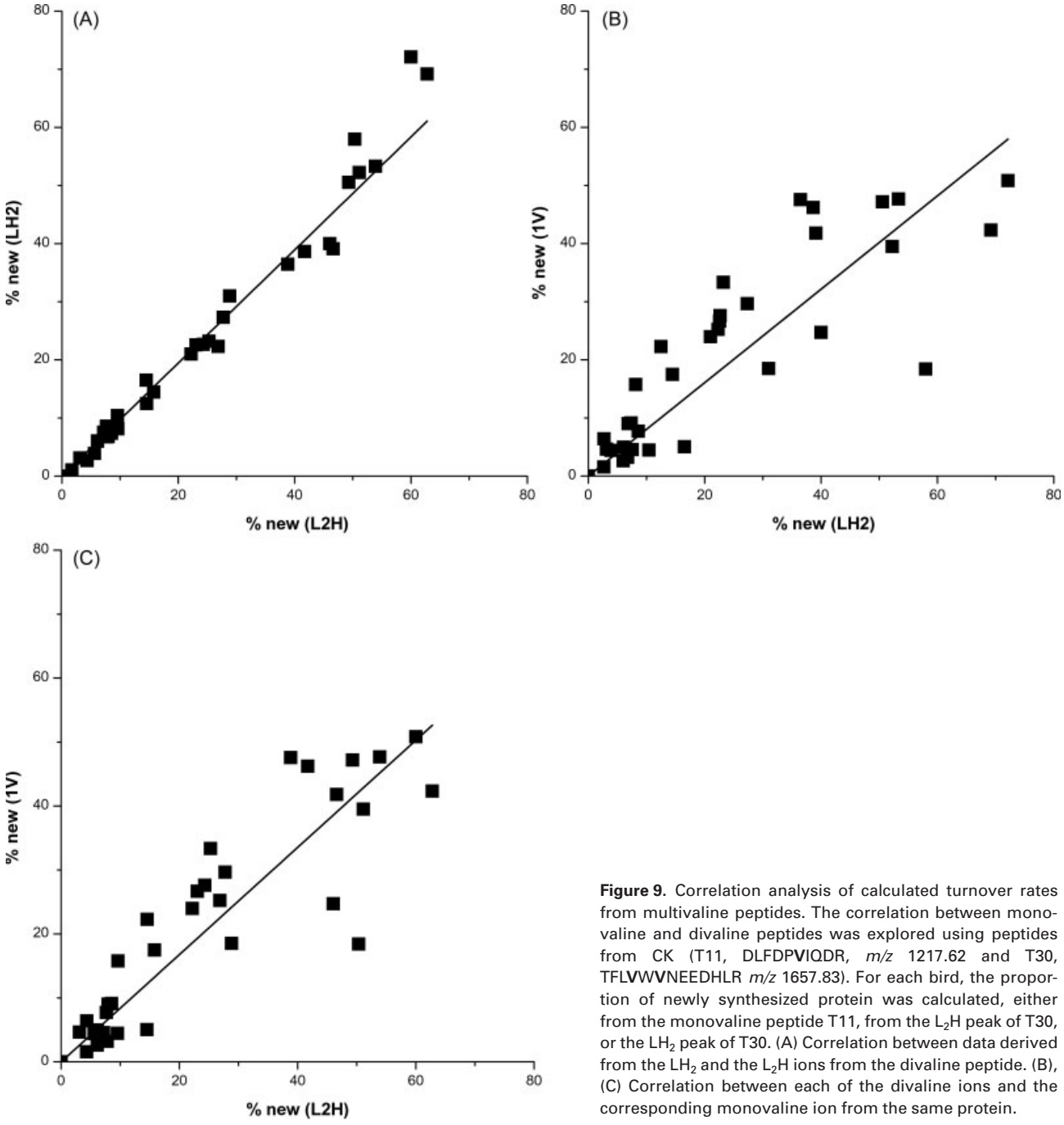

Figure 9. Correlation analysis of calculated turnover rates from multivaline peptides. The correlation between monovaline and divaline peptides was explored using peptides from CK (T11, DLFDP**V**IQDR, *m/z* 1217.62 and T30, TFL**V**W**V**NEEDHLR *m/z* 1657.83). For each bird, the proportion of newly synthesized protein was calculated, either from the monovaline peptide T11, from the L_2H peak of T30, or the LH_2 peak of T30. (A) Correlation between data derived from the LH_2 and the L_2H ions from the divaline peptide. (B), (C) Correlation between each of the divaline ions and the corresponding monovaline ion from the same protein.

tern that ensures almost continuous delivery of label, almost akin to continuous infusion experiments. Further, the crop and gizzard also conspire to even out the rate of delivery of ingesta to the digestive system. Extension of these studies to other animals may require novel approaches. Mammals, for example, are predominantly meal feeders, which might elicit short term variation in the RIA of the precursor pool. This need not necessarily be problematic, especially if rates of protein turnover are to be monitored over several days when such variation in the overall trajectory might be readily extracted from sufficiently finely grained data. Other experimental systems might be equivalently amenable to such studies. For example, *Caenorhabditis elegans* has been grown on bacterial lawns labelled to homogeneity with stable isotope amino acids [22]. Although the goal of this study was to obtain fully labelled *C. elegans* proteins for comparative proteomics studies, there is no reason *per se* why nematodes could not be harvested during the labelling period to monitor

the trajectory of protein labelling. Alternatively, prelabelled nematodes could be transferred to a lawn of unlabelled bacteria and the loss of label could be assessed, although reutilization of label would need to be assessed. Such experimental strategies have informed the thinking of those interested in protein turnover for several decades [15] although these studies have mostly used radiolabelled precursors. If stable isotopes are used instead, there are multiple gains, including lack of radiation hazards, experimental convenience, and the critical advantage of being able to report the isotopic enrichment of the immediate precursor – this parameter is very difficult to acquire with radiolabelling studies. The ability to address issues of intracellular proteome dynamics on a protein-by-protein, proteome-wide scale is a significant step forward, and extends our ability to understand the proteome in relation to the transcriptome and metabolome.

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