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Data availability statement: All mapped and analyzed proteomics data are included as Supplemental Information in this manuscript. All data will be publicly available upon publication via Dryad Digital Repository DOI: 10.5061/dryad.kh18932j0.

Funding statement: Author RWB was funded via an Institutional Research and Academic Career Development Award (IRACDA) made to Stony Brook University from the National Institute of General Medical Sciences of the National Institutes of Health [K12GM102778], and startup funding to NSV from Stony Brook University.

Experiments were paid for via startup funding to NSV from Stony Brook University

Conflict of interest disclosure: Authors have no conflicts of interest.

Ethics approval statement: Live animal experiments were conducted in accordance with guidelines and regulations from Stony Brook University Institutional Animal Care and Use Committee (IACUC). Research was conducted in accordance with plan and procedures in approved protocol SBU IACUC 2023-0014.

Patient consent statement: Not Applicable.

Permission to reproduce material from other sources: Not Applicable.

Clinical trial registration: Not Applicable.

Reduced Dietary Protein Induces Changes in the Dental Proteome

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[Graphical Abstract is in the Figures File]

Abstract

Experimental studies have demonstrated that nutritional changes during development can result in phenotypic changes to mammalian cheek teeth. This developmental plasticity of tooth morphology is an example of phenotypic plasticity. Because tooth development occurs through complex interactions between manifold processes, there are many potential mechanisms which can contribute to a tooth's norm of reaction. Determining the identity of those mechanisms and the relative importance of each of them is one of the main challenges to understanding phenotypic plasticity. Quantitative proteomics combined with experimental studies allow for the identification of potential molecular contributors to a plastic response through quantification of expressed gene products. Here, we present the results of a quantitative proteomics analysis of mature upper first molars (M1s) in *Mus musculus* from a controlled feeding experiment. Pregnant and nursing mothers were fed either a low-dietary protein (10%) treatment diet or control (20%) diet. Expression of tooth-related proteins, immune system proteins, and actin-based myosin proteins were significantly altered in our low-dietary protein

sample. The recovery of expression change in tooth development proteins was anticipated and consistent with previous proteomic studies. We also identified differential immune protein response along with systematic reduction in actin-based myosin protein expression, which are novel discoveries for tooth proteomics studies. We propose that studies which aim to elucidate specific mechanisms of molar phenotypic plasticity should prioritize investigations into the relationships between IGF regulation and tooth development and actin-based myosin expression and tooth development.

Research Highlights

A low-protein diet during development results in significantly altered protein expression for major dental building proteins, immune system proteins, and actin-based myosin proteins within *Mus musculus*.

Introduction

Phenotypic plasticity, the differential expression of a phenotype, is often invoked as a way that organisms respond to changing environments. Within lab and common garden experiments, nutritional changes such as changes in the quantity of dietary protein induce plastic changes to mammalian cheek tooth phenotypes (Paynter & Grainer, 1956; Shaw & Griffiths, 1963; (Patton & Brylski, 1987). Typically, these are changes in tooth size, size and shape of tooth cusps, and timing of eruption (Holloway et al., 1961; Paynter & Grainer, 1956; Searle, 1954; Shaw & Griffiths, 1963(Patton & Brylski, 1987). This demonstrates that there is plasticity in dental development which can provide a short-term, non-evolutionary response to changing environments (Levis & Pfennig,

2021). However, measures of plastic phenotypic response to environmental change often do not identify the molecular and genetic pathways which underlie those changes. The use of quantitative proteomics to quantify differences in protein expression could allow for the identification and study of pathways that are altered by an environmental change (e.g., are plastic). Here, we present a quantitative proteomics study characterizing protein expression variation in a sample of upper first molars (M1s) from lab mice fed a low-protein diet as part of a controlled feeding experiment. This experiment was explicitly designed to identify proteins and associated pathways which could underlie phenotypic plasticity. This allows us to characterize those pathways most impacted by exposure to low dietary protein during embryonic and early postnatal tooth development.

Proteomics

Proteomics is broadly the field focused on identifying, annotating, and quantifying variation of proteins. Quantification of variation includes both protein sequence variation and protein expression variation. Informatics approaches are applied to protein spectral data collected via tandem liquid-chromatography mass-spectrometry (LC-MS/MS) (Heck & Neely, 2020). Protein expression profiles are typically tissue- and developmental stage-dependent and must be interpreted within the specific spatiotemporal contexts in which samples were collected (Rebeaud, Mallik, Goloubinoff, & Tawfik, 2021 & Tawfik, 2021).

104 An assemblage of proteins that is expressed within a specific tissue or structure is often
105 referred to as a 'proteome' (Sharma et al., 2020). For example, within the dental
106 proteome enamel and dentin forming proteins are found in high abundance (Sharma et
107 al., 2020). However, outside of dental tissues, these proteins are detected in low
108 abundance and only at certain developmental stages (Ritchie, 2018, Bansal, Shetty,
109 Bindal, & Pathak, 2012 & Pathak, 2012).
110
111 If basic processes of tooth mineralization are impacted by dietary deficiency, we expect
112 a change in associated protein expression and a change in phenotype such as occlusal
113 pattern, size, and/or shape (Harjunmaa et al., 2014). It is possible that some aspects of
114 tooth mineralization are more easily perturbed by environmental change than others
115 and their associated pathways may more frequently underlie plastic changes.
116 Measuring the tooth proteome may allow us to identify potential candidates underlying
117 plastic changes in tooth phenotype.
118
119 Beyond stereotypical tooth-associated proteins, previous characterizations of the dental
120 proteome have suggested that teeth serve as reservoirs of more general patterns of
121 organismal protein expression during amelogenesis (tooth mineralization) (Green et al.
122 2019; Froment et al., 2021; Giovani et al., 2021; Sharma et al., 2020). For example,
123 Green et al. (2019) recovered immune system related proteins from enamel near the
124 enamel-dentin junction of mineralizing pig molars. These immune proteins currently
125 have no known function in amelogenesis. Their inclusion within enamel was inferred to
126 be because enamel cell proliferation and mineralization at the enamel-dentin junction

127 occurs in contact with salivary glands, putatively the source of the immune proteins
128 (Green et al. 2019) (Jagr et al.). If mineralized teeth serve as an archive of broader
129 organismal protein expression at the time of mineralization, it is possible that tooth
130 proteomic data could provide evidence of more generalized responses to environmental
131 perturbations.
132
133 **This Study**
134 To determine what pathways are altered by an impoverished diet, we conducted a
135 controlled feeding experiment and investigated protein expression in the cheek teeth.
136 Pregnant and nursing mouse dams were fed a low protein or control protein diet and
137 effects were measured in their offspring. Previous feeding studies on dietary protein
138 quantity in mice suggested that a threshold of 10-12% (by weight) dietary protein
139 reduction from a control of 20-24% would likely induce phenotypically plastic changes
140 (e.g., smaller tooth size and delayed dental development) (Holloway et al., 1961;
141 Paynter & Grainer, 1956; Paynter & Grainer, 1961; Shaw & Griffiths, 1963).
142
143 With this context we anticipated that reduction in dietary protein could disrupt normal
144 protein expression patterns during embryonic and early postnatal development. We
145 anticipated that there would be reduced expression for enamel- and dentin-forming
146 proteins. Previous studies suggested that halving dietary protein would likely increase
147 risk of infection and increase metabolic stress in low protein mice (Giovani et al., 2021;
148 Steward et al., 2023). Thus, we anticipated that there might be additional proteomic
149 signals related to stress or immune system function if those protein signatures are

preserved within dentition. Recovery of significant differential expression of proteins because of a dietary change allows us to identify and rank the molecular pathways most likely to be perturbed by environmental influences like dietary protein reduction.

Methods

Feeding Experiment

A breeding colony of inbred strain C57BL/6J (RRID: IMSR JAX:000664) mice was established at the Division of Laboratory Animal Research at Stony Brook University, in accordance with authorized IACUC protocol (SBU IACUC 2023-0014). Male and female mice were acquired at 8 weeks of age and housed in residence to acclimate until breeding began at 12 weeks of age. Males were placed with females for up to 72 hours (3 day-night cycles) and females were checked daily for presence of a copulatory plug. Once a plug was present or 72 hours had passed, the male and female were separated**.** Females, regardless of plug presence, were then randomly assigned to control or treatment diets to ensure that any developing embryos were on as consistent a diet as possible. Mice assigned to the control group were fed a 20% raw protein diet (PicoLab Rodent Diet 20, 5053). Mice assigned to treatment were fed a 10% raw protein diet (Mod LabDiet 5053 with ~10% Protein Red, 5BQM). Prior to this assignment, all specimens consumed the control diet.

If females were not pregnant, as evidenced by swollen abdomen after ~7 days post-mating attempt, low-protein females were cycled back to the control protein (20%) diet. They were re-acclimated to that diet for 14 days before additional mating was

attempted. To ensure that additional stress was not induced via single housing, pairs of females were housed together during acclimation and pregnancy. Mating timings were staggered to ensure that two females would not give birth at the same time. This allowed for the separation of females and their litters once they had given birth.

Pregnant and nursing females in the treatment group were fed only the low-protein diet for the remainder of their lifespan. Offspring were weaned at approximately ~21 days postnatal (P21). Siblings were housed with their respective sex and fed their respective diets until P28 to ensure full eruption of the third molars. At P28 dams and offspring were euthanized. Dams were not reused to avoid introducing bias related to improvement of maternal care from first to second litter (Weber & Olsson, 2008). A total of 74 offspring (treatment n=34, control n=30) from this feeding experiment were collected, eight of which (n=4 males from each group) were analyzed for protein expression in this study. Aside from the 74 collected offspring, there was postnatal attrition of one full litter of treatment pups (n=8), where the mother declined to nurse the pups. The smallest pup from each of five litters (treatment n=3; control n=2) also did not survive to weaning.

Sampling

The upper first molar (M1) was selected for protein extraction because of its ease of extraction and because it and its lower jaw counterpart are the first molars to develop and erupt. Left M1s were extracted from male offspring (treatment n=4; control n=4) immediately after euthanasia by dissecting away the maxillary gingiva and exposed

maxillary bone to reveal tooth roots. A blunt probe was used to lever the tooth out. Excess tissue, including any root bundle, was removed with forceps. Extracted teeth were then washed with 70% ETOH, wiped dry with a clean Kimwipe and stored in new cryotubes. Teeth were placed into a -80ºC freezer and maintained at -80ºC until preparation for protein quantification.

Protein analysis by LC-MS/MS

For protein analysis we first determined if our extraction and proposed sampling worked by initially sampling only two treatment and two control specimens. Once it was determined that our proposed protocols worked, we sampled the remaining two treatment and two control samples. Proteins were isolated in 5% SDS, 100mM TEAB, 10mM DTT using a Precellys bead homogenizer for two cycles and spun at 16,000 x G for 5 minutes. Supernatants were reduced at 55ºC for 30 minutes, and cysteines were alkylated with 25mM iodoacetamide for 30 minutes at room temperature in the dark. Proteins were subjected to digestion with trypsin, samples were acidified with phosphoric acid, proteins were then precipitated with 90% methanol, 50mM TEAB, and bound to S-Trap solid phase cartridges (Zougman, Wilson, & Banks, 2020). Protein precipitates were washed with 90% methanol, 50mM TEAB and digested with trypsin at 47ºC for two hours. Precipitates were then sequentially eluted with 50mM TEAB, 0.2% formic acid, and 50% acetonitrile, the 0.2% formic acid elution step was by centrifugation at 4000 x G for 1 minute each.

Peptides were analyzed by C18 reverse phase LC-MS/MS. HPLC C18 columns were prepared using a P-2000 CO2 laser puller (Sutter Instruments) and silica tubing (100µm ID x 15 cm) and were self-packed with 3u Reprosil resin. Peptides were separated using a flow rate of 300 nl/minute, and a gradient elution step changing from 0.1% formic acid to 40% acetonitrile (ACN) over 90 minutes, followed by a 90% ACN wash and re-equilibration steps. Parent peptide mass and collision-induced fragment mass information were collected using an orbital trap (Q-Exactive HF; Thermo) instrument followed by protein database searching using Proteome Discoverer 2.4 (Thermo). Electrospray ionization was achieved using spray voltage of ~2.3 kV. Information-dependent MS and MS/MS acquisitions were made using a 50ms survey scan (m/z 375 – 1400) at 60,000 resolution, followed by 'top 20' consecutive second product ion scans at 15,000 resolution. Peptide and spectra false discovery rates were set to 0.05. Peptide-specific label free quantitation (mapping) was performed using Proteome Discoverer 2.4, linking label free peptides to annotated mouse proteomes for standardized protein identification.

Filtering

To assess if dietary changes resulted in significantly altered protein expression, we first filtered our mapped proteomic data. Filtering was done in ProteoRE (Mehta et al., 2023). Because spectral ionization can vary between LC-MS/MS analyses we initially treated our dataset as two distinct datasets, representing the initial sampling (Group 1) and additional sampling (Group 2). There was no expectation that Groups 1 and 2 were substantially different from one another based on the experimental procedure: groups

differed only by the date of LC-MS/MS analysis. We chose a conservative approach of filtering each dataset individually and concatenating the resulting filtered datasets, ensuring that only proteins represented in both datasets were used for downstream analyses and interpretations. Each sample group was initially filtered by excluding all mapped proteins that had any one of the following criteria: A minimum false discovery rate (q-value) greater than 0.05, representation by fewer than two peptides, or three or fewer peptide spectral matches (PSMs). These criteria were selected based on proteomics field standard practices (Al-Amrani, Al-Jabri, Al-Zaabi, Alshekaili, & Al-Khabori, 2021).

Mapped Proteomics Analysis

To concatenate our results, a Venn analysis was performed to find the set of proteins identified as unambiguously present in both groups, resulting in a combined group (CG) dataset. This dataset reflected the shared mapped proteins between all eight (four control, four treatment) specimens in our sample. Groups 1 and 2 were further filtered to identify proteins with significant changes in protein expression between treatment and control specimens. First, proteins where at least one treatment specimen in each group had an abundance ratio adjusted p-value ≤0.05 were retained (n=135). Then, proteins which had missing data for two or more specimen comparisons were discarded (n=15). Those two datasets were subsequently concatenated via a Venn analysis to form a combined group significant (CGSig) dataset. Every protein in CGSig is significantly differentially expressed in at least one treatment/control comparison in Group 1 and at least one treatment/control comparison in Group 2.

Abundance ratio is the ratio of estimated protein abundance for one sample over another sample, in our case a treatment specimen over a control specimen. Because protein abundances can vary significantly in terms of magnitude between samples, the estimated protein abundances are commonly transformed on a Log2 scale to make cross-specimen comparisons possible (Liu & Zhang, 2021). A result of this transformation is that some calculated abundance ratios will effectively become -∞ or ∞ but are represented in the dataset as values of -3.32 or 3.32. We calculated the average Log2 fold change reported in Table 1 by taking the mean of the estimated abundance ratios from samples which had significant p-values, excluding significant samples which were equal to -3.32 or 3.32.

To identify protein functions, associated interactions, and general biological profiles represented by CGSig we performed Gene Ontology (GO) enrichment analysis via the ClusterProfiler tool of ProteoRE and Pathway Enrichment Analysis via REACTOME (Wu et al., 2021) (Croft et al., 2011). For GO enrichment analyses, we queried at two ontology levels, using cutoffs for p-value of 0.05 and q-value of 0.05. Outputs for GO analyses were used to assign broad categorical function to proteins (Table 1) based on Metabolic Functions, Cellular Component, and Biological Processes categories (Figure 1).

For pathway enrichment analysis via REACTOME we queried the *Mus musculus* REACTOME for the proteins found in the CGSig dataset. To calculate enrichment, the

number of entities (in our case proteins) identified as belonging within a specific pathway are identified. Then the total number of entities (proteins) which could be contained in that pathway is calculated and divided by the total number of entities (proteins) from the organism (in our case *Mus*). The resulting 'Entity Ratio' is used to correct for pathway size to determine which pathways are overrepresented compared to a random distribution. From this result, a p-value is calculated on a 95% confidence interval and pathways with Entities p-value ≤ 0.05 are significantly enriched. Enriched pathways were then ranked based on the Entity Ratio, which represents the percentage of the total number of *Mus* proteins represented within that pathway (i.e., a pathway with an Entity Ratio of 0.04 contains 4% of the total number of proteins known from *Mus*). Pathways with higher entity ratios are ranked higher.

Preliminary Test of Developmental Archive

Prior research and the design of our study allowed us to conduct a preliminary investigation of whether enamel and dentin proteomes represent an archive of protein expression during mineralization, rather than proteins expressed earlier in tooth development or at the time of specimen collection. We queried the Mouse Gene eXpression Database (GXD) to determine if a subset of proteins was expressed at developmental stages prior to the onset of mineralization, during mineralization, or after mineralization was complete. First, we investigated 20 proteins known to be expressed during tooth mineralization (Pandya et al. 2017). Second, we investigated proteins related to immune and actin-based myosins from our CGSig dataset. A protein's associated gene is expressed during mineralization, but not earlier or later in time would

support the argument that tooth proteomes represent a specific window of development during amelogenesis.

Results

Within G1, a total of 2189 unique proteins were mapped (SI 1). 1622 unique proteins were mapped for G2 (SI 1). The combined group (CG) of proteins shared between G1 and G2 was 1469 unique proteins (SI 1). Of the combined group, there were a total of 120 proteins with significant differential expression (fold change) (CGSig; Table 1).

Gene ontology profiling revealed that significantly differential proteins are primarily associated with the Binding (in Molecular Function), the Cell generally (as opposed to a specific cellular component), and the Biological Process of Metabolism (Figure 1; SI 2). Pathway enrichment analysis via REACTOME identified 387 biological pathways associated with significantly differentially expressed proteins (SI 1). Of those 387, 35 were significantly enriched ($p \leq 0.05$) (Table 2). Approximately 47% of matched proteins are represented in the top 6 enriched pathways (Entity Ratio ≥ 0.01) (Table 2; SI 1).

Odontogenesis and Osteogenesis Proteins

Seven proteins associated with odontogenesis, and osteogenesis had significant differential expression (Log2 fold change) between treatment and control groups (Table 1). In low-protein treatments, the major enamel-forming protein, Amelogenin X (AMELX) had an average -1.52-fold change in expression. One of the two major dentin-forming proteins, Dentin sialophosphoprotein (DSPP) had a 1.77-fold increase in expression.

The other dentin forming protein, Dentin matrix acidic phosphoprotein 1 (DMP1) did not have a clear direction of differential expression; three treatment comparisons showed an increase in protein expression, while two had a decrease in expression. For osteogenesis, Integrin alpha-V (ITGAV) had -2.66-fold change and Tartrate-resistant acid phosphatase type 5 (ACP5) had -1.71-fold change. The remaining two osteogenic proteins Collagen alpha-1(II) chain (COL2A1) and Galectin-7 (LGALS7) had variation which made interpretation unclear. For COL2A1, one specimen had increased and two had decreased expression. For LGALS7, three were increased and two decreased.

Inflammation and Immune Response

Nine proteins associated with inflammation and immune system response had significant differential expression (Log2 fold change) between treatment and control groups (Table 1). Two of the nine proteins, S100-A8 (S100A8) and Coagulation factor X (F10), had a 1.27 and 0.23 decrease in expression in treatment groups, respectively. Five of the nine proteins had increases in expression: 0.92 for Platelet factor 4 (PF4), 1.10 for Galectin-3 (LGALS3), 1.50 for BPI fold-containing family A member 2 (BPIFA2), 2.30 for Protein AMBP (AMBP), and 2.40 for Arginase-1 (ARG1). Two of the immune proteins, Apoptosis-associated speck-like protein containing a CARD (PYCARD) and Calmodulin-4 (CALM4), lacked consistent signal between pairwise specimen comparisons. For PYCARD, one specimen showed increased expression and one showed decreased expression. For CALM4, two specimens were increased in expression and one decreased in expression.

Muscle Contraction

We recovered 13 proteins associated with muscle contraction, all of which had decreased expression for treatment specimens (Table 1). Seven of the 13 proteins are actin-based myosins (Myosins 1, 4, 7B, 8, Myosin Light chain 1, Tropomyosin 2, and Myosin regulatory light chain 2), with the remaining six being actin-specific proteins (Troponin T, Actins Alpha 1 and Alpha 2, Tintin, and Four and a half LIM domains protein 1).

Preliminary Test of Developmental Archive

Genes associated with two of 20 tooth proteins previously found during mineralization (Pandya et al., 2017) are also found in tooth tissues prior to the onset of amelogenesis based on a query of GXD. Those genes are *Alpl* and *Itgb1*. Both are found only in the tooth developmental stage immediately prior to amelogenesis (TS21 associated with embryonic days 12.5-14). The genes associated with the remaining 18 proteins are found only in developmental stages associated with molar amelogenesis (TS22+, embryonic day 15 through postnatal day 8). Querying genes associated with our sample's differentially expressed immune and actin-based myosin proteins within GXD revealed that immune genes are universally expressed within tooth tissues during all reported stages until adulthood. Actin-based myosins were present from the onset of mineralization forward. Two of the actin-based myosins (*Actn2* & *Fhl1*) were reported as definitively absent from tooth tissues at E14.5 (i.e., just prior to mineralization) (Visel et al. 2004). Additionally, one gene (*Myh1*) was present in mineralization stages but was

definitively absent from postnatal week 6-8 aged mouse specimens (Freeman et al. 1998).

Discussion

Developmental Archive

Results from GXD queries support the argument that our tooth proteome dataset primarily represents protein/gene expression during mineralization and not prior to mineralization. For example, genes for actin-based myosins of interest within our dataset are not expressed in mouse teeth prior to the onset of amelogenesis. While it is not possible to say whether the measured immune-system proteins in our sample were expressed during mineralization or at the time of euthanasia, previous studies (e.g., Green et al. (2019) and Jagr et al. (2019)) have identified immune and inflammation related proteins incorporated within mature enamel. In our case, we recover several of the immune related proteins reported by those studies, including S100A8 and CALM4 supporting the idea that molar enamel represents an archive of gene and protein expression during amelogenesis. Further evidence for this conclusion is supported by the fact that the gene for differentially expressed MYH1 in our dataset is not expressed after mineralization, indicating our MYH1 signal likely represents protein expression during mineralization rather than at a later time point (Freeman et al. 1998). Further, we would not expect expression of amelogenesis specific genes or proteins after the end of mineralization, because of the cessation of proliferation of ameloblasts and lack of vascularization within the fully mineralized tooth (Nanci 2007; Alghadeer et al. 2023).

While this hypothesis requires further validation, our results support the concept that the recovered proteome represents a limited window of development.

Odontogenic Proteins

We predicted that proteins associated with enamel and dentin formation would be altered by our feeding experiment, and specifically that they would be decreased in expression. The major enamel forming protein, AMELX, was significantly reduced in expression for treatment specimens (Table 1). We anticipated that our dietary protein reduction would impact body and possibly tooth size, and thus predicted that AMELX expression would likely be decreased, because AMELX is a necessary component for the formation of enamel. While changes in AMELX met our expectations, changes in dentin forming proteins did not.

In the case of DMP1, there is not a clear signal to interpret whether expression was increased or decreased in our sample. This highlights the challenges of drawing interpretations from proteomic data where individual variation can influence the overall interpretation. This challenge is recognized by the field of quantitative proteomics, but still represents an area where increased research efforts will be needed (Al-Amrani et al., 2021; Chantada-Vazquez et al., 2022; Liu & Zhang, 2021; Steward et al., 2023). A clearer interpretation of DMP1 expression, would be helpful for constructing future hypotheses. For instance, decreased expression of DMP1 should lead to decreased expression of DSPP and dentin hypomineralization, suggesting that DMP1 and DSPP expression contribute significantly to dentinogenesis imperfecta (Orsini et al., 2014) (Shi

et al., 2020). Being able to robustly identify such patterns or, at minimum, make supported interpretations based on the variable evidence, will enhance the utility of future quantitative proteomic studies.

Our finding of increased DSPP expression seemed initially counterintuitive. However, this result is supported by a recent study of protein expression in a hypomineralized enamel defect found in humans (Mukhtar et al., 2022). In this study of hypomineralized molars, the enamel defect impacts the first permanent molars of children and results in a significant reduction in mineral density from normal teeth (Mukhtar et al., 2022). This reduced density was associated with downregulation of AMELX, upregulation of DSPP, but no reported differences in DMP1 expression (Mukhtar et al., 2022).

The causal mechanism for the pattern of increased DSPP expression is unknown. The general role of DSPP is to control the conversion of dental pulp cells into odontoblasts via binding with Integrin beta 6 (ITGB6) (Ritchie, 2018; Wan et al., 2016). Previous work indicated that mice with either a DSPP heterozygous ($DSPP^{+/-}$) or DSPP knockout ($DSPP^{-/-}$) genotypes experience dentin dysplasia and dentinogenesis imperfecta due to haploinsufficiency of DSPP (Shi et al., 2020). Haploinsufficiency suggests that dentin is impacted when DSPP expression is decreased but does not indicate what phenotype results from elevated DSPP expression. Amelogenesis imperfecta enamel is typically thin and chalky while dentin appears to be normally mineralized, suggesting that DSPP overexpression does not result in dentin hypermineralization, but this has not been experimentally validated and dentin structure was not reported by Mukhtar et al. (2022).

Our proteomic results appear consistent with protein expression patterns associated with amelogenesis imperfecta and not dentinogenesis imperfecta, based on the shared expression changes for AMELX and DSPP (Mukhtar et al., 2022; Shi et al., 2022; Orsini et al., 2014). It is unlikely that both amelogenesis and dentinogenesis imperfecta are simultaneously present within a single specimen's dentition. Only a single study has reported compounded presence of amelogenesis and dentinogenesis imperfecta, which occurred in an MSX2 knockout transgenic line (Aioub et al., 2007). It remains to be tested if the mouse molars from our study have mineralized structures consistent with amelogenesis imperfeca. If so, this would suggest that development of thinner and less mineralized dental enamel isa phenotypically plastic response to reduced dietary protein during early development.

Immune and Inflammation Proteins

Previous work on mapping protein expression across micro-sampled enamel sections of pig molars had suggested that there was possibility of recovering immune and inflammation related proteins from mineralized dentition (Green et al., 2019). In their case, Green et al. (2019) were constructing a detailed map of proteomic expression associated with amelogenesis. Their reported immune system proteins were localized from enamel which came from along the enamel-dentin junction (Green et al., 2019). Their study was not designed nor attempted to induce differential protein expression based on experimental procedures. In our case, we were unsure if we would recover immune and inflammation proteins because we created a tissue-averaged signal by

crushing and processing the entire M1. By finding these proteins in our sample and recovering differential expression of them, we present a novel result of immune response to an environmental change. Of the nine immune or inflammation response proteins with significant fold change, five were increased in expression for treatments relative to controls, two were reduced in expression, and two had mixed interpretations (Table 1).

Seven of the nine proteins are primarily associated with neutrophil degranulation, including transport and proliferation of neutrophils. Of these seven, five were increased in expression. However, Calmodulin 4 (CALM4) had a mixed interpretation and coagulation factor X (F10) was reduced in expression. Neutrophils function as critical, but specialized, immune system response molecules. Neutrophils contain granules of multiple types (azurophilic, specific, ficolin-rich, tertiary, and secretory), which target specific threats and/or regulate immune system response to specific infectious threats (Eichelberger & Goldman, 2020; Othman, Sekheri, & Filep, 2022 2022; Yin & Heit, 2018).

The precise nature of what was being targeted by immune system activation is unknown. However, the result of F10 being reduced in expression may provide some insight into the potential infectious threat. Deficiency of F10 has been implicated as part of an immune response to the common, antibiotic-resistant, bacterium *Acinetobacter baumannii* (Choby et al., 2019). In those cases, F10 deficiency is indicative of an increased abundance of neutrophils and macrophages (Choby et al., 2019). Thus,

492 though F10 is decreased in expression, relative to the increase in five other neutrophil
493 degranulation proteins, the combination of patterns supports a conclusion that our
494 treatment group had higher immune system response than our control group. Future
495 research efforts to systematically compare immune-related proteomic signatures from
496 mineralized structures to standardized health monitoring tools should prove fruitful.
497
498 We also recovered changes in PYCARD and S100A8 within this sample, which are
499 indicative of an inflammation response (Table 1). Studies have indicated that PYCARD
500 directly mitigates inflammation when upregulated and contributes to inflammation when
501 downregulated (Sartoretto et al., 2019; Wittmann, Behrendt, Mishra, Bossaller, & Meyer-
502 Bahlburg, 2021 Bossaller, & Meyer-Bahlburg, 2021). In our sample, one treatment
503 specimen showed a moderate increase in PYCARD expression over controls, and one
504 showed a moderate decrease (Table 1). Importantly, deviation in either direction
505 suggests that there is either an increased response to inflammation (increased
506 expression) or increased inflammation present (decreased expression) (Sartoretto et al.,
507 2019; Wittmann et al., 2021). Reduced expression is recovered for S100A8, which is
508 consistent with an ongoing physiological response to inflammation (Wang et al., 2018).
509 Thus, we recover a signal consistent with increased inflammation response, which
510 indicates that there was increased inflammation in treatment specimens versus controls.
511
512 Because of our controlled experimental design, we postulate that reduced dietary
513 protein is the cause of increased inflammation. A recent study investigating the impact
514 of low dietary protein during gestation indicates that intrauterine inflammation can occur

and result in increased inflammation present in the offspring of Syrian golden hamsters (Mohammed et al., 2023). While Mohammed et al. (2023) focused on measuring inflammation of the liver of the offspring, the connection between low dietary protein during embryonic and postnatal development and increased inflammation was strongly established. Our study and results further support this connection. Future studies should aim to systematically investigate proteomic signals of mineralized structures along with standardized inflammation panels. This would further establish the connection between dietary protein, inflammation, and the signals archived within mineralized structures.

Muscle Contraction Proteins

All thirteen significantly modified Muscle Contraction pathway proteins were reduced in expression. They were predominantly actin-based myosins, which play a critical role in cellular movement and structure formation by acting as motor molecules (Guhathakurta et al., 2018). Actin-based myosins are broadly implicated in the proper development of many tissues, including dentition Du et al., 2024; Guhathakurta et al., 2018; Luis & Schnorrer, 2021). During dental development actin-based myosins contribute to the proper formation of enamel rods by transporting ameloblasts (Duverger & Morasso, 2018). Lower expression of actin-based myosins, including some of those which are differentially expressed in our study (e.g., Myosin-1, Myosin-4, Myosin-8, and Tropinin 1) are associated with the syndromic form of amelogenesis imperfecta (Duverger & Morasso, 2018).

Lower expression of actin-based myosins have also been associated with smaller body size (Luis & Schnorrer, 2021). Smaller body size is an anticipated and often postulated phenotypically plastic response to reduced dietary protein (Holloway et al., 1961; Paynter & Grainer, 1956; Paynter & Grainer, 1961). To date, no proteomic study of dentition has recovered differential expression of actin-based myosins. however no dental proteomic study has attempted to experimentally induce differential expression of these proteins. Future investigations should center on understanding the distribution of actin-based myosins within the tooth and the correlation between protein expression and body size. One potential way is to directly investigate changes in actin-based myosin expression of other craniofacial tissues which are systematically reduced when body size is reduced. We propose this future direction because if such an approach is validated, it could serve as an independent proxy of growth. That such a proxy could be derived from proteins contained within a mineralized tooth could be a powerful tool for studies of phenotypic plasticity of deceased or extinct organisms.

Potential Pathways for Phenotypic Plasticity

We recovered 35 significantly enriched REACTOME pathways from our 120 significantly differentially expressed proteins. Among those pathways, two of the top six most enriched pathways, regulation of insulin-like growth factor and muscle contraction, have proteins that play many roles during morphogenesis.

Insulin-like growth factor plays a role in a number of different developmental processes, including odontogenesis, bone development, organ development, and brain

development (Oyanagi et al., 2019; Baroncelli et al., 2017; S. M. Brown, Peters, & Lawrence, 2017 2017; Chen, Martin-Gronert, Tarry-Adkins, & Ozanne, 2009; Dodington et al., 2021; Luo, Liu, Luo, Wang, & Tao, 2020 Wang, & Tao, 2020; Montivero et al., 2021; Vassilakos et al., 2019). Thus, regulation of IGF is a complex set of processes with many different factors involved in regulating expression in different tissues. This includes regulation by thyroid growth hormone (Yakar & Isaksson, 2016) and regulation via phosphorylation of binding proteins to aid in transport, activation, and inhibition (Chrudinova et al., 2024; Dong et al., 2022; Huttlin et al., 2010; Palma-Lara et al., 2023; Tagliabracci et al., 2015). Given the importance of IGF to multiple developmental processes, regulation of IGF is a probable candidate for a mechanism underlying phenotypic plasticity. Experimental manipulation of IGF1 and IGF2 during odontogenesis has revealed systematic changes to the size and number of cusps of developing molars (Oyanagi et al., 2019). The connection between IGF1 and IGF2 gene expression and proteins associated with IGF regulation, which includes Amelogenin X, is not currently well understood (Bansal et al., 2012; Oyanagi et al., 2019; Pandya & Diekwisch, 2021). However, regulation of insulin-like growth factor offers a promising pathway for future investigation, particularly in elucidating responses of IGF gene expression and associated regulatory factors to environmental perturbations, such as poor diet.

Similarly, our collection of proteins from the muscle contraction pathway are primarily actin-based myosins. The function of myosins as motor proteins is well documented, but the specific nature of how myosins interact during odontogenesis is poorly understood

(Du et al., 2024; Guhathakurta et al., 2018). Previous proteomic studies of teeth have not reported differential expression of actin-based myosins, making this result unexpected. Simultaneously, those studies were not attempting to experimental induce plastic responses like our study. The connection of actin-based myosins with body size and potentially with amelogenesis imperfecta suggests that future investigations may be fruitful. These future studies would hopefully confirm the results we recovered and validate a link between protein expression and body size variation.

Conclusions

Proteomic expression results supported our prediction that halving dietary protein during embryogenesis and early postnatal development would alter expression of proteins recovered from mouse molars. Specifically, we identified 120 differentially expressed proteins associated with a reduction of dietary protein during embryonic and early postnatal development. Changes in dietary protein have been proposed to result in phenotypically plastic changes to tooth and body size and shape. Our study provides quantification of the proteomic pathways which could underlie these plastic changes. We recovered significant changes in proteins associated with dental development, which are primarily within the pathway associated with regulation of Insulin-Like Growth Factor. The connection between IGF and dental proteins remains to be further investigated, but changes in expression in this pathway could directly influence tooth size and shape. We also identified systematically reduced expression for proteins in the Muscle contraction pathway, specifically actin-based myosins, a novel discovery for

tooth-derived proteomics data. Actin-based myosins are broadly implicated in vertebrate development and are correlated with body size and tooth development.

Our controlled feeding experiment induced increased immune system activation and inflammation response, as evidenced by increased expression of proteins in the Neutrophil degranulation pathway. While this result does not directly inform us about phenotypic plasticity, that we are able to derive such information from a fully mineralized tooth could prove useful for studying the biology of deceased or extinct organisms.

We propose that proteomic quantification of non-experimental organisms will prove fruitful and predict that dietary changes in wild settings will change enamel and dentin formation by altering aspects of the IGF regulatory pathway and potentially expression of actin-based myosins. Future research efforts then should focus on elucidating the connection between IGF gene expression and enamel and dentin protein expression; determining the role of actin-based myosins in tooth and skeletal development and body size; and comparing the immune and inflammation signals in proteomic data to classically used tools (e.g., blood panels and cortisol screening). With these efforts it will become clearer precisely which sections of these pathways underlie this kind of phenotypically plastic response and enhance the utility of quantitative proteomics for investigating organismal biology more broadly.

Acknowledgments

We thank the Stony Brook Division of Laboratory Animal Research (DLAR), particularly Laurie Levine, for animal husbandry and management. We thank Dr. John Haley of the Stony Brook University Proteomics Core Facility, for sample preparation and initial post-

processing. We thank the Stony Brook University Center for Inclusive Education staff: Lisa Ospitale, Karian Wright, Diana Champney, and Erica Valdez, for their support. Author RWB was funded via an Institutional Research and Academic Career Development Award (IRACDA) made to Stony Brook University from the National Institute of General Medical Sciences of the National Institutes of Health [K12GM102778], and startup funding to NSV from Stony Brook University. The content is solely the responsibility of the authors and does not necessarily represent official views of the National Institutes of Health.

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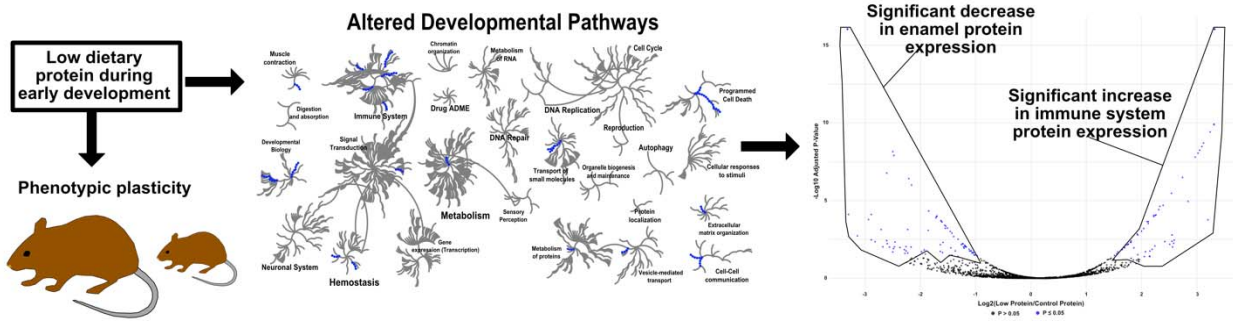
Graphical Abstract:



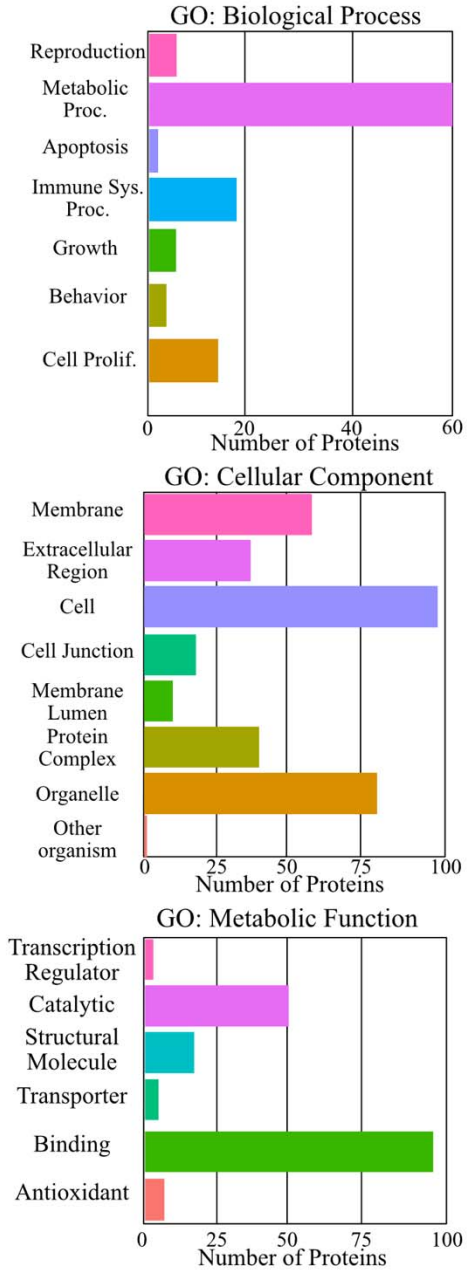


Figure 1: Most enriched pathways from Gene Ontology (GO) analysis of the 120 significant differentially expressed proteins between treatment and control specimens.