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Postmortem muscle proteomics reveals breed specific responses to environmental enrichment and broiler meat quality

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The meat industry faces growing pressure to adopt sustainable and welfare-friendly practices. This study used mass spectrometry-based proteomics to examine the effects of genetics and on-farm environmental enrichment on broiler performance and meat quality. Slower-growing (SG; Hubbard S757N) and faster-growing (FG; Hubbard JA787) broilers were raised in enriched and non-enriched environments within higher-welfare systems. The SG broilers showed higher expression of detoxification and cytoskeletal proteins, supporting robust muscle architecture, higher protein content and reduced moisture retention. Enriched environments further enhanced immune function, metabolic resilience and physical health in SG broilers. Conversely, FG broilers prioritised anabolic pathways, driving rapid muscle growth and intramuscular fat accumulation. Growing in enriched conditions led to reduced breast yield in FG broilers, likely due to higher proteasome activity. These findings highlight the importance of breed-specific strategies to support sustainable farming, as only SG broilers benefited from environmental enrichment, potentially improving meat quality while supporting welfare outcomes.

Meat has been a key component of the human diet for millions of years and remains an important source of high-quality protein[1]. Poultry is projected to supply 43% of the global protein intake from meat by 2033, positioning it as the fastest-growing segment in meat production and consumption[2]. This demand is driven by poultry's favourable combination of high-quality protein, low-fat content[3], lower production costs, sustained productivity and the lowest environmental impact compared to other meat sources[2]. To address rising global demand and optimise production systems, genetic advances have led to hybrid, faster-growing breeds[4], that reach weights of 1.5–2.5 kg within 28–42 days[5], representing a three-fold increase in growth rate compared to unselected, slower-growing breeds[4]. However, this rapid growth raises concerns regarding meat quality[6], as evidenced by increased incidences of muscular myopathies, which may reflect the physiological limits of animal biology imposed by intensive selection[7].

As breeders and producers strive to balance profitability with quality, there is a growing focus on husbandry factors to enhance animal welfare and intrinsic meat quality[8–10] without reducing too much economic efficiency[11,12]. This transition promotes the adoption of lower-input, higher-welfare farming systems that integrate slower-growing genetics[13,14], reduced stocking density[15–17] and environmental enrichments[18]. These can include perches, platforms, and materials that encourage foraging and dustbathing, aimed at promoting species-specific behaviours that may improve the physical and mental well-being of animals[19]. These factors have been collectively indicated as "extensification factors"[20], some of which can be linked to meat quality.

Intrinsic meat quality is shaped by physiological changes in the muscle during husbandry and biochemical alterations during muscle-to-meat conversion[21]. These changes modify the protein composition of the muscle, which can be studied to reveal molecular-level adaptations. Advancements in mass spectrometry (MS)-based proteomics combined with bioinformatics[22] have emerged as powerful tools for characterising these molecular changes[21]. While many current biomarkers are derived from beef

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and pork[23], extending their applicability to poultry presents an exciting research opportunity. Despite the rapid growth of broiler production, only a few studies have investigated the chicken proteome across different breeds and farming systems (organic vs. non-organic)[24–26] to discover authenticity markers. These markers are particularly important in meat from "extensified" systems, which are more costly to produce but add value to the product, making it vulnerable to food fraud[27]. However, there is a paucity of research that considers individual animals alongside their production data in a large controlled experimental setup to study the effect of extensification factors.

This study, conducted as part of the *mEATquality* European Union project, used a large intervention experiment to examine the main effects and interactions of extensification factors in higher-welfare, non-organic systems with relatively low stocking density. As illustrated in Fig. 1, the study focused on how genetics and on-farm enrichments (as two extensification factors) influence broiler performance, intrinsic meat quality and the molecular mechanisms underlying these traits with the aim of supporting improved production strategies.

Results and discussion

Performance, yield and meat quality characteristics in broilers

Broiler performance data were analysed on the pen level and summarised in Table 1, comparing slower-growing (SG) and faster-growing (FG) broilers raised under non-enriched and enriched conditions. Significant breed effects on performance were observed, with no interactions or enrichment effects. Body weight, feed conversion rate (FCR), daily growth, and feed intake were significantly influenced by breed ($p < 0.001$). SG broilers consumed less feed daily, showed lower daily growth and had higher FCR, consistent with previous findings[13,28]. Mortality rates were low overall, with a non-significant trend toward higher mortality in enriched conditions ($p = 0.07$).

Eight male birds per pen (40 per treatment, 160 total) were randomly selected for further analysis. Meat yield and post-mortem pH values are illustrated in Fig. 2. The dressing percentage was higher ($p = 0.03$) in FG broilers (67.4 ± 0.9%) than in SG broilers (66.4 ± 0.8%), resulting in a greater overall meat yield (Fig. 2a), which is consistent with previous findings[13]. A higher breast muscle yield (20.4 ± 0.9%, $p < 0.001$) was also observed in FG broilers, reflecting a larger allocation of muscle mass to the breast area (Fig. 2b). In contrast, drumstick contribution (%) to carcass weight did not differ significantly (16.2 ± 0.6%) (Fig. 2c). These findings align with selective breeding practices in FG broilers, aimed at promoting rapid muscle growth and enhancing breast muscle yield to optimise meat production[29]. Enrichment also significantly ($p < 0.01$) influenced breast muscle development, with a genetic-enrichment interaction ($p = 0.03$) (Fig. 2b). In enriched environments, FG broilers showed lower relative breast weights (19.7 ± 0.4%) than those raised in non-enriched environments (21.1 ± 0.5%), highlighting the pronounced negative impact of enrichment on FG broilers. Conversely, SG broilers showed similar breast weight contributions (%) in both enriched (14.5 ± 0.8%) and non-enriched (14.7 ± 0.7%) environments.

Post-mortem pH was measured at 24 h and 48 h (Fig. 2d). At 24 h post-mortem, the average pH was 5.92 ± 0.04, with no significant differences across treatments. At 48 h, SG broilers (5.86 ± 0.06) exhibited a significant decrease, unlike FG broilers (5.92 ± 0.05). All pH values remained within the normal range for quality chicken breast[30]. This breed-specific trend supports previous findings showing that SG broilers undergo a higher post-mortem

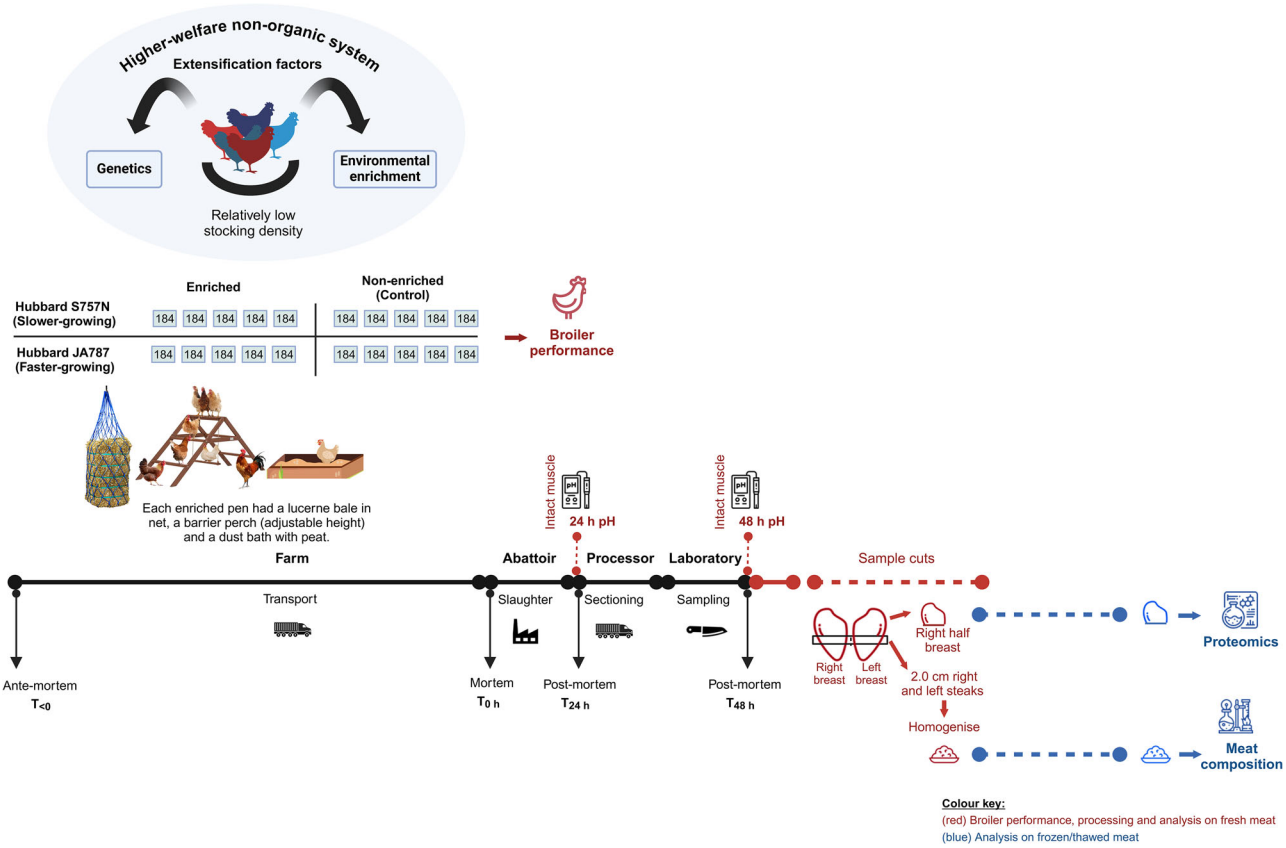


Fig. 1 | Workflow of the study from farm to laboratory. The timeline from the farm (ante-mortem) through transport, slaughter (T_0), sectioning, sampling and pH measurements at 24 h ($T_{24\,h}$) and 48 h ($T_{48\,h}$), highlighting sample cuts designated for each analysis. Small boxes represent pens, each replicated five times on the farm. A total of 184 day-old chicks (50% male, 50% female) were placed in each pen, resulting in 3680 broilers. The study tested four treatments using two breeds: Hubbard S757N (slower-growing) and Hubbard JA787 (faster-growing) under two environmental conditions: non-enriched (control) and enriched, all with relatively low stocking density. Figure created with BioRender.com.

Table 1 | Broiler performance in slower-growing (SG) and faster-growing (FG) broilers under non-enriched (NE) and enriched (E) conditions ($n = 5$ pens per treatment group)

	SG-NE	SG-E	FG-NE	FG-E	SE
Final body weight (g)	1876.2[a]	1882.0[a]	2074.2[b]	2085.7[b]	8.8
Mortality (%)	0.33	0.87	0.22	0.87	0.3
Feed Conversion Rate	2.23[a]	2.24[a]	1.73[b]	1.72[b]	0.01
Growth (g/day)	29.1[a]	29.2[a]	48.4[b]	48.6[b]	0.3
Feed Intake (g/day)	65.0[a]	65.5[a]	83.9[b]	83.9[b]	0.6

The table presents values as LS means. Within each row, different superscript letters (a, b) indicate significant differences between treatment groups (at least $p < 0.05$).

pH decline than FG broilers[31], resulting in higher final pH values in FG broilers[11].

Muscle myopathies were assessed to evaluate their implications on meat quality. The incidence of white striping (WS) in modern broilers ranges from 5% to 98%, influenced by multiple factors such as breed, age, body weight, sex and nutrition[6,7]. In this study, WS incidence (provided in Supplementary Table 1) was 62.3%, with no severe cases. Consistent with previous findings linking WS to breed type[32] and rapid growth rates[4,33,34], FG broilers exhibited a higher incidence. The lowest incidence (45.0%) occurred in SG broilers under non-enriched conditions, whereas enriched conditions in FG broilers were associated with the highest prevalence (77.5%, including 5.0% moderate WS). As noted above, all birds included in downstream analyses were males. Since male broilers are known to exhibit a higher incidence of WS than females, this may have contributed to the prevalence and severity observed in this study, although it remains unclear whether this effect is independent of body weight[6]. Spaghetti meat (SM) evaluation

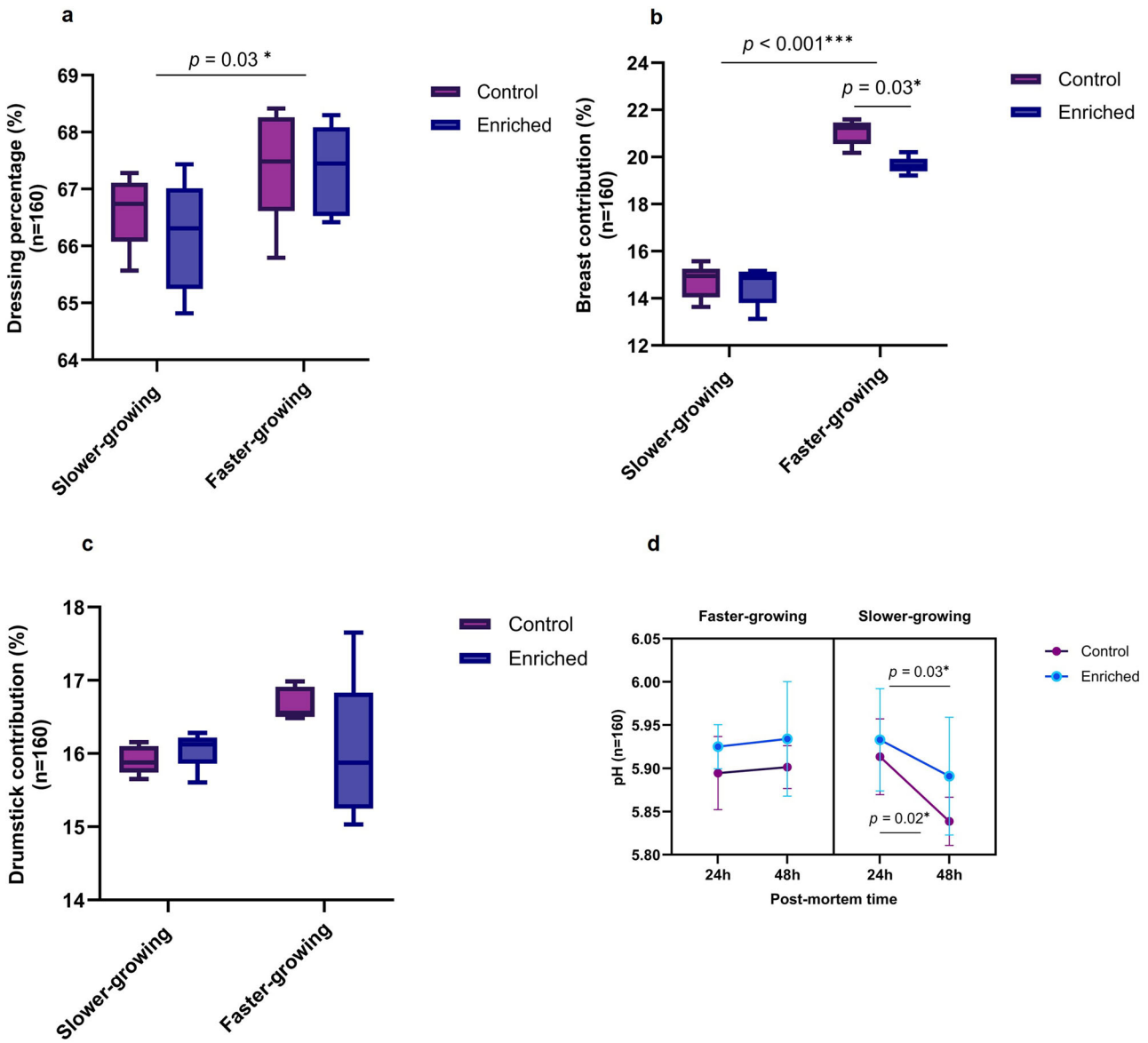


Fig. 2 | Meat yield and post-mortem pH in slower-growing and faster-growing broilers under non-enriched and enriched conditions ($n = 160$). **a** Dressing percentage (%). **b** Breast weight contribution (%) to the carcass. **c** Drumstick weight contribution (%) to the carcass. **a**–**c** ANOVA for a randomised complete block design, followed by Tukey's post hoc test, was used to compare the effects of the tested factors. **d** pH values of breast muscles at 24 and 48 h post-mortem. A paired *t*-test was applied to compare pH values for each sample between the two time points, with significant *p* values indicated on the graph. **a**–**d** Graphs present mean values with standard deviations. Stars indicate significance levels: $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

Table 2 | Meat composition of slower-growing (SG) and faster-growing (FG) broilers under non-enriched (NE) and enriched (E) conditions

Treatments	Moisture (%)	Protein dry basis (%)	Protein wet basis (%)	Fat dry basis (%)	Fat wet basis (%)
Genetics					
Faster-growing (FG)	74.71 ± 0.11 (n = 80)	92.11 ± 1.02 (n = 80)	23.29 ± 0.28 (n = 80)	2.26 ± 0.75 (n = 30)	0.57 ± 0.19 (n = 30)
Slower-growing (SG)	74.20 ± 0.24 (n = 80)	93.40 ± 0.84 (n = 80)	24.10 ± 0.36 (n = 80)	0.75 ± 0.2 (n = 30)	0.19 ± 0.05 (n = 9)
p value	<0.001***	<0.01**	<0.001***	<0.001***	<0.001***
Enrichment					
Control (NE)	74.52 ± 0.20 (n = 80)	92.89 ± 0.89 (n = 80)	23.67 ± 0.32 (n = 80)	1.47 ± 0.87 (n = 30)	0.37 ± 0.22 (n = 30)
Enriched (E)	74.39 ± 0.41 (n = 80)	92.61 ± 1.35 (n = 80)	23.73 ± 0.68 (n = 80)	1.55 ± 1.06 (n = 30)	0.39 ± 0.27 (n = 30)
p value	0.06	0.46	0.63	0.74	0.63
Interaction					
FG-NE	74.67^{a} ± 0.07 (n = 40)	92.60 ± 1.22 (n = 40)	$23.46^{b,c}$ ± 0.29 (n = 40)	2.10 ± 0.79 (n = 15)	0.53 ± 0.20 (n = 15)
FG-E	74.76^{a} ± 0.13 (n = 40)	91.62 ± 0.50 (n = 40)	23.13^{c} ± 0.16 (n = 40)	2.42 ± 0.77 (n = 15)	0.61 ± 0.20 (n = 15)
SG-NE	74.38^{b} ± 0.18 (n = 40)	93.19 ± 0.29 (n = 40)	$23.88^{a,b}$ ± 0.18 (n = 40)	0.84 ± 0.28 (n = 15)	0.20 ± 0.07 (n = 15)
SG-E	74.02^{c} ± 0.13 (n = 40)	93.60 ± 1.18 (n = 40)	24.32^{a} ± 0.36 (n = 40)	0.67 ± 0.07 (n = 15)	0.17 ± 0.02 (n = 15)
p value	<0.01**	0.08	<0.01**	0.31	0.34

The table presents values as mean ± standard deviation. ANOVA for a randomised complete block design, followed by Tukey's post hoc test, was used to compare the effects of the tested factors. Stars indicate significance levels: ***p< 0.001, **p < 0.01 and *p < 0.05. Significant differences are indicated by different superscript letters (a,b,c) within the same column when interactions are significant.

revealed no cases in any treatment group (SM1 Sum = 0%, Supplementary Table 1).

Meat composition analysis results are presented in Table 2. It revealed that the FG broilers exhibited significantly higher (p < 0.001) moisture content (74.7 ± 0.1%) than the SG broilers (74.2 ± 0.2%) with a genetics-enrichment interaction (p < 0.01). The lowest water retention was observed in SG broilers raised in enriched environments. Higher moisture content in FG broilers corresponded to 1.3% lower dry-based protein content than SG broilers, reflecting a trade-off between muscle water retention and protein concentration by slaughter age[11]. Additionally, the FG broilers had three times higher fat content than SG broilers on both dry and wet bases.

The influence of muscle myopathy levels on meat composition was further analysed and the results are shown in Supplementary Tables 2 and 3. Significant effects were observed only in FG broilers raised under enriched conditions, where moderate-scored samples showed three times higher dry fat content than normal-scored samples, resulting in a fourfold increase in the fat-to-protein ratio (p < 0.05). These findings align with previous studies linking WS severity to muscle composition[6,7]. Moreover, the meat composition of normal, non-myopathic samples from each treatment group (Supplementary Table 3) continued to reflect the patterns observed in the full dataset (Table 2). This supports the interpretation that treatment effects are the primary determinants of meat composition, with limited additional influence from myopathy severity.

Features of the proteomics analysis

The proteomics workflow (as shown in Fig. 3) analysed 20 pectoralis major muscle samples, with one broiler selected per pen. Muscles were sampled 48 h post-mortem, aligning with industry practices to best represent consumer-facing products from farm to fork. The workflow incorporated a sensitive MS-based approach using the protein aggregation capture (PAC) method optimised for meat proteomics, which improves peptide recovery and increases the identification of unique peptides and proteins in muscle tissue[35]. The "match between runs" option in MaxQuant was also used to leverage accurate liquid chromatography (LC) retention time alignments, facilitating the transfer of identifications from a peptide library and improving the identification of low-abundant muscle peptides[36]. This approach identified ~1600 protein groups with 11,927 unique peptides (Supplementary Data 1) (*Methods*).

The completeness of measurements was assessed for each treatment (shown in Supplementary Fig. 1a) by assessing proteins with no missing values (valid values) and counting the number of proteins that could not be quantified in all samples (missing values). The dependency between the number of missing values for each protein with respect to protein intensities was also illustrated in Supplementary Fig. 1b, revealing, as expected, a higher density of missing values at lower protein intensities. Applying stringent identification criteria (min. three valid values in at least one treatment), 975 protein groups and 10,830 unique peptides were identified with a median sequence coverage of 37% (Supplementary Data 2).

Sample correlations were examined (Supplementary Fig. 2), and one FG broiler sample from enriched environments (block 4) was clustered separately from the rest. This sample had a mild muscle myopathy score of 0.5 and was initially included only because of the absence of non-myopathic samples in block 4. After it was concluded that the impact of myopathy made the sample a clear outlier compared to the main experimental factors, this sample was removed from the dataset for further imputation, which could impact statistical tests. As a result, only normal, non-myopathic samples were retained for the final analysis.

The newly obtained dataset revealed a high median Pearson's correlation (r = 0.90) across biological replicates from the remaining 970 protein groups (n = 19) (Supplementary Data 3), with the principal component analysis (PCA) plot illustrating the distribution across all treatments (Supplementary Fig. 3). Sample correlation coefficients ranged from 0.83 to 0.94, reflecting the least to most similar subjects. At the proteome level, genetic differences had a more substantial effect than on-farm enrichment, with interaction effects also observed, as shown in Supplementary Fig. 4.

Proteomics analysis reveals markers specific to slower- and faster-growing broilers

This study examined the quantitative post-mortem muscle proteome differences between SG and FG broilers raised in non-enriched environments, as illustrated in Fig. 4. PCA plot revealed a separation of the protein profiles between breeds (Fig. 4a). Differentially expressed proteins (DEPs) were identified and ranked using a posteriori information fusion scheme, which combines the biological relevance (fold change) and the statistical significance (p value) into one score[37]. This analysis revealed 56 DEPs (significant fusion score ≤ 0.05; Fig. 4b and Supplementary Data 4). Functional enrichment on DEPs, performed using Gene Ontology (GO) annotations and KEGG pathways (Fisher Exact Test, Benjamini–Hochberg correction; $p \leq 0.05$), highlighted breed-specific metabolic and structural differences (Fig. 4c, d and Supplementary Data 5).

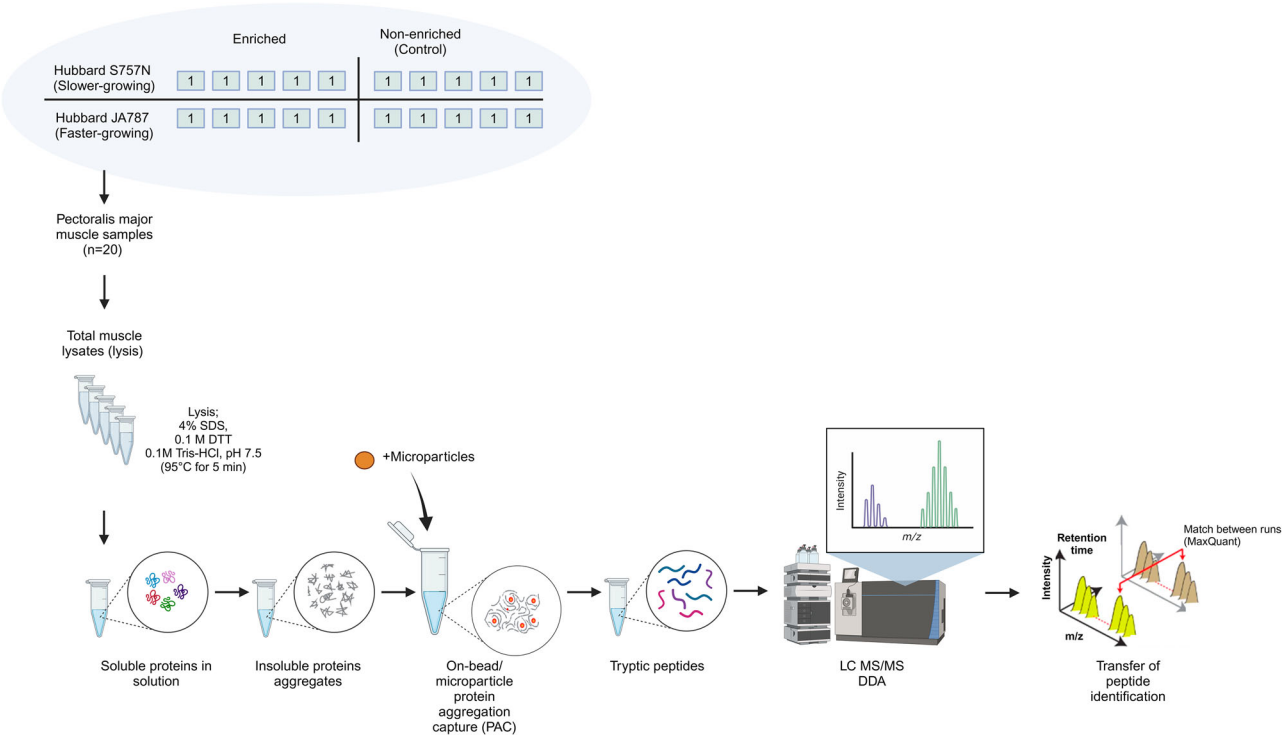


Fig. 3 | Workflow of the proteomics analysis. Each small box represents a pen, with one broiler per pen selected for proteomics analysis ($n = 20$). Pectoralis major muscle samples were dissolved in lysis buffer and processed using protein aggregation capture (PAC) on microparticles. Proteins were digested by trypsin, followed by LC-MS/MS with data-dependent acquisition (DDA) mode and computational analysis using MaxQuant with the "match between runs" feature. Figure created with BioRender.com.

In SG broilers, 36.8% of the DEPs were involved in actin filament binding (GO:0051015) and 26.3% were associated with actin filament-based processes (GO:0030029) (Fig. 4c). This was driven by proteins such as filamin A (FLNA), myosin light chain kinase 2 (MYLK2), myosin heavy chain 11 (MYH11), tropomyosin 1 (TPM1), transgelin (TAGLN), SH3 domain containing kinase binding protein 1 (SH3KBP1) and annexin A6 (ANXA6). In SG broilers, actin filament-based processes contribute to structural and muscle resilience by supporting focal adhesions (KEGG:04510) at the cell-extracellular matrix interface. FLNA is a large scaffolding protein that crosslinks the orthogonal actin filament at the Z-disk[38]. MYLK2 activates muscle contraction through phosphorylation of myosin II regulatory light chains by binding myosin cross bridges to actin filaments[39]. They are associated with cellular architecture and structural kinetics believed to be regulated by complex cross-talk signalling molecules[40]. The higher expression of centrosome and spindle pole-associated protein 1 (CSPP1; a newly discovered microtubule end-capping protein) further supports enhanced muscle structure integrity, as it stabilises both the positive and negative ends of cell microtubules, which are essential for the shape of the cell skeleton and motility[41]. These findings suggest that the stronger muscle architecture of the SG broilers potentially enables them to better handle physical demands such as increased activity, balance, and coordination. This is consistent with Dixon, who reported superior motor skills and welfare in SG broilers[13]. Additionally, ANXA6, a $Ca2^{+}$-regulated membrane-binding protein, plays an important role in protein homo-oligomerisation (GO:0051260) (Fig. 4c), a process through which ANXA6 functions as a sensor responding to cellular and organismal stress and involves in muscle and myofiber membrane repair[42]. This further supports muscle resilience in SG broilers, potentially reducing muscle damage and the associated myopathies, as evidenced by the lower incidence of WS (Supplementary Table 1).

Additionally, the SG broilers also showed a higher expression of proteins related to glutathione metabolism (KEGG:00480) and detoxification (GO:0098754; GO:1990748) processes (Fig. 4c). These specifically included glutathione peroxidase 3 (GPX3), glutathione S-transferase like (LOC100859645), glutathione S-transferase alpha 3 (GSTA3) and aldo-keto reductase family 1 member A1 (AKR1A1). The glutathione system is a key player in redox regulation and a major antioxidant at the cellular level[43]. This suggests that SG broilers may better manage oxidative stress, potentially leading to healthier muscle tissue and improved welfare outcomes. This aligns with previous studies reporting higher blood antioxidant levels in SG genotypes[44,45]. While considerable research has explored the role of anti-oxidants in improving animal health and their ability to cope with on-farm stress, it remains unclear whether tissue-level antioxidants improve the functional aspects of meat[46,47]. Our data suggest there is a straightforward interplay among animal welfare, tissue-level glutathione metabolism, and meat quality.

In FG broilers, 49.6% of the DEPs were involved in skeletal myofibril assembly (GO:0014866) (Fig. 4d). Key proteins, including actin alpha 1 (ACTC1), leimodin 3 (LMOD3), myotrophin (MTPN), myosin binding protein C (MYBPC2), smoothelin like 2 (SMTNL2) and thymosin beta 4X (TMSB4X) were involved in cytoskeletal protein binding (GO:0008092), and actin cytoskeleton organisation (GO:0030036). Unlike SG broilers, actin filament-based processes (GO:0030029) in FG broilers emphasise dynamic remodelling of the cytoskeleton to support rapid muscle growth, aided by proteins involved in muscle cell development (GO:0055001) and striated muscle cell differentiation (GO:0051146). These proteins regulate actin filament length (GO:0030832), assemble ribonucleoprotein complexes (GO:0022618) and organise protein-containing complexes (GO:0065003). Their activities reflect the dynamic nature of muscle filament assembly, as they are involved in actin filament polymerisation (GO:0030041) and depolymerisation (GO:0030042), and both the regulation (GO:0030834) and negative regulation (GO:0030835) of depolymerisation. Additionally, these proteins are also prominently associated with actin filament capping (GO:0051693) (Fig. 4d), which further regulates filament length and cytoskeleton restructuring[48].

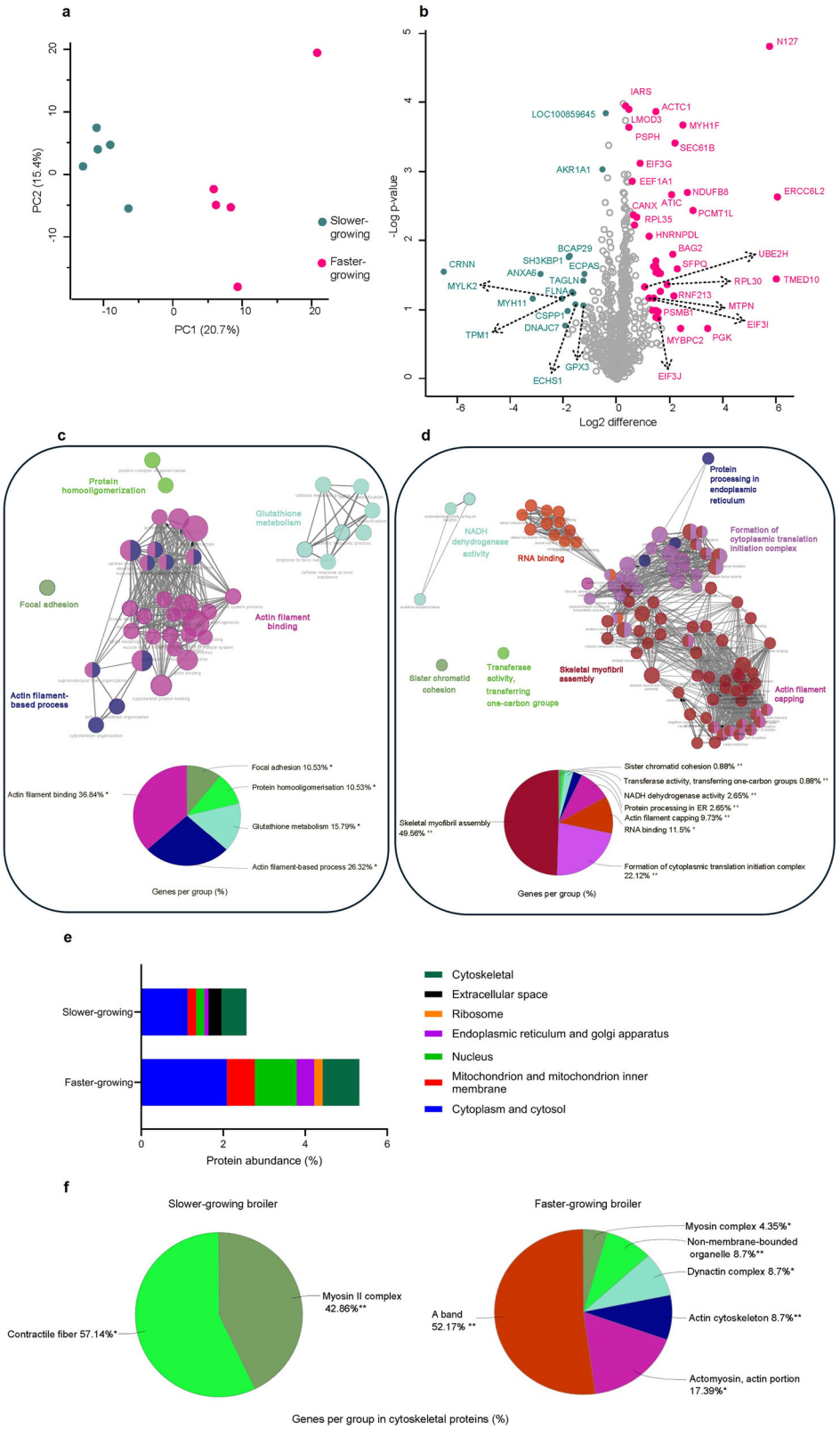


Fig. 4 | Quantitative post-mortem muscle proteome differences between slower-growing (SG) and faster-growing (FG) broilers. a Principal component analysis (PCA) plot performed on 946 proteins detected in SG (green dots) and FG (pink dots) broilers. **b** Volcano plot comparing the protein abundance in SG and FG broilers. Differentially expressed proteins (DEPs) (significant fusion score ≤ 0.05) are marked in green (higher in SG) and pink (higher in FG). Selected protein gene names are displayed. **c** ClueGo functional group annotation network of DEPs in SG broilers, based on the similarity of their associated genes. Group names are assigned based on the most significant term (Fisher Exact Test, Benjamini–Hochberg correction; $p \leq 0.05$). The pie chart shows functional groups with the percentage of genes found relative to the total associated genes. Significance levels are indicated as **: if the group $p < 0.001$, *: $0.001 < p < 0.05$ and. (dot): $0.01 < p < 0.05$. **d** ClueGO functional group annotation network of DEPs in FG broilers, with annotations as described for (**c**). **e** Comparative protein abundance of major organelles in DEPs from SG and FG broilers, shown as an average of corresponding proteins. **f** Pie charts showing the cellular component distribution of cytoskeletal proteins in DEPs from both SG and FG broilers, with group sections corresponding to the number of terms included. Significance levels are marked as described in (**c**).

The FG broilers also exhibited higher expression of proteins involved in the cytoplasmic translation initiation complex (GO:0001732) (Fig. 4d), such as EIF3G, EIF3I and EIF3J, which initiates the selective translation of a subset of mRNAs involved in cell proliferation[49]. Both in vivo and in vitro studies in eukaryotes indicate that EIF3I and EIF3G help to facilitate the scanning and recognition of the start codon[50,51], whereas EIF3J stimulates translation termination[52] and may coordinate a transition to a new cycle of translation[53]. Considering previous results of deleting EIF3J in *S. cerevisiae*, which resulted in a leaky scanning and slow-growth phenotype[54], it has been suggested that EIF3J might be required for efficient protein synthesis[55]. The EIF3 complex has also been related to regulating skeletal muscle mass and hypertrophy via the mechanistic target of rapamycin (mTORC1) upon

mitogen/growth factor/amino acid stimulation[56]. However, the phenotypic function of the translation initiation complex has not yet been investigated in FG broilers.

Along with the cytoplasmic translation initiation complex, heterogeneous nuclear ribonucleoprotein D-like (HNRNNPDL), isoleucyl-tRNA synthetase (IARS), splicing factor proline and glutamine-rich (SFPQ), ribosomal protein L30 (RPL30) and ribosomal protein L35 (RPL35) are all associated with the RNA binding (GO:0003723) (Fig. 4d) with functions in mRNA processing[57,58], translation accuracy[58,59] and ribosome assembly[60]; all of which ensure the efficient production of proteins necessary for muscle growth. Additionally, proteins in this group exhibit binding affinities for small molecules (GO:0036094), carbohydrate derivatives (GO:0097367), and heterocyclic compounds (GO:1901363), underscoring their versatile roles in interacting with various biomolecules. Their ability to bind nucleoside phosphates (GO:1901265), nucleotides (GO:0000166), and ribonucleotides (GO:0032553) highlights their significant involvement in nucleotide metabolism and energy transfer processes. Furthermore, these proteins demonstrate binding capabilities with purine nucleotides (GO:0017076), adenyl nucleotides (GO:0030554), and ATP (GO:0005524), emphasising their critical roles in energy-dependent processes and signal transduction pathways.

Proteins involved in protein processing in the endoplasmic reticulum (ER) (KEGG:04141) (Fig. 4d), such as BAG2, calnexin (CANX) and sec61 translocon beta subunit (SEC61B), were also upregulated in the FG broilers. These proteins are essential for ensuring protein quality and proper progression along the pathway. The BAG2 functions as a molecular cochaperone interacting with the heat shock protein 70 family molecular chaperones (Hsp70/Hsc70)[61] while CANX functions as a molecular chaperone[62], and both contribute to efficient protein folding[61,62]. The SEC61B assists in translocating proteins across the ER membrane by associating microtubules[63]. Increased expression of proteasome subunit beta 1 (PSMB1), ring finger protein 213 (RNF213), and ubiquitin-conjugating enzyme E2 H (UBE2H) in this group suggests an enriched role in protein turnover and catabolic process (GO:0051603; GO:0010498), crucial for tagging and proteasomal degrading of damaged or abnormal proteins, thereby maintaining protein quality within the cells[64,65].

Additionally, the FG broilers exhibited increased expression of proteins related to NADH dehydrogenase activity (GO:0003954), transferase activity (transferring one-carbon groups, GO:0016741) and sister chromatid cohesion (GO:0007062) (Fig. 4d), further highlighting their essential roles in ATP production and cellular energy metabolism[66], purine biosynthesis and amino acid metabolism[67], as well as genomic stability during cell-cycle progression[68].

The organelle composition (Fig. 4e) was assessed by calculating the percentage of DEP intensities located in different organelles using the Gene Ontology Cellular Component (GOCC) (Supplementary Data 6). As expected, the FG broilers exhibit a higher proportion of proteins associated with mitochondria, nucleus, ER and Golgi apparatus, reflecting their enhanced capacity for rapid muscle growth and high metabolic demands. In contrast, SG broilers showed an abundance of proteins in the extracellular space, primarily supporting detoxification and oxidative stress management. These distinct organelle associations between the two breeds are likely key to their differing metabolic profiles. Notably, several studies have reported that broiler breast muscle is predominantly composed of glycolytic fibres (type II, fast-twitch), regardless of breed[69], indicating that these metabolic differences are independent of fibre-type specific metabolism.

Furthermore, variations in the cellular localisation of cytoskeletal proteins (Fig. 4f) emerged between SG and FG broilers (0.6% vs. 0.9% among the DEPs, respectively; Supplementary Data 7). In SG broilers, these proteins were predominantly associated with contractile fibres (GO:0043292) and myosin II complex (GO:0016460), which are crucial for muscle contraction and integrity. FG broilers still emphasised these fundamental components but also displayed additional associations with more dynamic elements, including the dynactin complex (GO:0005869) and non-membrane-bounded organelles (GO:0043232), reflecting enhanced cellular reorganisation, transport and cytoskeletal restructuring required for rapid growth[70,71], as supported by the performance data (Table 1).

Proteomics response to on-farm environmental enrichment in slower- and faster-growing broilers

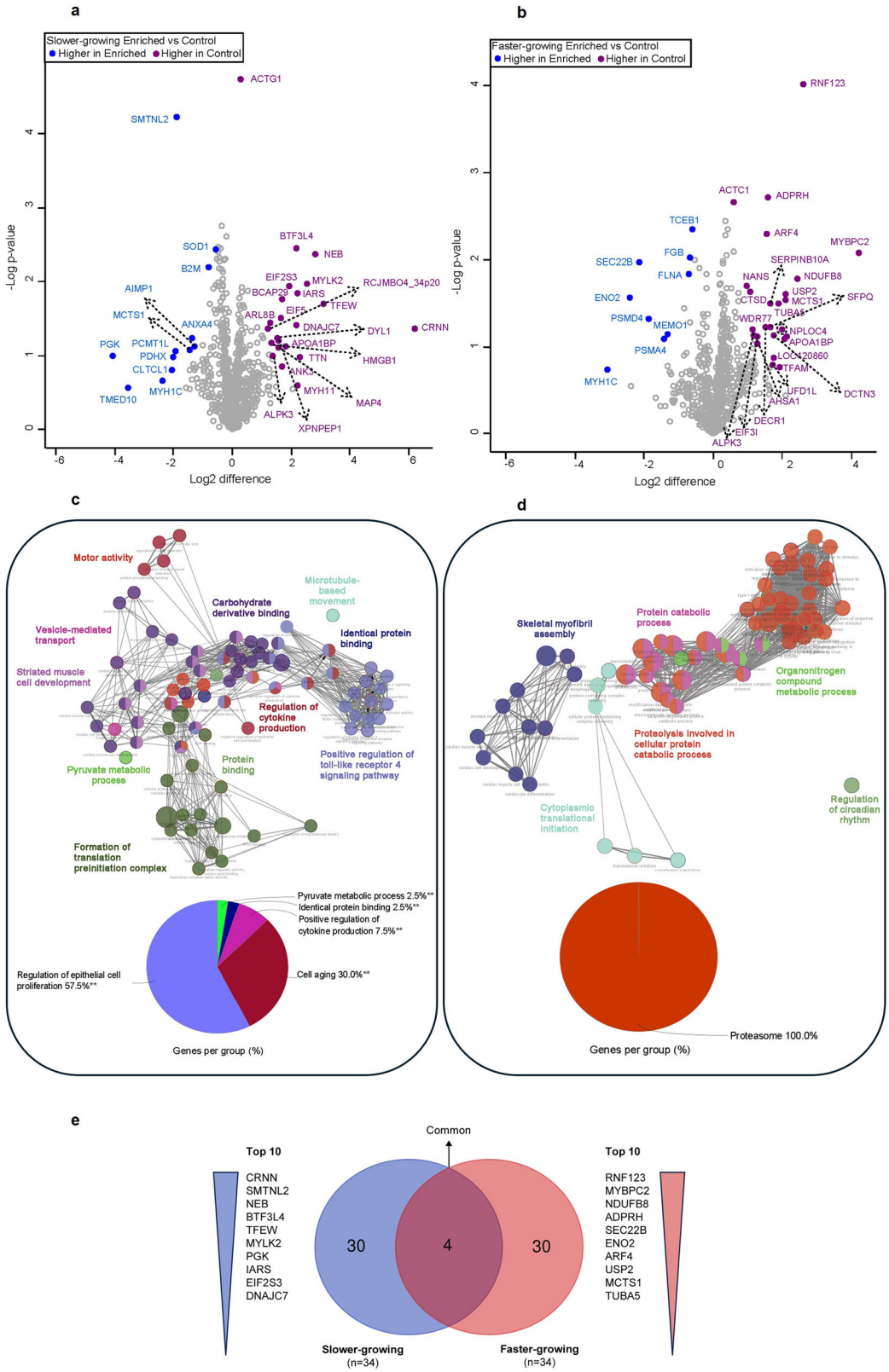
Building on the analysis of proteome differences in non-enriched environments, we investigated the adaptive response of SG and FG broilers to on-farm enrichment (Supplementary Data 8). The differential regulation of 34 proteins (significant fusion score ≤ 0.05) in response to enrichments is illustrated in Fig. 5a, b.

The functional group annotation network (Fisher Exact Test, Benjamini–Hochberg correction; $p \leq 0.05$; Supplementary Data 9) presented in Fig. 5c, d highlights the enrichment-regulated proteins in SG and FG broilers, respectively, based on the similarity of their associated genes. The pie charts in these figures represent only the functional groups for genes with higher expression in enriched environments. In SG broilers, 57.5% of these proteins (Fig. 5c) were associated with regulating epithelial cell proliferation (GO:0050678). In this process, aminoacyl tRNA synthetase complex-interacting multifunctional protein 1 (AIMP1) plays a critical role in maintaining tissue integrity and repair[72] while beta-2-microglobulin (B2M) ensures rapid replacement of damaged cells[73]. This indicates that environmental stimuli, provided by the enrichment elements, enhance tissue turnover and repair mechanisms. These adaptations are likely to improve the resilience of broilers to environmental challenges and may also promote wound healing. This finding should be further investigated, although environmental enrichment has previously been shown to enhance resilience in other meat-producing animals[74].

The B2M and superoxide dismutase 1 (SOD1) represent together two key molecular players in cellular ageing (GO:0007569) and the positive regulation of cytokine production (GO:0001819) (Fig. 5c). The B2M contributes to immune-related aspects of ageing[73], while SOD1 mitigates oxidative damage through its function as a frontline antioxidant enzyme[75]. Cytokines help control the immune response and inflammation[73,76], suggesting that enriched environments improve the immune response in SG broilers, potentially improving their ability to manage infections and stressors. Research in pigs has shown that enriched housing reduces disease susceptibility to infections[77] and stress indicators[78], but these effects remain largely unexplored in broilers. This may be attributed to the short lifespan of FG broilers, which limits the window for welfare and health manifestations. However, the dual benefit of supporting both physical health and immune function underscores the importance of environmental enrichment in SG poultry farming, which could lead to healthier and more sustainable flocks with reduced disease outbreaks, such as avian influenza. Additionally, proteins involved in identical protein binding (GO:0042802) (Fig. 5c) highlight the critical role of stable protein complex formation in maintaining cellular resilience. Notably, whereas FG broilers appeared to rely on sister chromatid cohesion to safeguard genomic stability during cell-cycle progression under fast-growth pressure (Fig. 4d), SG broilers enhance genomic stability through enrichment-induced mechanisms, including controlled epithelial turnover, antioxidant defence and immune-modulatory factors (Fig. 5c).

Proteomics results indicate that while SG broilers benefited from enrichments through enhanced overall resilience, physical health and immune responses, FG broilers adapted to enrichments by increasing proteasome pathway (KEGG:03050) (Fig. 5d) activity, reflecting enhanced protein degradation processes. Proteins like proteasome subunit alpha 4 (PSMA4) and proteasome 26S subunit, non-ATPase 4 (PSMD4) ensure efficient and specific proteolysis[79]. However, this adaptation may also contribute to the highest incidence of WS observed in the study (Supplementary Table 1).

Additionally, enrichment elevated the pyruvate metabolic process (Fig. 5c) (GO:0006090) in SG broilers, including pyruvate dehydrogenase complex component X and phosphoglycerate kinase, reflecting increased cellular metabolism and energy demands[80]. Although no significant differences in feed intake were observed, as presented in Table 1, these



findings suggest that environmental stimuli may promote other physiological adaptations that support increased metabolic demands.

The overlap between enrichment-regulated proteins in SG and FG broilers was investigated next (Fig. 5e). Several proteins, including alpha kinase 3 (ALPK3), apolipoprotein A-I binding protein (APOA1BP), myosin heavy chain 1C (MYH1C) and MCTS1 re-initiation and release factor (MCTS1) were commonly regulated. However, their regulation patterns differed between breeds. The intensity of ALPK3 and APOA1BP was lower in both breeds. This suggests a reduced need for cytoskeletal kinase activity[81] and apolipoprotein A-1 binding related to fat transport[82], potentially as a response to altered metabolic demands by enrichment. Conversely, the intensity of MYH1C was higher in both breeds under enriched

Fig. 5 | Quantitative proteomics responses of slower-growing (SG) and faster-growing (FG) broilers to on-farm environmental enrichment. a Volcano plot comparing protein abundance in SG broilers. Differentially expressed proteins (DEPs) (significant fusion score ≤0.05) are marked in blue (higher in broilers raised in enriched (E) environments) and purple (higher in broilers raised in non-enriched (NE) environments). **b** Volcano plot comparing protein abundance in FG broilers. DEPs (significant fusion score ≤ 0.05) are marked in blue (higher in E environments) and purple (higher in NE environments). **c** ClueGO functional group annotation network of enrichment-regulated proteins in SG broilers, based on the similarity of their associated genes. Group names are given by the most significant term in each group (Fisher Exact Test, Benjamini–Hochberg correction; $p \leq 0.05$). The pie chart shows functional groups for the user genes that are higher in enriched environments. The label displayed on the chart shows the percentage of genes found compared to all the genes associated with the group. Significance levels are marked as **: if the group $p < 0.001$, *: $0.001 < p < 0.05$ and. (dot): $0.01 < p < 0.05$. **d** ClueGO functional group annotation network of enrichment-regulated proteins in FG broilers, with annotations as described for (**c**). **e** Overlap of on-farm enrichment-regulated proteins in SG and FG broilers. The gene names of the top 10 proteins are shown for both breeds, listed in order from highest to lowest fusion scores.

environments. Interestingly, MCTS1 showed divergent patterns between the breeds. In SG broilers, higher MCTS1 might support enhanced cellular growth and protein synthesis[83]. Conversely, lower MCTS1 in FG broilers might reflect a shift toward sufficient resource utilisation, balancing rapid growth with metabolic demands.

Slower- and faster-growing broilers respond differentially to environmental enrichment

After examining within-breed responses to environments, we analysed between-breed responses to on-farm enrichment to elucidate the underlying mechanisms in a breed-dependent manner (i.e., breed-specific responses). The interaction between on-farm enrichment and genetic factors was reported in Fig. 6a, b. We identified 80 proteins regulated in a breed-specific manner (significant fusion score ≤ 0.05) (Fig. 6a and Supplementary Data 10). Proteins quantified exclusively in one breed were classified as "exclusive". For this analysis, only proteins quantified at least three times in at least one breed were included (Fig. 6b; Supplementary Data 10).

In the FG broilers, 19 proteins were quantified as exclusive, with NADH oxidoreductase subunit B8 (NDUFB8) being the only protein significantly regulated by enrichment. As a key component of the mitochondrial complex I, NDUFB8 is essential for ATP production through oxidative phosphorylation[84]. Its exclusive presence in FG broilers underscores the high mitochondrial activity required to sustain their accelerated growth. Its lower expression in enriched environments may reflect an adaptive response to the environment. In SG broilers, three proteins were classified as exclusive, including cornulin (CRNN), which was significantly regulated by enrichment and exhibited lower expression in enriched environments. However, it is important to note that the identification of CRNN was limited based on a modification site and a single unique peptide, which may impact annotation and quantification accuracy. While its role remains unclear, this result warrants cautious interpretation.

Functional annotation and enrichment analyses (Fisher exact test, Benjamini–Hochberg correction; $p < 0.05$) of the 80 proteins regulated in a breed-specific manner revealed significant protein categories (Fig. 6c and Supplementary Data 11). SG broilers in the enriched environments exhibited significantly higher protein quantities related to mitochondrial inner membrane and amino acid biosynthesis than FG broilers in enriched environments. These findings suggest that environmental enrichment may enhance energy metabolism and protein synthesis, likely contributing to better overall health in SG broilers. In FG broilers, there was a notable emphasis on the cytoplasmic proteins. However, enrichment analysis did not return any significant molecular functions or biological processes associated with these cytoplasmic proteins.

At baseline, FG broilers allocate a higher fraction of DEPs to the mitochondrion and its inner membrane compared to SG broilers (Fig. 4e). Soumeh et al. reported that the mitochondrial volume in the breast muscle of modern FG broilers has halved as a result of genetic selection[85]. Taken together, these findings suggest that in FG broilers, a relatively undersized mitochondrial compartment is being overloaded to meet the metabolic demands of rapid growth. Environmental enrichment appears to further stress this limited oxidative capacity in FG broilers, which exhibit increased reliance on proteasome-mediated muscle catabolism, as evidenced by higher expression of proteasome subunits (Fig. 5d) and lower expression of the complex-I protein NDUFB8 (Fig. 6b). In contrast, SG broilers respond to the same environmental stimulus with coordinated upregulation of proteins related to the mitochondrion inner membrane and biosynthesis of amino acids (Fig. 6c), suggesting an expansion of oxidative capacity rather a shift toward muscle catabolism.

Linking proteomics to phenotypic and intrinsic meat quality traits

Proteins involved in skeletal myofibril assembly, cytoplasmic translation initiation, RNA binding and protein processing in the ER highlight the rapid muscle growth characteristic of FG broilers. The higher expression of translation regulators (EEF1A1, EIF3G, EIF3I, EIF3J) and ribosomal proteins (RPL30, RPL35) underscores an increased protein synthesis capacity, supporting increased muscle mass (Fig. 2a) and breast yield (Fig. 2b).

Figure 7 visualises some motor proteins and cytoskeleton detected among the DEPs between breeds in the muscle structure. Elevated actin filament-related proteins (e.g., ACTC1, LMOD3) reflect the need for efficient myofibril organisation in FG, but rapid hypertrophy may overwhelm cellular homeostasis, potentially contributing to muscle myopathies like WS[7]. Similarly, increased ER activity for protein folding and trafficking, evidenced by the higher detection of proteins like BAG2 and CANX, may induce ER stress and trigger unfolded protein responses when growth exceeds cellular capacity[86]. These findings suggest that FG broilers allocate significant metabolic resources to anabolic pathways, supporting rapid muscle growth (Fig. 2a, b), but this prioritisation may come at a physiological cost. This trade-off between growth performance and muscle quality becomes particularly evident in enriched environments, where FG broilers exhibit reduced breast muscle yield (Fig. 2b). This reduction may be linked to higher proteasome pathway activity (KEGG:03050), suggesting that higher protein turnover through enhanced proteolysis limits muscle deposition. While proteolysis is essential for skeletal muscle breakdown and mass regulation[87], it may hinder FG broilers' ability to sustain optimal muscle growth under these conditions. Despite skeletal muscle's adaptability to environmental stimuli[88], the adaptive proteome remodelling in response to farm-based enrichment has not been studied in broilers. Four publications in the past decade have examined only the use of perches as a form of enrichment, reporting no significant impact on body weight, carcass yield, or meat quality[20].

The higher moisture content in FG broilers (Table 2) may result from their rapid muscle mass accumulation, which retains more water and likely reflects less mature muscle fibres. In contrast, SG broilers exhibit a lower moisture content (Table 2), particularly in enriched environments, which may result from their enhanced muscle resilience. This resilience is supported by the higher abundance of proteins involved in detoxification and oxidative stress management (GPX3, LOC100859645, GSTA3, AKR1A1), contributing to a muscle composition that retains less water and contains a higher density of structural proteins. Additionally, SG broilers' elevated expression of stress-response and cellular repair proteins in enriched environments, such as AIMP1, B2M, and SOD1, further enhances muscle function and integrity, reducing water retention. Furthermore, the robust and organised muscle structure in SG broilers is supported by proteins involved in actin filament binding and cytoskeleton organisation (FLNA, MYLK2, MYH11, TPM1, ANXA6, TAGLN, SH3KBP1), some of which are illustrated in Fig. 7. These proteins contribute to well-developed muscle

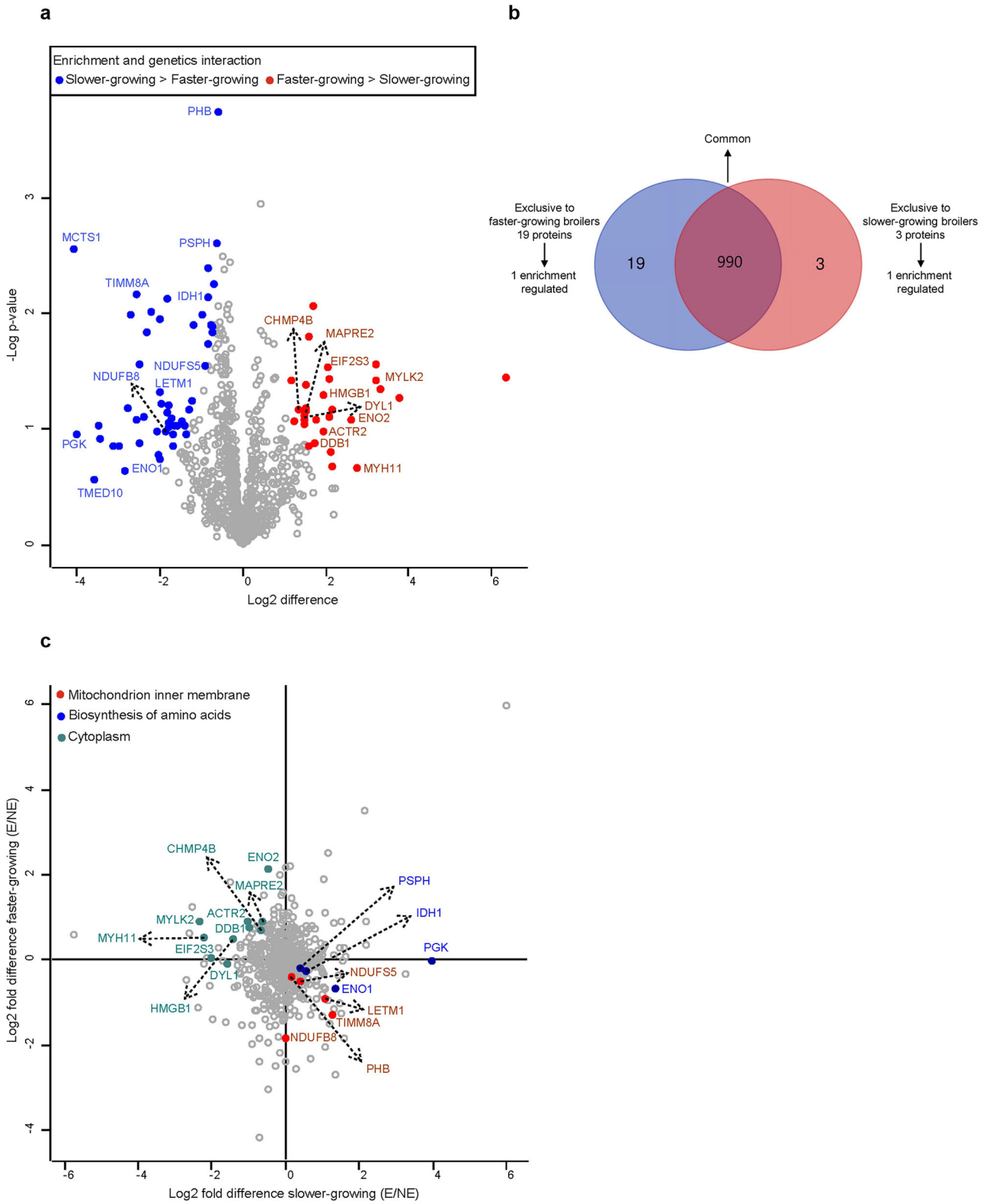


Fig. 6 | Slower-growing (SG) and faster-growing (FG) broilers adapt differentially to on-farm environmental enrichment. a Volcano plot comparing fold differences between enriched (E) and non-enriched (NE) environments across both breeds. Differently regulated proteins are marked in blue (change in slower-growing > faster-growing) and red (change in faster-growing > slower-growing). The gene names of a selected set of proteins are shown. **b** Venn diagram illustrating the number of proteins exclusively and commonly quantified in SG and FG broilers, with the number of enrichment-regulated proteins within the exclusively detected proteins. **c** Scatter plot comparing $\log_2$ fold changes (E vs. NE) between SG and FG broilers. Significant protein categories (Fisher exact test, Benjamini–Hochberg correction; $p < 0.05$) identified by DAVID and their gene names from both breeds are shown.

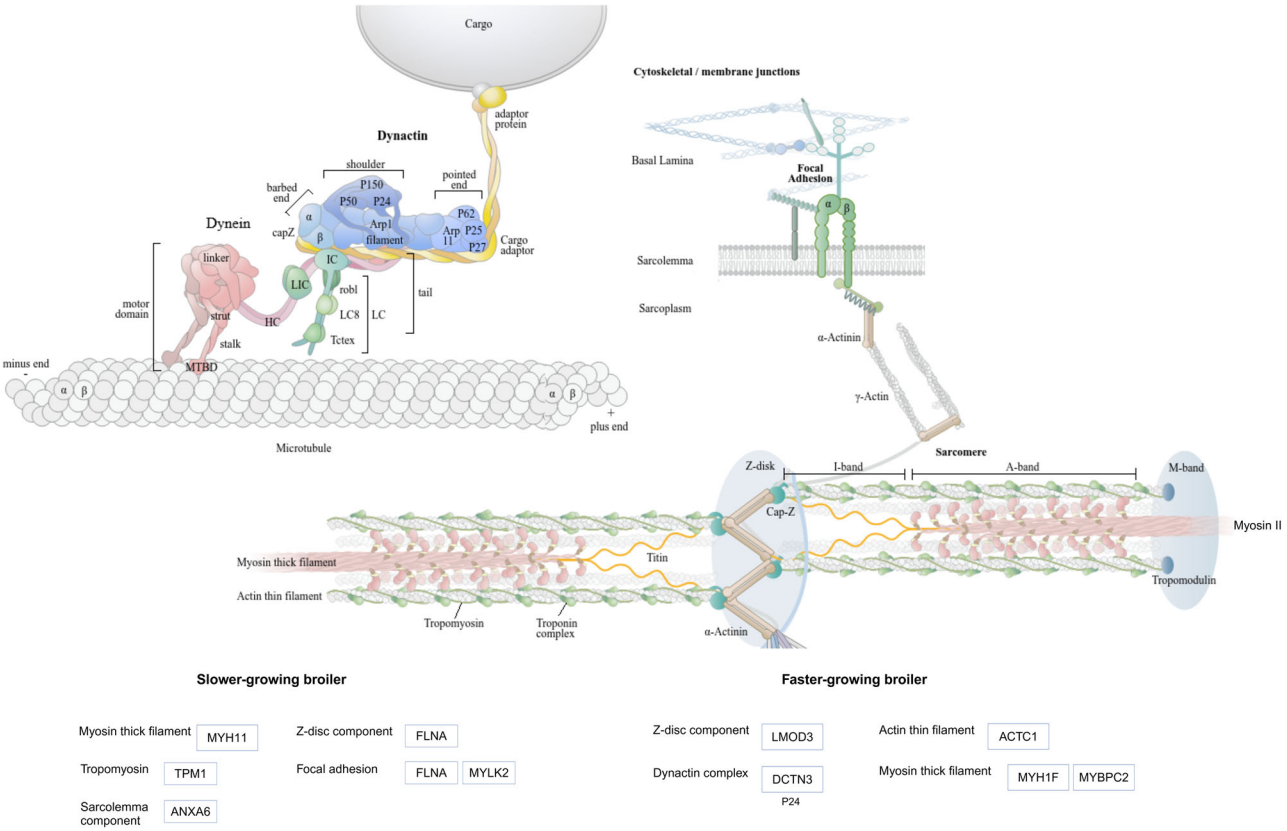


Fig. 7 | Motor proteins and cytoskeletal components in muscle. Proteins are extracted from KEGG pathways, showing only some DEPs identified in both breeds. The illustration combines elements from the cytoskeleton in muscle cells (KEGG:04820) and motor proteins (KEGG:04814), with annotations based on data provided by Kanehisa Laboratories. Modified from KEGG PATHWAY Database (kegg.jp/kegg/pathway.html).

fibres with higher protein content (Table 2). Together, these findings confirm a trade-off between muscle water retention and protein concentration that is, at least in part, attributable to differences in slaughter age between the breeds[11].

In FG broilers, the higher expression of the EIF3 complex that plays a central role in the mTOR pathway likely drives both protein synthesis and lipogenesis, which contributes to increased fat storage in the muscle alongside muscle growth[56,89,90], thereby shaping their distinct phenotypic traits[20]. In addition, FG strains are typically characterised by higher feed intake[28], as was also observed in the current study (Table 1) and lower locomotor activity[16], which may result in higher metabolisable energy availability. These behavioural and metabolic predispositions may further contribute to the elevated fat content observed in their breast muscle (Table 2). This increased fat content may, in return, contribute to improved meat quality as fat plays a favourable role in sensory attributes such as tenderness and perceived flavour[91].

These findings highlight the value of proteomics in uncovering protein expression profiles that drive the phenotypic differences in broiler yield and meat composition. While the smaller sample size in the proteomics subset may limit statistical power, as shown in Supplementary Table 4, it offers focused insights into the molecular mechanisms driving these traits observed in the full dataset. Notably, the impact of sample size may be more relevant for FG broilers, considering the higher variability observed in their muscle proteome profiles compared to SG broilers (Fig. 4a and Supplementary Fig. 3), which suggests a more heterogeneous muscle metabolic status, potentially reflecting underlying physiological variability related to rapid growth.

Our study comprehensively explored the differential regulation of key proteins in SG and FG broilers in response to on-farm environmental enrichments. Data were collected in the frame of a very well-controlled intervention experiment. The findings underscored the trade-offs between growth efficiency and muscle quality, with SG broilers demonstrating superior muscle structural integrity, stress resilience, and overall adaptability. Enrichment in SG broilers enhanced tissue repair, cellular health, immune function, stable protein interactions, and energy metabolism. Given that these birds are typically raised in outdoor systems[92], it is plausible that they are naturally equipped and adapted to cope with the environmental challenges often found in such systems. In contrast, FG broilers exhibited a proteome optimised for rapid muscle growth. Under enriched conditions, this led to increased proteasome activity, which likely contributed to reduced breast yield. Consequently, FG broilers do not benefit from environmental enrichments.

In the long term, these findings can guide breeders and producers in developing strategies that meet societal demands for sustainable and welfare-friendly poultry farming. This study provides a deeper molecular understanding of the sources of variation and how they influence product authenticity. The proteomics data provide a foundation for developing authentication approaches for broiler meat based on husbandry practices. Future research should focus on validating these molecular associations and further exploring the impact of extensification factors on other aspects of meat quality.

Methods

The workflow of the study is presented in Fig. 1.

Husbandry factors and experimental design

A randomised block design of a 2 × 2 factorial was employed. A relatively low stocking density was applied, with a target of 30 kg/m^2, comparable to higher-welfare systems[5]. The experiment was conducted at the broiler facility of Agrifirm (Proefboerderij Laverdonk, Heeswijk Dinther, The Netherlands). The facility had a single room containing 36 pens, of which 20 pens (arranged in five blocks of four pens each, across two rows) were used

for this experiment. Each pen measured 13.5 m^2 (4.45 m length × 3.14 m width). Four treatments were investigated using two breeds: naked-neck Hubbard S757N (used for Label Rouge production), a slower-growing breed (SG)[5], and Hubbard JA787, a faster-growing breed (FG)[5], as well as two environmental conditions: non-enriched (NE)-control and enriched (E). Each enriched pen had a lucerne bale in a net, a barrier perch (adjustable height) and a dust bath with peat at the back of the pen (1 × 3.13 m). Apart from the dustbathing area, wood shavings were used as bedding material (1.5 kg/m^2 per pen). Each treatment combination was replicated five times. In each pen, 184 day-old chicks were placed (50% male, 50% female), resulting in 3680 broilers in the study. The day-old SG chicks were sourced from a 36-week-old parent stock, while the FG chicks were from a 47-week-old parent stock. Broilers were reared until they reached a target weight of ~2.2 kg, at which point they were processed. The experiment lasted up to 42 days for the FG and 63 days for the SG. Birds that were clinically ill or were not vital were excluded from the study.

Climate and light requirements

The ambient temperature was set at 35 °C on Day 0 and gradually reduced to 19–21 °C from Day 42 onwards. Relative humidity was kept at a minimum of 40% during the first week and maintained at 50–70% after Day 14. The lighting schedule followed commercial standards for SG broilers, with artificial light at a minimum intensity of 20 lux. The dark periods were scheduled as follows: on Day 0–4 from 21:00 to 00:00 h; on Day 5–10 from 20:00 to 00:00 h; on Day 10–13 from 20:00 to 00:00 h and from 04:00 to 06:00 h; and from Day 14 onwards, from 20:00 to 04:00 h.

Feed and water

Each pen was equipped with five pan feeders and 24 nipple drinkers with cups, providing *ad libitum* access to feed and water. Birds were fed a commercially available four-phase broiler diet programme as applied for slower-growing strains under the Dutch *Beter Leven* (*Better Life*) concept, a one-star welfare label. The feeding programme (ABZ, Leusden, The Netherlands) consisted of a starter diet (crumble, Day 0–14, raw protein content 200 g/kg), grower 1 (pellet, Day 14–25, raw protein content 186 g/kg), grower 2 (pellet, Day 25–35, raw protein content 175 g/kg), and a finisher diet (pellet, Day 35–63, raw protein content 173 g/kg). Coccidiostats were incorporated into all diet phases.

Animal care

Broilers were checked twice daily to monitor their health status. A standard vaccination schedule was followed, including Innovax ND IBD (Gumboro), Marek Rispens, and IB primer at the hatchery, followed by an IB vaccine administered at 14 days of age. No antibiotic treatment was applied throughout the experimental period.

Broiler performance

Feed and water intake were recorded daily via automated systems, and body weights were measured with automated scales. A sample of 50 chickens per pen was manually weighed by the caretakers upon placement and when changing the diet programme. All birds per pen were manually weighed upon depopulation. These data, corrected for mortality per pen, were used to calculate the daily growth rate, FCR and water: feed ratio.

Transportation and slaughter

On the slaughter day, feed withdrawal was 6 h before transportation. The birds were transported to a slaughter and processing plant in Diessen, the Netherlands, about 35 min away from the production facility. After a 1 h rest, the birds were electrically stunned (240 mA for 6 s), then water scalded (60 °C for 90 s) and subjected to the plucker to remove the feathers. Thereafter, any remaining feathers, the intestines, tails, feet and heads were removed. After processing, hot carcass weights (with skin) were recorded, and carcasses were chilled (air chiller set to 1 °C).

Broiler sampling and muscle myopathy identification

Eight male birds per pen (40 per treatment, 160 total) were randomly selected for further analysis. At 24 h post-mortem, cold carcass weights (with skin) and breast muscle pH were recorded. pH was measured three times and the average value was reported. Measurements were taken using a portable meat pH metre equipped with an insertion glass electrode (HI99163, Hanna Instruments Nederland). Prior to measurement, the electrode was calibrated using two standard buffer solutions (pH 4.01 and 7.01), and calibration was re-checked after every ~10 birds. The samples were always measured at the same place. The chilled carcasses were vacuum-packed and transported under refrigerated conditions (4 °C) to a laboratory (Wageningen University, The Netherlands), where the pH measurements were repeated 48 h post-mortem to reflect the physiological condition of the muscle at the time of downstream analyses. At 48 h post-mortem, the pectoralis minor muscles were separated from the pectoralis major, and the weights of the right and left pectoralis major as well as the right and left drumsticks (with skin) were recorded. Samples from the upright pectoralis major were collected for proteomics analysis, while mid-point samples from the right and left pectoralis major were homogenised and collected for moisture, protein and fat analysis. Proteomics samples were flash-frozen in liquid nitrogen and stored at −80 °C for further analysis. Samples for proximate analysis were stored at −20 °C for 3–4 months. Protein and fat analyses were performed on freeze-dried samples.

Two muscle myopathies, i.e., WS and SM, were evaluated by visually examining images (IRIS-Alphasoft electronic eye) of whole breasts and cut surfaces, captured during the experimental period, according to established criteria[93,94]. WS was scored on a scale of 0 (normal) to 3 (severe). SM was rated as absent (SM0) or present (SM1). To minimise scoring variations, three trained team members independently evaluated the myopathies. One sample was excluded from the evaluations due to over-scalding. Wooden breast was not assessed in this study due to practical constraints during the experimental days, in order to minimise handling time and prevent tissue degradation across analyses.

Meat proximate composition

The moisture content of homogenised pectoralis major ($n = 160$) was determined following the AOAC method 950.46 (AOAC, 2005). The samples were thawed at 4 °C overnight. A 2–3 g portion of the thawed meat was weighed into aluminium containers (50 mm in diameter) and dried at 105 °C overnight. After drying, the samples were placed in a desiccator for 2 h before weighing to calculate the weight difference before and after drying. All analyses were performed in duplicate.

The crude protein content was measured ($n = 160$) using the AOAC method 992.15 (AOAC, 1992). Test portions (5–6 mg) of freeze-dried ground samples were weighed into tin cups and analysed by a nitrogen analyser (Flash EA 1112 Protein Analyzer, Thermo Fisher Scientific) using the Dumas combustion (850 °C). The protein content was calculated with a conversion factor of 6.25.

Three broiler samples per pen were selected for fat analysis across all treatments ($n = 60$), with samples chosen to reflect the incidence of muscle myopathy within each pen. This selection strategy was deliberately designed to capture the natural variation in myopathy severity and its potential impact on meat composition traits. Visible fat was removed from the samples, and fat extraction was performed following the AOAC Official Method 991.36. One gram of freeze-dried sample was weighed into a thimble, topped with cotton wool, and placed in an extraction cup containing 90 mL of petroleum ether. The samples were processed using a Soxtherm apparatus (C. Gerhardt. SOX 416 Macro, Germany) with the following settings: T-classification temperature of 200 °C, extraction temperature of 150 °C, 30-min hot extraction phase, reduction intervals of 4 min with 2-s pulses, followed by extraction time for 60 min and evaporation steps (Evaporation A: 5 intervals, B: 3–4 intervals, C: 4 min). After extraction, the cups were dried at 103 °C ± 2 °C for at least 1 h, cooled in a desiccator, and weighed. All steps were performed in duplicate.

Statistical analysis for performance, yield, and meat quality characteristics in broilers

Dressing percentage (%) was calculated as the portion of the live animal weight over the hot carcass weight. Breast meat contribution (%) was calculated as the portion of the total pectoralis major muscle weight relative to the cold carcass weight, and drumstick contribution (%) was the drumstick weight relative to the cold carcass weight.

A Randomized Complete Block Design for two factors and their interactions was implemented for the data analysis. Performance data were analysed on the pen level using SAS (version 9.4) and the GLIMMIX procedure. Phenotype data were analysed using the "doebioresearch" package (version 0.1.0) in R software (version 4.4.0). A two-way ANOVA was performed to identify factors contributing to differences in phenotype data. The assumptions of normality and homogeneity of variance were verified using the Shapiro–Wilk and Levene's tests, respectively. Tukey's test was applied for post hoc analysis. A p value of less than 0.05 was considered statistically significant.

Sample selection and processing for proteomics analysis

The workflow for the proteomic analysis is presented in Fig. 3. The proteomics experimental design comprised one selected broiler from a subset of eight broilers per pen initially chosen for meat quality analysis. To minimise confounding effects, samples with moderate muscle myopathies, as well as those with larger residuals than their group averages for colour (L*, a* and b* values), shear force (colour and shear force data not reported here) and the live bird weight/carcass weight, were excluded. The selection of the 20 pectoralis major muscles was based on the interquartile range that captures 50% of the data points in the aforementioned dataset. Where multiple options were available, samples within the 2.5–97.5% and 5–95% percentiles, respectively, were prioritised. This approach ensured a robust representation of the genetics and environmental effects under study.

Protein extraction was performed as previously described[95,96] with modifications. Liquid nitrogen-frozen pectoralis major muscle samples were cryo-milled. Approximately 50 mg of sample (in triplicate) was homogenised using Ultra-Turrax (IKA T25) in 750 µL lysis buffer (0.1 M Tris-HCl, 0.1 M DTT, 4% SDS, pH 7.5) and heated (Eppendorf ThermoMixer C) at 95 °C for 5 min. Lysates were cooled down at room temperature and cold sonicated (Sonation ultrasonic bath) in ten ultrasound periods of 10 s each with 10 s intervals. Lysates were then clarified by centrifugation at 16,000 × g for 15 min. Proteins were then precipitated using cold acetone (x4 sample volume) and incubated overnight at −20 °C. Samples were later centrifuged (3 × 10 min, 4 °C, 16,000 × g) and washed in 80% cold acetone. The precipitates were dissolved in 1 mL UREA buffer (8 M Urea in 0.1 M Tris-HCl, pH 8.5) and incubated at 4 °C while mixing at medium speed. Cell debris was removed by centrifugation (20 min, 4 °C, 15,000 × g), and the protein concentration of the supernatants was determined using the Pierce BCA (Thermo Fisher Scientific) with a bovine serum albumin standard (yielding an average of 5.8 µg/µL protein). The replicate with the protein concentration closest to the average of the triplicates was chosen for downstream analysis (total of 20 samples).

Total muscle lysate sample processing with Protein Aggregation Capture (PAC)

For each sample, 100 µg of protein was reduced with 15 mM dithiothreitol in the low-binding Eppendorf tubes and incubated at 45 °C for 30 min. Proteins were then alkylated by adding up to 20 mM acrylamide and incubated at 21 °C for 30 min. The pH was adjusted to 7.0 using 10% trifluoroacetic acid (TFA). The PAC[35] with modifications was employed. Proteins were captured by adding 1-µm-diameter magnetic carboxylate-modified beads (GE Healthcare). The aggregation was induced by adding 2.5 times the working volume of 100% acetonitrile, followed by 20 min shaking at room temperature. The beads were separated using a magnetic rack, and the liquid was removed. The beads were washed with 70% ethanol in water and 100% acetonitrile. Proteins were digested overnight at room temperature by adding 100 µL bovine sequencing grade trypsin (0.5 µg/µL in 1 mM HCl) within 50 µL of 50 mM ammonium bicarbonate. The digested peptides were acidified to pH 3.0 using 10% TFA. Samples were pulse-centrifuged, and the bead-free liquid was transferred to new low-binding Eppendorf tubes.

Liquid chromatography (LC) and mass spectrometry (MS)

The peptide of 0.5 µg per sample was loaded directly onto a 0.10- × 250-mm ReproSil-Pur 120 C18-AQ 1.9-µm beads (Dr. Maisch, Germany, Ammerbuch-Entringen) analytical column (prepared in-house) at a constant pressure of 925 bar (flow rate of circa 700 nL/min) with 1% formic acid in water and eluted at a flow of 0.5 µL min^{-1} with a 50 min linear gradient from 9% to 34% acetonitrile in water and 1% formic acid with a Thermo Vanquish Neo nanoLC (Thermo Fisher, Waltham, MA). An electrospray potential of 3.5 kV was applied directly to the eluent via a union connecting the nLC outlet tubing and the analytical column. On the connected Orbitrap Exploris 480 (Thermo Fisher, Waltham, MA), the MS and MS/MS automatic gain control targets were set to 300% and 100%, respectively, or maximum ion injection times of 50 ms (MS) and 30 ms (MS/MS) were used. Higher energy collisional dissociation (HCD) fragmented (isolation width 1.2 m/z and 28% normalised collision energy) MS/MS scans in a cycle time of 1.1 s, the most abundant 2–5+ charged peaks in the MS scan were recorded in a data-dependent mode (Resolution 15,000, threshold 2e4, 15 s exclusion duration for the selected $m/z \pm 10$ ppm).

Computational proteomics

The raw MS files were analysed with MaxQuant (version 2.0.3.0)[36]. The MS/MS spectra were searched by the Andromeda search engine (integrated into MaxQuant) against the UniProt *Gallus gallus* database (version year 2023) and a database of frequently observed contaminants. The search included variable modifications of methionine oxidation, N-terminal acetylation, deamidation of Asparagine (N) and Glutamine (Q) and fixed modification of cysteine to propionamide. The minimal peptide length was set to seven amino acids, and a maximum of two missed cleavages was allowed. The false discovery rate (FDR) was 1% for peptide and protein identifications. The MS runs were analysed with the "match between runs" option. Matches to the reverse database and potential contaminants were excluded. Protein identification was based on detecting at least two peptides, with proteins identified by only one peptide retained if it was unique. For comparative proteome analysis, normalised intensities from label-free quantification (LFQ) were obtained using the MaxLFQ algorithm in MaxQuant software[97]. It is important to note that the Uniprot *Gallus gallus* database also contained an actin sequence highly similar to the corresponding *Gallus gallus* sequence, annotated as actin (Fragment) from *Cryptosporidium sp*. To ensure accuracy, this sequence was excluded from this analysis. The MS proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD066390[98].

Bioinformatics and statistical analysis for proteomics data

Data files generated by MaxQuant were analysed with *prolfqua R*-Package (version 1.2.5)[99] and Perseus software (version 2.0.11)[100]. For comparative analysis, proteins quantified at least three times in at least one group (Groups: SG-NE vs FG-NE, SG-NE vs SG-E, FG-NE vs FG-E and all four groups for interactions) were included. Before group comparisons, log_2-transformed data were imputed to fill missing abundance values by drawing random numbers from a Gaussian distribution with a standard deviation of 30% compared to the standard deviation of measured protein abundances and 1.8 standard deviations downshift from the mean. These parameters have been adjusted to simulate the low-abundant protein distributions best. To estimate protein-specific differences between breeds and breed-specific responses to on-farm environmental enrichment, a series of linear models was performed on the proteome data. The analysis aimed to estimate (1) breed-type differences at baseline (SG-NE vs FG-NE), (2) within-breed responses to the enrichment factor (SG-E vs SG-NE, FG-NE vs FG-E) and (3) between-breed responses to the enrichment factor (breed-type-specific responses). Imputation was performed before each linear model

comparison (provided as Supplementary Figs. 5–8). Pearson's correlation across biological replicates within treatments was based on LFQ intensities. In Fig.4a, the PCA was performed on imputed values. To evaluate interactions, a two-factor ANOVA was used with genetics and on-farm enrichment as the main effects in a full factorial design. DEPs were identified and ranked using an a posteriori information fusion scheme, which combines the biological relevance (fold change) and the statistical significance (p value) into one score, as described by Xiao et al.[37] and applied to proteomics data by Deshmukh et al.[96]. We chose this fusion scheme to generate a robust protein significance score (π value), enabling the reliable identification of DEPs while minimising the risk of false discoveries. This method integrates both aspects, complementing other approaches in recent broiler meat proteomics studies that focus primarily on either statistical significance (p value) alone[25] or consider both fold change and statistical significance (p value) separately[26]. For this study, a significant fusion score cut-off of 0.05 was selected.

To identify enriched biological processes within the set of DEPs, the Cytoscape plugin ClueGO[101] was employed, using annotations from all GO evidence levels and KEGG pathways. The enrichment analysis was performed using the hypergeometric test and FDR correction for multiple testing (Benjamini–Hochberg; $p \leq 0.05$). The enriched terms were presented as a simplified network. Categories with enrichment of a single GO term/pathway selection were excluded, and the group name was assigned based on the most significant term within each cluster. Node sizes reflect the statistical significance of the terms, while edges indicate the connectivity between terms, which is calculated using kappa statistics. Pie charts in Figs. 4c, d and 5c, d. present the functional groups for DEPs, with labels displaying the percentage of found genes relative to the total genes associated with each group. The significance level for the groups is indicated on the charts.

In Fig. 4e, the percentage protein intensities of DEPs by organelle composition were calculated using the sum of LFQ intensities for proteins annotated to their respective categories in GOCC. Briefly, the LFQ intensity of each protein was divided by the total LFQ intensity of all proteins and expressed as a percentage of protein abundance. Data presented in Fig. 6c include analysis of functional annotations, GO and KEGG pathway using Database for Annotation, Visualisation and Integrated Discovery (DAVID)[102]. Benjamini–Hochberg adjusted $p < 0.05$ values in DAVID were considered statistically significant.

Data availability

All data supporting the findings of this study are available within the manuscript and its Supplementary Information files.

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Author contributions

S.Y., I.C.J., S.B. (Shai Barbut), V.F. and S.W.E. conceptualised and designed the study, and contributed to methodology. I.C.J. conducted the experimental design on the farm and carried out the broiler performance analyses and performed their associated statistical tests. S.Y. and S.W.E. carried out sample collection and processing. S.Y. established the proteomics methodology for this study and performed the experiment,

statistical tests and bioinformatics analysis. S.B. (Sjef Boeren) contributed proteomics expertise and generated the LC-MS/MS data output. All authors contributed to supervising the execution of the study and the analysis process. S.Y. drafted the original manuscript and prepared the visualisations; all authors provided edits and revisions. V.F. promoted funding for proteomics analysis. All persons designated as authors qualify for authorship, and all those qualifying for authorship are listed. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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