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Lactylation Dynamics in Myxozoan Proteins

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Abstract

Lactylation has recently emerged as a novel post-translational modification with critical implications for gene regulation and host–parasite interactions, as demonstrated in species of *Plasmodium* and *Trypanosoma*. Here, we applied an integrative approach combining in-silico predictive and experimental analyses to detect protein lactylation in parasitic cnidarians in the class Myxozoa. In-silico analyses were conducted using data from seven myxozoan species, six obtained from public databases and one generated in this study. Experimental validation was based on Oxford Nanopore Technologies (ONT) genome sequencing and Western blotting of *Myxobolus macroplasmoidal* samples. This integrative approach enabled us to validate the in-silico analysis, to identify conserved lactylation sites in key stress-response proteins (including HSP70 and several metabolic enzymes), and to map electrochemical signatures of lactylation sites in the ONT data for *M. macroplasmoidal*. Comparative analyses across species, supported by functional enrichment and network-based approaches, demonstrated conserved regulatory roles of lactylation alongside species-specific variation in the biological processes it controls. Functional enrichment analyses further implicated lactylation in critical pathways, including glycolysis, oxidative phosphorylation, and immune signaling, supporting its integration into core regulatory networks underlying the functional biology of parasitism.

Keywords: Myxozoa; Epigenetics; Oxford Nanopore sequencing; Host–parasite interactions, Cnidaria.

1 Introduction

Epigenetic phenomena, such as methylation and acetylation, allow cells to regulate gene expression without modifying the underlying DNA sequence. Accordingly, these processes play crucial roles in the biology of hosts and parasites. They may orchestrate host-parasite interactions, including those involving host immune responses to parasite challenges and the development of virulence of parasites within hosts [1, 2, 3, 4]. The recent demonstration of histone lysine lactylation [5] as a novel epigenetic modification in mammalian cells expands our understanding of epigenetic mechanisms [6]. The process consists of the addition of a lactyl group, derived from lactate, to the lysine residues on histone tails [5], and hence the regulation of chromatin structure and gene expression. Lactylation has been shown to upregulate the transcription of certain genes related to macrophage polarization during inflammation [5, 7] and to enhance the expression of genes that promote tissue repair and maintain homeostasis [8].

Subsequent research has revealed lactylation in parasitic protozoans, including *Trypanosoma brucei* [9] and *Plasmodium falciparum* [3], the causative agents of African trypanosomiasis and malaria, respectively. Zhang et al. [9] identified 387 lysine-lactylated sites across 257 proteins with diverse cellular localizations and biological functions in *T. brucei*. Their study also revealed that glucose metabolism plays a crucial role in regulating protein lactylation, since the parasite lacks lactate dehydrogenase. Jabre et al. [4] reported the presence of histone lactylation in four *Plasmodium* species, under both *in vitro* and *in vivo* conditions, and demonstrated species-specific differences in the magnitude and responsiveness of this epigenetic modification. In addition to histone tail lactylation, numerous non-histone proteins were demonstrated to be lactylated, and there was evidence for species-specific patterns. It was therefore suggested that species-specific evolution of the Lactyl Post-Translational modification (PTM) in the *Plasmodium* lineage has entailed epigenetic control of gene transcription via histone tail marks. This process involves chemical modifications of histone proteins and their associated nucleic acids that regulate chromatin accessibility and transcriptional activity, co-transcriptional control of RNA processing, and post-translational modulation of protein activity [10, 4]. Because of this recent research, lactylation is regarded as a new frontier for investigating post-translational modifications [8].

Although both histone and non-histone protein lactylation have been examined since the modification was first described [5], the enzymes responsible for catalyzing these modifications remain incompletely characterized. Investigations of lactylation in mammalian systems proposed that certain histone acetyltransferases might exhibit relaxed substrate specificity, enabling transfer of lactyl groups in addition to acetyl groups [5, 4]. Such reactions require appropriate metabolic donors. However, although acetyl-CoA is abundant in eukaryotic cells, lactyl-CoA has been detected only at low levels [11], raising questions about the plausibility of the mechanism. More recently, alanyl-tRNA synthetase 1 (AARS1) has been proposed as a candidate lactyltransferase in mammals. Experimental evidence indicates that AARS1 can sense elevated cytosolic lactate levels, translocate to the nucleus, and catalyze histone lactylation in an ATP-dependent manner [12]. Additional support for this role was provided by Zong et al. [13], who demonstrated that β -alanine reduces histone lactylation by competitively inhibiting lactate binding to AARS1. Nevertheless, it remains uncertain whether AARS1 alone accounts for all lactylation events, particularly those affecting non-histone proteins, or whether additional enzymes contribute in different cellular contexts or taxa. These findings highlight that the enzymatic bases of lactylation are still unclear and underscore the importance of investigating potential lactyltransferases across diverse organisms.

Myxozoa are early diverging metazoans that have undergone extensive morphological simplification and size reduction compared to their free-living cnidarian relatives [14, 15]. Their complex life cycles involve invertebrate (annelids and freshwater bryozoans) and vertebrate (primarily fish but also amphibians, reptiles, waterbirds and mammals) hosts [16]. Myxozoans comprise some 20% of described cnidarians and are causative agents of fish diseases with ecological and economic impacts [17, 18, 19]. Concerns about disease impacts on wild and farmed fish have promoted a body of research on disease ecology, epidemiology, and potential control [20, 21, 22, 23, 24]. Recent research has focused on identifying epigenetic processes in myxozoans. Understanding such processes could then offer strategies to control commercially significant fish pathogens. Evidence for cytosine methylation in myxozoans was recently demonstrated using long-read sequencing technology (Oxford Nanopore Technology, ONT), which enabled genome-wide detection of methylation signals across multiple

species [25]. These findings challenge earlier conclusions that myxozoans lack cytosine methylation [26], whose analyses were based on alternative sequencing approaches. This contrast highlights the importance of methodological differences in detecting epigenetic modifications and underscores the need for further investigation using high-resolution sequencing technologies. Evidence for regulation of gene silencing by microRNA was obtained by analyzing a transcriptome of one myxozoan (*Myxobolus bejeranoi*) [27].

Here we carried out an integrative study based on both in-silico and experimental analyses for further evidence of lactylation in myxozoans. For the in-silico analysis we used the recently developed software AGAE-Layzer [28] which predicts lysine lactylation sites from DNA, RNA, or protein sequences obtained from any sequencing platform, analyzing genomic and proteomic datasets from several myxozoans. Our experimental analyses entailed examination of ONT sequencing data and validation via Western Blot analysis. For the former we used a recently developed signal-analysis tool (the software JEAN [29] designed to identify epigenetic modifications from ONT sequencing data. Our experimental analyses focused on data obtained from samples of the myxozoan *Myxobolus macroplasmoidal* parasite of the South American bryconid fish *Salminus brasiliensis*. We further assessed whether proteins predicted to be lactylated in *M. macroplasmoidal* show differential expression patterns. Comparative analyses across species were then conducted to evaluate interspecific variation in lactylation, identify associated biological pathways, and infer potential roles in host–parasite interactions. Finally, computational prediction tools were applied to estimate the impact of lactylated proteins on host cellular processes and to determine whether the implicated pathways may represent candidates for targeted therapeutic intervention.

2 Material and Methods

Bioinformatic analyses were employed to characterize lactylation in myxozoan parasites of fish hosts. The study was carried out on seven myxozoan genomes. These included the following five myxozoan genomes retrieved from the NCBI database (30-Sayers et al. 2022): *Ceratomyxa* sp. from Amazonian sciaenids (*Plagioscion squamosissimus*) (GCA_040939075.1), *Ceratonova shasta* from North American

salmonids (*Oncorhynchus mykiss*) (GCA_918559645.1), *Ellipsomyxa* sp. from Amazonian curimatids (*Curimata inornata*) (GCA_040938985.1), *Henneguya salminicola* from North American salmonids (*Oncorhynchus tshawytscha*) (GCA_009887335.1), and *Myxobolus squamalis* from North American salmonids (*Oncorhynchus kisutch*) (GCA_010108815.2). A sixth genome of *Tetracapsuloides bryosalmonae*, which parasitizes European salmonids (*O. mykiss*), was obtained using protein information, retrieved from the NCBI database (GSE198859). A seventh genome was obtained for this study from *M. macropasmodialis*, using ONT sequencing (PRJNA1449577). Analyses of lactylation sites in *Chironex yamaguchii*, a cnidarian from the class Cubozoa, was included for comparative purposes (GCA_024741275).

The Expasy Translate tool [31] was used to translate genomic sequences into predicted protein sequences by selecting the most appropriate open reading frames. The resulting protein dataset was then mapped back to the genomic sequence dataset from the previous step, allowing us to establish links between predicted proteins and their corresponding coding regions, and to prioritize proteins according to their predicted biological function. For benchmarking purposes, the reference genome of *C. shasta* (GCA_918559645.1) was used, with the annotated proteins serving as a comparative dataset to validate annotation quality and mapping accuracy across the focal myxozoan species.

To predict putative lactylation sites in the proteomes, the AGEA-Lyzer tool (<https://agea-layzer-v1.netlify.app> [28], which incorporates models that utilize sequence-based features and curated reference datasets to identify candidate post-translational modifications (PTMs), was used.

A comparative site-frequency analysis was performed to evaluate the distribution of predicted lactylation sites across histone proteins. Predicted modification sites generated by the AGEA-Layzer tool were mapped to reference histone sequences, and the frequency of modified lysine residues at each position was calculated. Site counts were normalized to protein length and total number of predicted sites to allow comparison among residues and across species. Positions showing elevated modification density relative to background levels were interpreted as candidate enrichment sites. This

analysis enabled identification of preferentially modified residues, including H3K18 and H3K27, which were subsequently examined in a functional context.

Differential expression analysis was performed to identify proteins that were significantly upregulated or downregulated in infected samples compared to uninfected controls, using statistical software R [32] and Python [33]. To interpret the biological processes affected and to determine enriched pathways, functional enrichment analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [34] and the Gene Ontology (GO) database [35]. Protein–protein interaction (PPI) networks were then constructed using STRING (*Search Tool for the Retrieval of Interacting Genes/Proteins*) [36], enabling visualization of functional relationships among proteins and identification of potential hub proteins. GO terms were also used to obtain detailed descriptions of the biological functions of differentially expressed proteins.

A comparative analysis of our results was performed against existing proteomic and transcriptomic datasets from the same set of myxozoan species analyzed in this study, namely *Ceratomyxa* sp., *C. shasta*, *Ellipsomyxa* sp., *H. salminicola*, *M. squamalis*, *T. bryosalmonae*, and *M. macropasmodialis*. Furthermore, custom computational scripts implemented within the AGAE-Layzer framework (<https://agae-layzer-v1.netlify.app> [28]) were used to simulate the potential effects of protein lactylation on host cellular processes, thereby enabling the identification of candidate molecular targets potentially involved in host–parasite interactions.

To identify candidate orthologs of alanyl-tRNA synthetase 1 (AARS1), we used known protein sequences from *Homo sapiens* and *Escherichia coli* as queries in BLASTp searches against the predicted proteomes of the myxozoan species. The search criteria included an E-value threshold of 0.05. Protein hits with significant similarity were subsequently examined by pairwise alignment using ClustalW [37] to confirm the presence of conserved domains characteristic of AARS1. Visualization using the Integrative Genomics Viewer (IGV) [38] was used to reveal the lactylation marks.

ONT sequencing was performed on a sample of *M. macropasmodialis* (see materials and methods in the Supplementary File S1). The spatiotemporal mapping of epigenetic and post-translational modifications were inferred from electrical signal shifts in the ONT sequencing data associated with lactyl group binding using the computational

framework JEAN (<https://jean-v2-0.netlify.app/> [29]. In addition to the predictions using AGAE-Layzer and electrochemical signature confirmation using JEAN, lactylation was further validated using the classic molecular biology approach (Western Blot) also performed on the *M. macroparasmodialis* sample (see materials and methods in the Supplementary File S2).

A comparative score distribution analysis was conducted to assess concordance between computational predictions and experimental measurements of lactylation. Confidence scores generated by the AGAE-Layzer predictive framework and corresponding experimental scores derived from JEAN analysis of ONT data were compiled for matched sites. A dataset of 1,000 observations spanning the full confidence range (0.0–1.0) was used for comparison. Score distributions were visualized using density-normalized histograms to enable direct comparison independent of sample size. Descriptive statistics, including mean and standard deviation, were calculated for each dataset. Agreement between predicted and experimental scores was quantified using Pearson correlation analysis. All statistical analyses and visualizations were performed using R (version 4.5) with standard scientific libraries.

3 Results and Discussion

3.1 Predictive modeling of lactylation and methylation sites

Our in-silico prediction analysis, using the predictive tool AGAE-Lyzer identified lysine residues within myxozoan histones and associated proteins that are potentially recognized as targets for lysine-lactylation. Lysine-lactylation sites were detected in protein sequences derived from gene models of *T. bryosalmonae*. Lysine residues predicted with statistical significance in *T. bryosalmonae* provide evidence that specific histone regions are enriched in lactylation signatures. The chemical conversion of lysine to lactyllysine supports the biochemical feasibility of this post-translational modification in *T. bryosalmonae*. Together, these computational predictions and biochemical identifications highlight the presence of potential histone lactylation sites in myxozoans (Figs 1 and 2).

A comparative site-frequency analysis revealed a higher density of predicted

lactylation sites on H3K18 and H3K27 in *M. macropasmodialis* compared with other residues (Supplementary File S3). This enrichment provides predictive evidence that these loci may represent preferential targets of lactylation. Such localization is consistent with the established association of H3K18 and H3K27 modifications with transcriptionally active chromatin and regulatory elements, including promoters and enhancers [39]. In contrast, predicted methylation marks were predominantly localized to regions such as H3K9 and H3K27me3, which are commonly linked to transcriptional silence [40]. The predicted co-occurrence of lactylation (associated with gene activation) and methylation (associated with repression) on histones leads us to hypothesize that a finely tuned regulatory mechanism exists in myxozoans. Such an interplay, which is well-documented in other host-parasite systems for balancing gene activation and repression, could be a critical mechanism for enabling adaptation to changing host conditions. These PTMs are hypothesized to regulate essential biological processes such as protein stability and metabolic adaptation, which are critical for parasite survival. Lactylation, for instance, has been shown to enhance transcription [5], and in parasites it may be particularly important for immune evasion, while concurrent methylation can silence host defense-related genes [41]. This precise modulation of gene expression likely facilitates infection establishment and persistence, supporting the broader hypothesis that parasite PTMs, such as lactylation and methylation, fine-tune host–pathogen interactions and manipulate host cellular processes to their advantage [4, 41, 42, 43]. Recent studies reinforce this view. H3K18 lactylation (H3K18la) has been found to be enriched at active promoters and tissue-specific enhancers in human and mouse tissues [44], and glycolysis-dependent histone lactylation has been shown to facilitate chromatin accessibility at enhancer regions, thereby activating transcriptional programs in chick embryos [39]. Methylation marks are generally associated with transcriptional repression, particularly cytosine methylation at CpG-rich promoter regions, which blocks transcription factor binding and stabilizes gene silencing [45, 46]. Histone modifications, such as H3K9, provide an additional repressive layer by establishing heterochromatin states linked to DNA methylation [47]. Collectively, these findings variously demonstrate that lactylation promotes transcriptional activation at regulatory elements, whereas methylation functions as a hallmark of repressed chromatin, highlighting their complementary roles in shaping

parasite–host interactions.

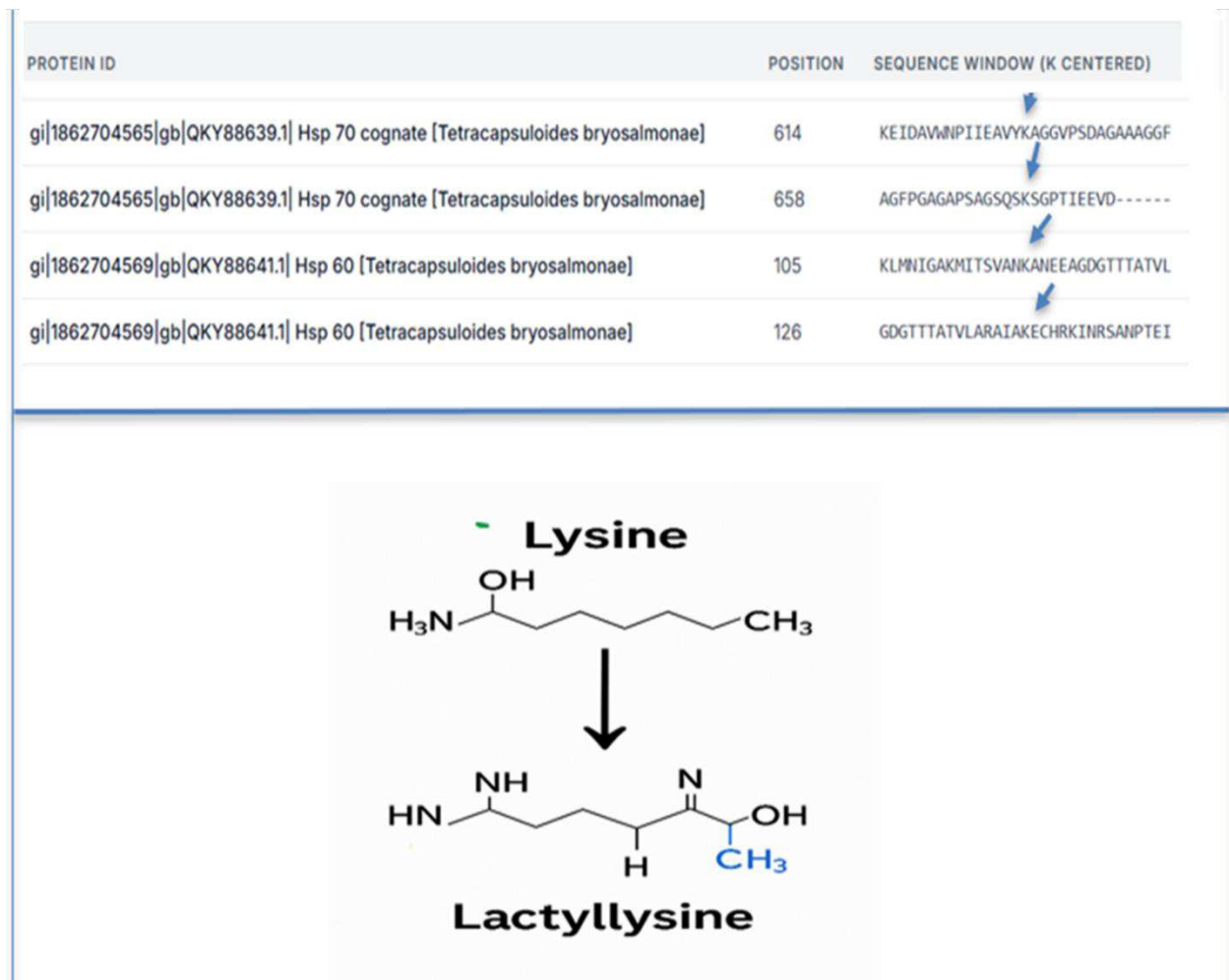


Fig. 1 Prediction and biochemical representation of histone lactylation. The upper panel shows examples of predicted lactylation sites (blue arrows) within protein sequences derived from gene models in myxozoan genomes (*Tetracapsuloides bryosalmonae*). The lower panel illustrates the chemical transformation of lysine into lactyllysine

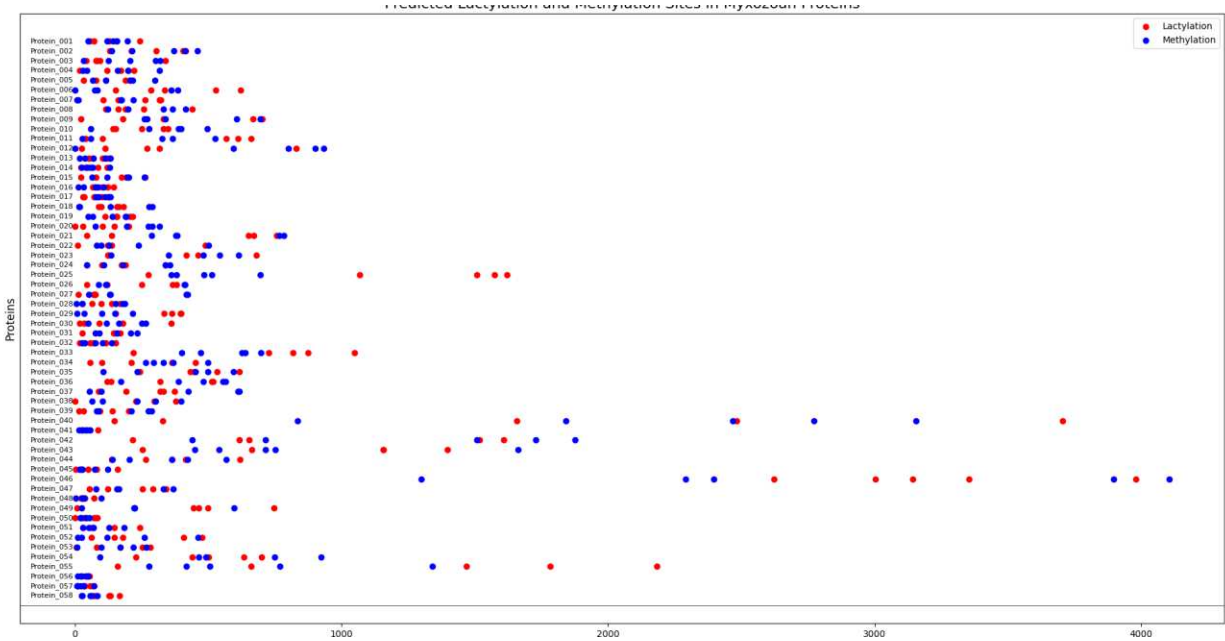


Fig. 2 Predicted lactylation (red dots) and methylation (blue dots) sites across conserved proteins identified in the seven myxozoans species involved in this study. The x-axis represents the residue positions within each protein. The analyzed proteins are listed on the y-axis. Lactylation and methylation marks are dispersed across the protein sequences, but regions with higher densities of these modifications may be functional hotspots

3.2 Concordance of predictive and experimental analyses

To validate our in-silico analysis lactylation predictions, we analyzed ONT sequencing data using the JEAN tool and performed Western blotting, in both cases using *M. macropasmodialis* samples. Signal profiles obtained from ONT analysis provided a comprehensive characterization of epigenetic modifications in *M. macropasmodialis* (Supplementary Table S1). Comparative score distribution analysis demonstrated a strong concordance between experimental results and computational predictions. The overlapping histogram analysis presents density-normalized score distributions for experimental measurements and computational predictions (n = 1,000 data points) across the complete epigenetic modification confidence scoring range (0.0-1.0) (Fig. 3). Experimental scores demonstrate a right-skewed distribution with mean $\mu = 0.798$ and standard deviation $\sigma = 0.154$, indicating modest, but consistently high confidence in experimental detection capabilities. The predictions exhibit a similar distributional pattern with mean $\mu = 0.751$ and standard deviation $\sigma = 0.168$, showing slightly broader variance but comparable central tendency.

The highly significant Pearson correlation coefficient ($r = 0.978$; $p < 0.001$) signifies a strong correlation between independent predictive and experimental methodologies, as visualised by the substantial overlap in predictive (AGE-Layzer analysis) and experimental (JEAN analysis) (Fig. 3). This cross-validation indicates that both experimental measurements and computational predictions capture equivalent underlying biological phenomena. Density normalization enables direct comparison of distributional characteristics while accounting for potential variation in sample size, thereby ensuring robust statistical interpretation of cross-method agreement.

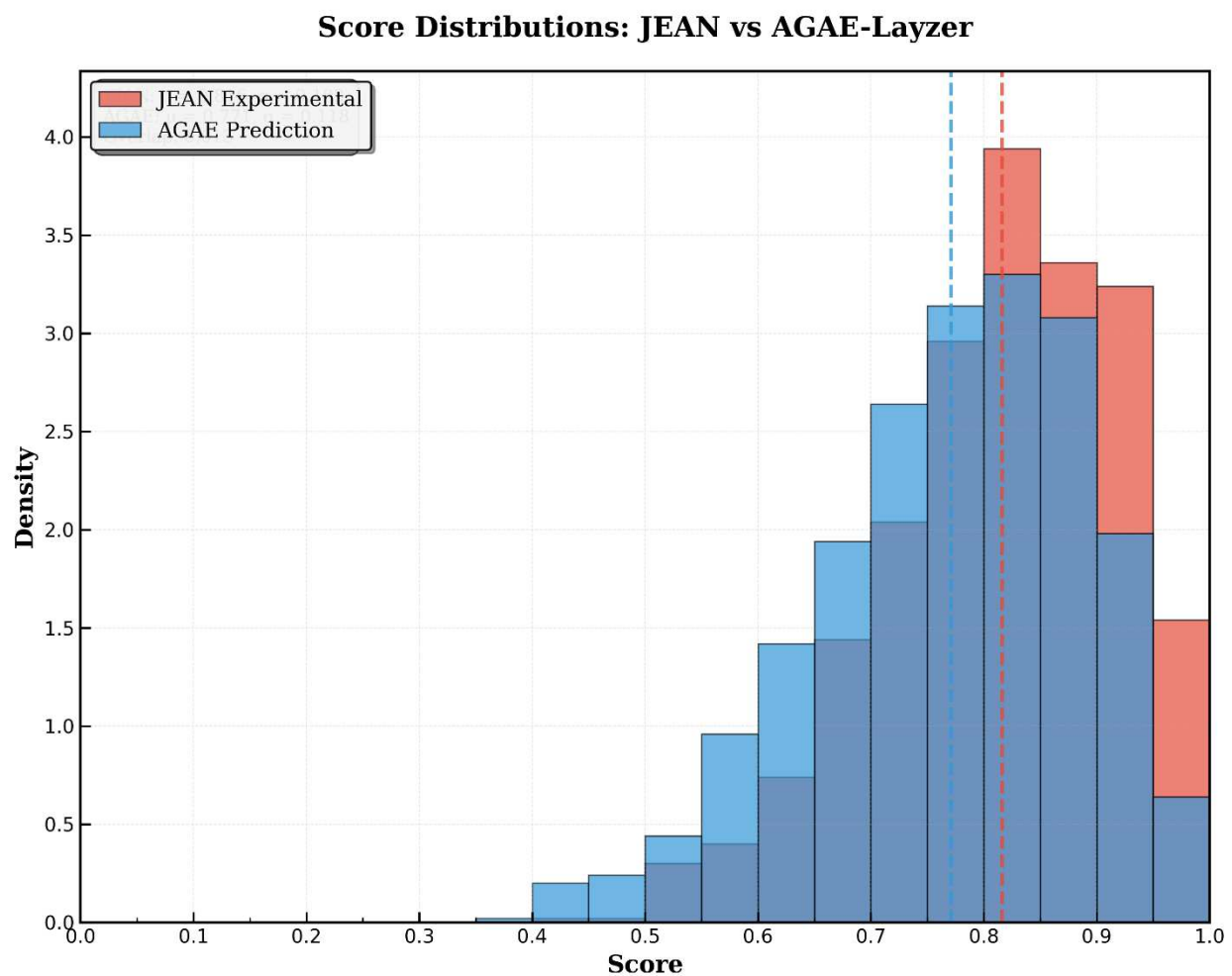


Fig. 3 Comparison of score distributions for experimental analysis (red) and predictive analysis (blue). Both methods show highly overlapping distributions with similar variance, and experimental analysis exhibits a slightly higher mean score. Vertical dashed lines mark the respective distribution means, highlighting the modest but consistent offset between experimental and computational approaches

Western Blot analysis provided evidence for histone lactylation, confirming predictive work. Pan-lactyl-lysine immunoblotting revealed widespread protein lactylation across multiple molecular weights. Acid-extracted fractions showed enrichment of histone-sized proteins and robust detection of histone H3 (Fig. 4). Site-specific lactylation at H3K18 was detected, confirming histone lactylation in *M. macroparasmodialis*. Concurrent detection of H3K18la and H3K9ac indicates coexistence of lactylation and acetylation on histone H3 (Fig. 5), supporting a combinatorial epigenetic regulatory model responsive to metabolic cues. This analysis provides experimental evidence for global protein lactylation and site-specific histone lactylation in *M. macroparasmodialis*. The coexistence of lactylation and acetylation marks suggests a multi-layered, metabolically sensitive epigenetic system contributing to parasite gene regulation and adaptation. The detection of Histone H3 is similar to the first detection of lactylation [5], providing evidence of post-translational changes in the lactylation mark.

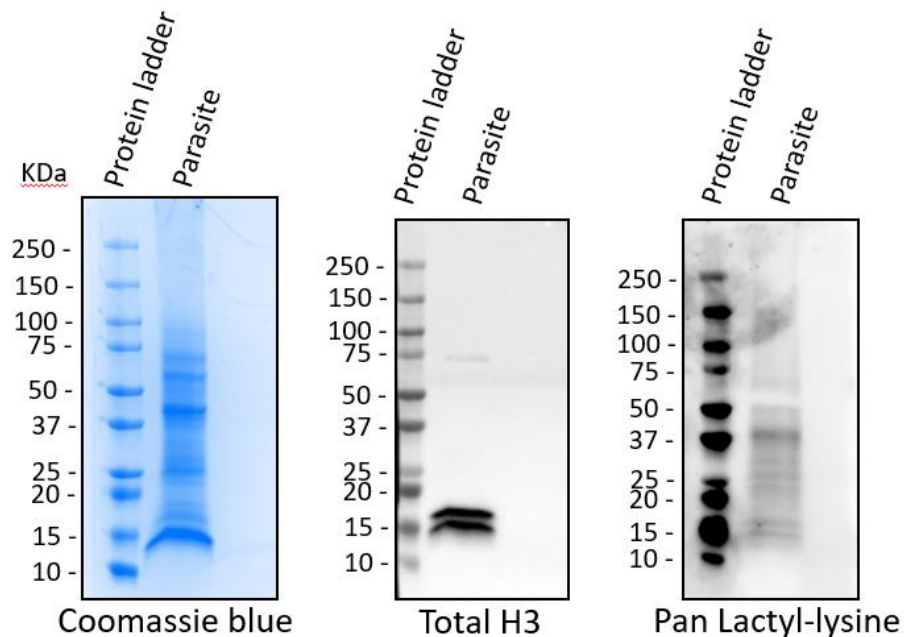


Fig. 4 Coomassie blue-stained gel showing the proteins present in the parasite and immunoblots for total H3, Pan Lactyl-lysine in the whole cell lysate from the parasite. To access original figures, see Supplementary Fig. S1.

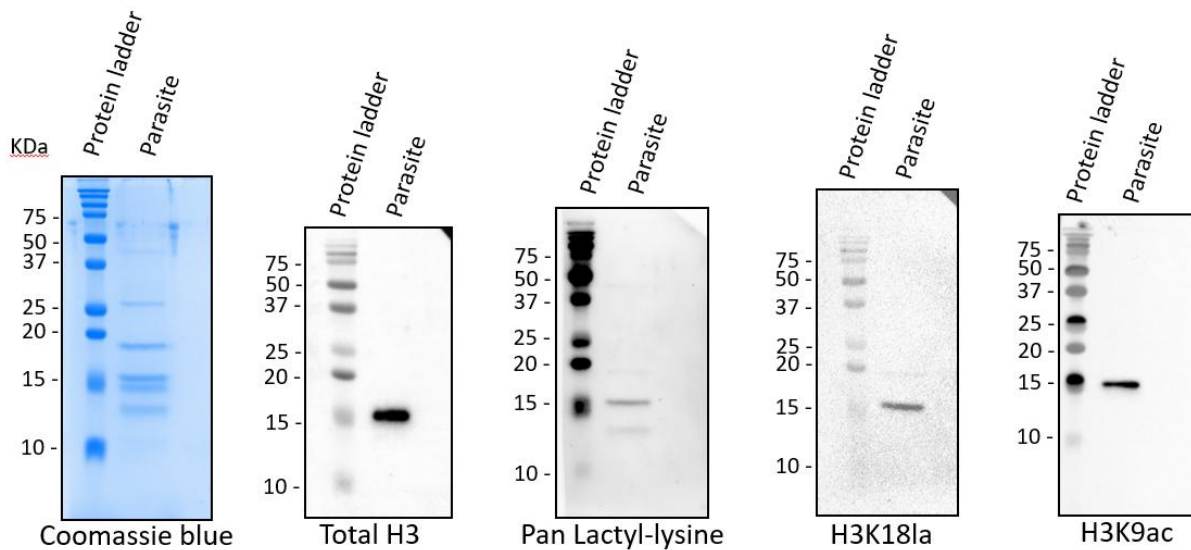


Fig. 5 Coomassie blue-stained gel showing the enrichment for histone proteins and immunoblots for total H3, Pan Lactyl-lysine, H3K18la and H3K9ac in the acid extracted histones of parasite. To access original figures, see Supplementary Fig. S1.

3.3 Differential localization of lactylation and methylation marks

Visualization using IGV revealed that predicted lactylation marks frequently localize to promoter regions and active enhancer elements, genomic loci that are generally associated with transcriptional regulation. A representative example at the Heat shock cognate 70 kDa protein locus showed a clear overlap between predicted lactylation peaks and RNA-seq coverage patterns (Supplementary File S4). Notably, at this specific locus, lactylation marks coincided with regions of reduced transcriptional signal, whereas predicted methylation marks overlapped regions of higher expression. This observation indicates that the functional consequences of lactylation may be context-dependent rather than uniformly activating, potentially reflecting interactions with other chromatin modifications or regulatory mechanisms. Such differential localization suggests that lactylation and methylation can exert distinct, and possibly antagonistic, influences on gene regulation. The enrichment of lactylation sites within promoter and enhancer regions nevertheless supports a role in modulating transcription of genes involved in metabolic adaptation and parasitism. Collectively, these findings indicate that histone lactylation

contributes to fine-scale regulation of gene expression in myxozoans rather than acting as a simple on/off activation mark.

The regulatory impact of lactylation is reflected in transcriptional activity differences observed in RNA-seq coverage tracks from original data of *C. shasta* (Supplementary File S4).

3.4 Lactylation influences expression of key proteins

Proteomic analysis, summarized in the volcano plot (Fig. 6), identified a set of key proteins whose expression is likely to be significantly influenced by lactylation. Differential expression analysis revealed enrichment of proteins involved in several important biological processes, including glycolysis, immune evasion, and stress adaptation. We specifically highlight elevated lactylation levels on the inducible HSP70 isoform (HSPA1A/B). The proteins belonging to the HSP70 family can differ in expression dynamics, with HSP70 being stress-inducible and HSC70 constitutively expressed [48, 49].

Our analysis revealed that lactylation of histones is associated with the activation of transcription of metabolic genes, establishing a direct link between lactate production and the functional biology of parasites (Supplementary File S5). These findings highlight the versatility of lactylation, demonstrating an ability to modulate both structural proteins, like HSP70, and regulatory proteins, like histones. This dual role underscores the potential importance of lactylation in the biology of myxozoans and other cnidarians (Supplementary File S5).

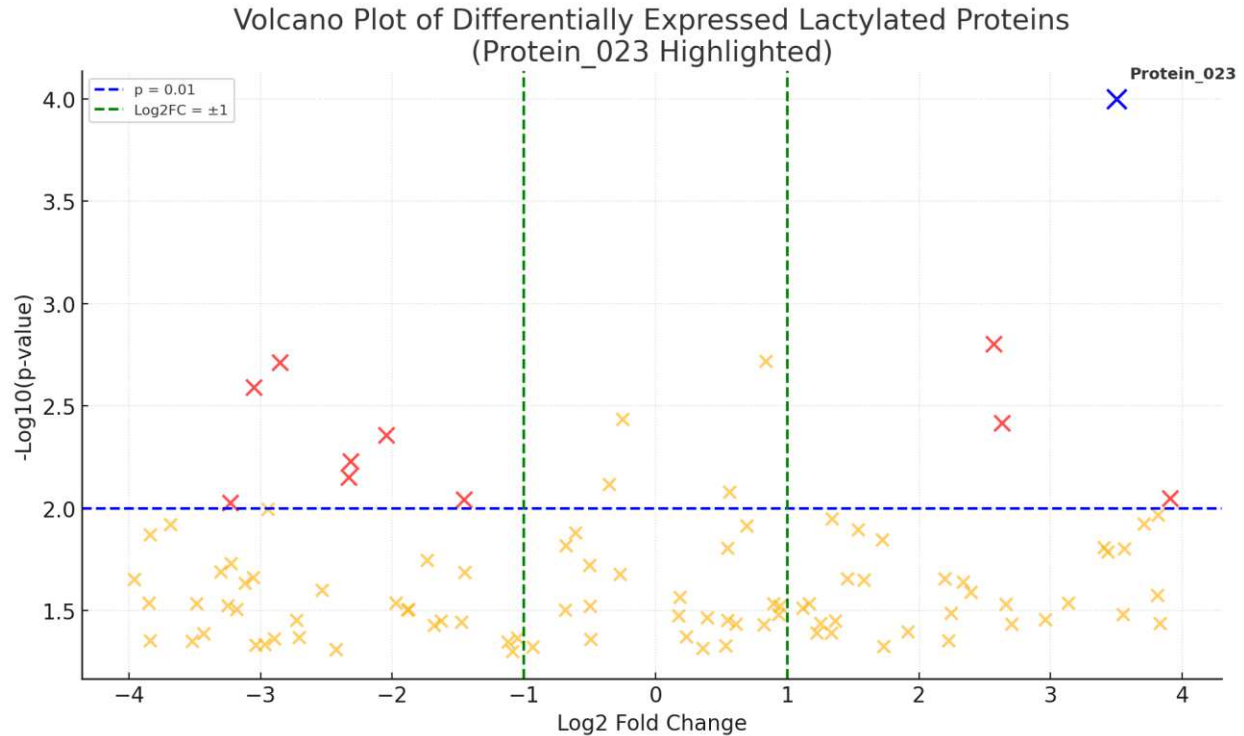


Fig. 6 Volcano plot of differentially expressed lactylated proteins in the seven species studied. Red markers denote proteins meeting significance criteria ($p < 0.01$ and $|\text{Log}_2 \text{fold change}| > 1$); yellow markers are non-significant. Dashed blue and green lines indicate the p-value (0.01) and Logoffs (± 1) thresholds, respectively. The key outlier, Protein_023 identified as AARS1 (Alanine-tRNA ligase), is highlighted in blue for emphasis which shows a high level of expression

3.5 Lactylated proteins are associated with protein networks

The PPI network analysis of myxozoan proteins revealed the presence of lactylated proteins in protein networks, particularly HSP70 and histones (Fig. 7), implying that lactylation plays a crucial role in coordinating various myxozoan cellular processes. For example, the observed interactions between lactylated HSP70 and histones suggest a coordinated regulation of stress responses and transcriptional activity (Supplementary file S6) that may be critical for parasite survival within the host. These findings further support the differential gene expression results and identify potential candidate drug targets. The network analysis highlights clusters of pathways involved in metabolic adaptation (reprogramming of glycolysis and energy metabolism under host stress) and immune modulation (parasite-driven suppression or redirection of host immune responses), providing further support for a central role of lactylated proteins in the functional biology of parasites.

Heuristic Protein-Protein Interaction Network (Random Connections)

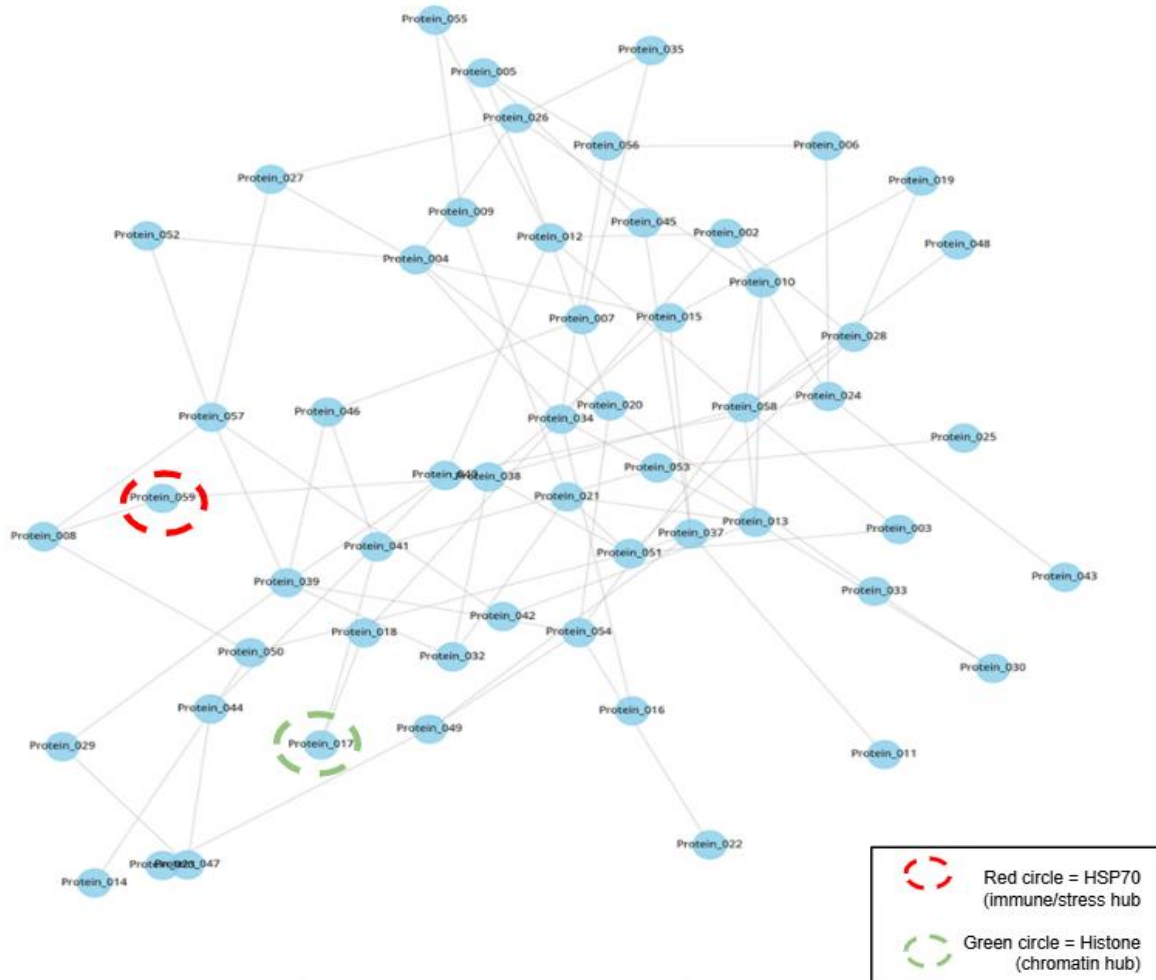


Fig. 7 Heuristic protein–protein interaction (PPI) network of 59 myxozoan proteins predicted to undergo lactylation. Nodes represent individual proteins, and edges denote potential interactions. The network highlights two central lactylation hubs: Protein_059 = HSP70 (red dashed circle), a molecular chaperone involved in proteostasis and immune modulation, and Protein_017= histone (green dashed circle), which regulates chromatin accessibility and transcription. Note that these hubs occupy central positions in the network, underscoring their potential role in metabolic adaptation, immune modulation, and stress response pathways. Proteins significantly modified by lactylation are therefore highlighted as candidate intervention targets for host–parasite interaction studies

3.6 Analytical approaches collectively identify lactylation as an integral process

Our comparative analysis reveals lactylation across myxozoan species and suggests variation in lactylation levels amongst species (Figs 8–14). This variation is consistent with earlier reports in *Plasmodium* species [4] and may reflect species-specific differences in lactylation patterns. Alternatively, such variation could arise from

developmental stage–specific regulation in fish hosts or from environmental influences such as temperature and oxygen availability.

The global Protein-Protein Interaction network of 59 proteins (Fig. 8) revealed modular clusters, while hierarchical clustering analysis (Fig. 9) grouped proteins with similar lactylation profiles into conserved functional modules. A hub network analysis (Fig. 10) identified central nodes such as HSP70 (Protein_059) and histones (Protein_017), which anchor interaction clusters associated with stress adaptation, proteostasis, and chromatin regulation. These network-based insights provide stronger evidence for functional targeting of lactylation than that gained by considering average levels of each protein alone. Building on these results, we extended the analysis to include both parasitic and free-living cnidarians, revealing conserved enrichment of lactylated proteins in core regulatory pathways. This suggests that lactylation is a mechanistic feature broadly conserved across cnidarian taxa (Supplementary File S5).

Protein-Protein Interaction Network for All 59 Proteins
(Cosine ≥ 0.8)

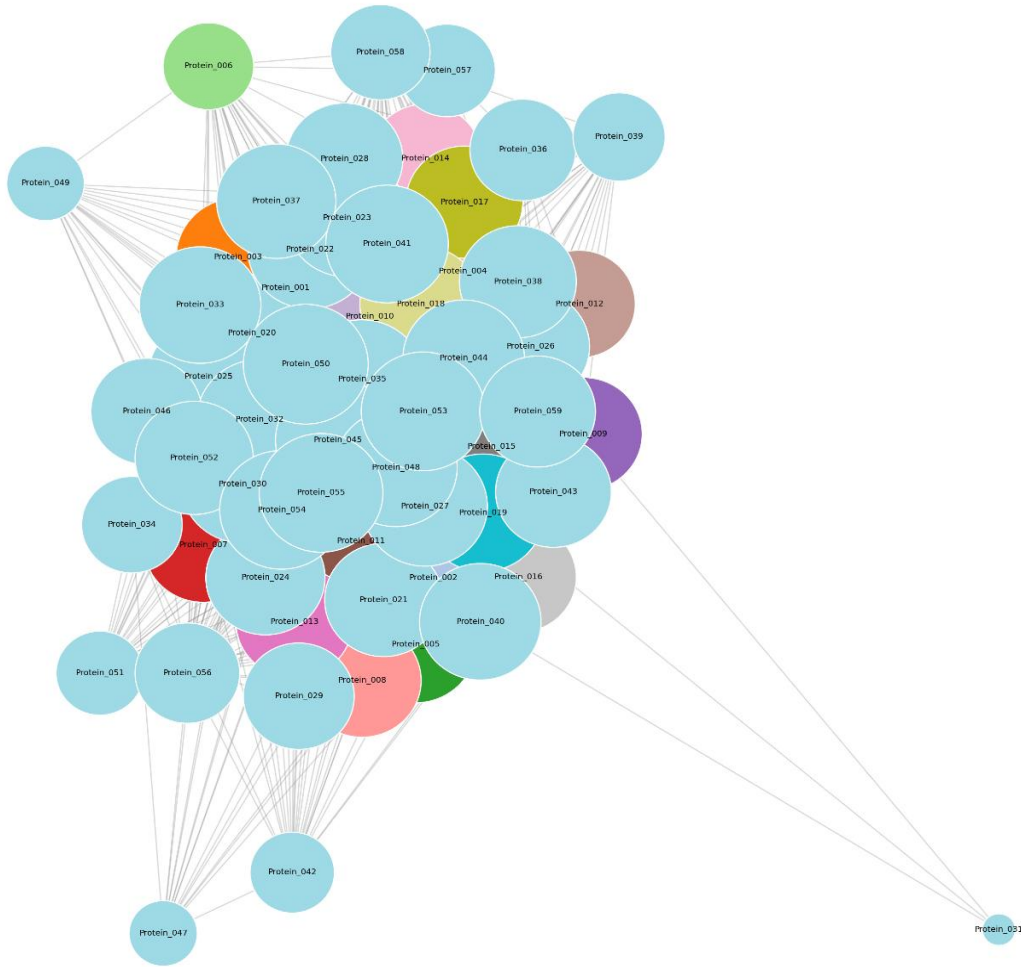


Fig. 8. Global protein-protein interaction (PPI) network of lactylated proteins across the species. Nodes represent 59 conserved proteins predicted to undergo lactylation. Node size corresponds to the number of interaction partners (degree), while node colors highlight distinct clusters. Edges denote cosine similarity ≥ 0.8 based on amino acid composition. The spring layout shows network modularity, indicating functional groupings of lactylated proteins within parasite proteomes

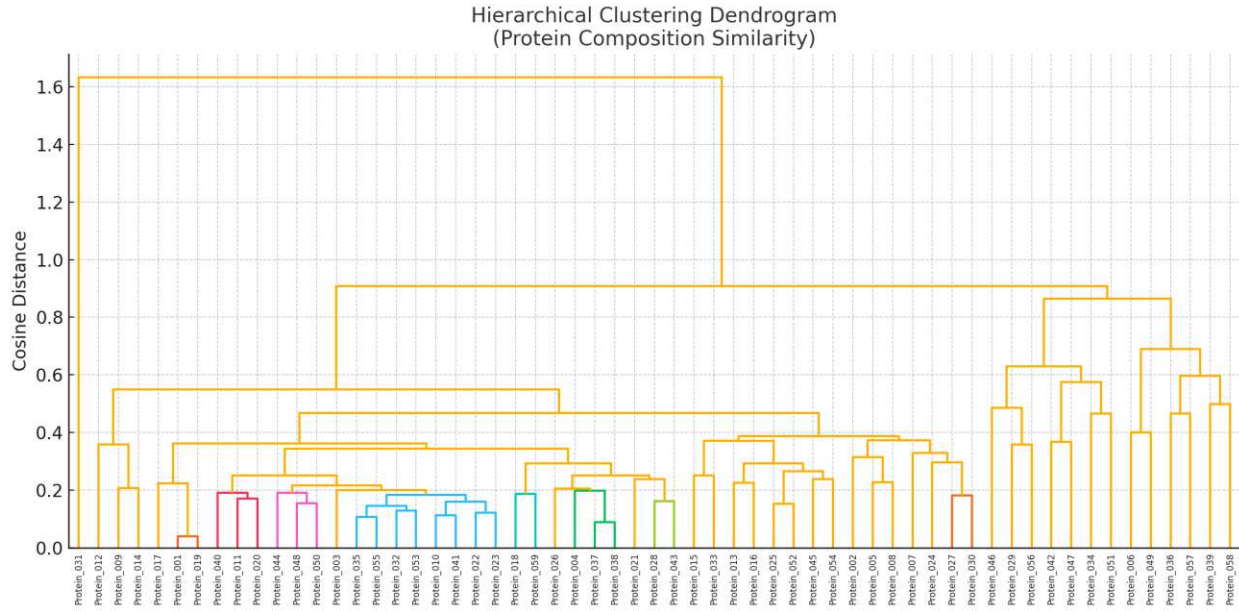


Fig. 9 Hierarchical clustering of lactylated proteins in studied species. The dendrogram groups proteins according to similarity in lactylation-level profiles, measured by cosine-distance. Shorter branch lengths indicate more similar lactylation signatures. Colored clusters denote putative functional modules of proteins that may be co-regulated by lactylation, pointing to conserved biological pathways across species

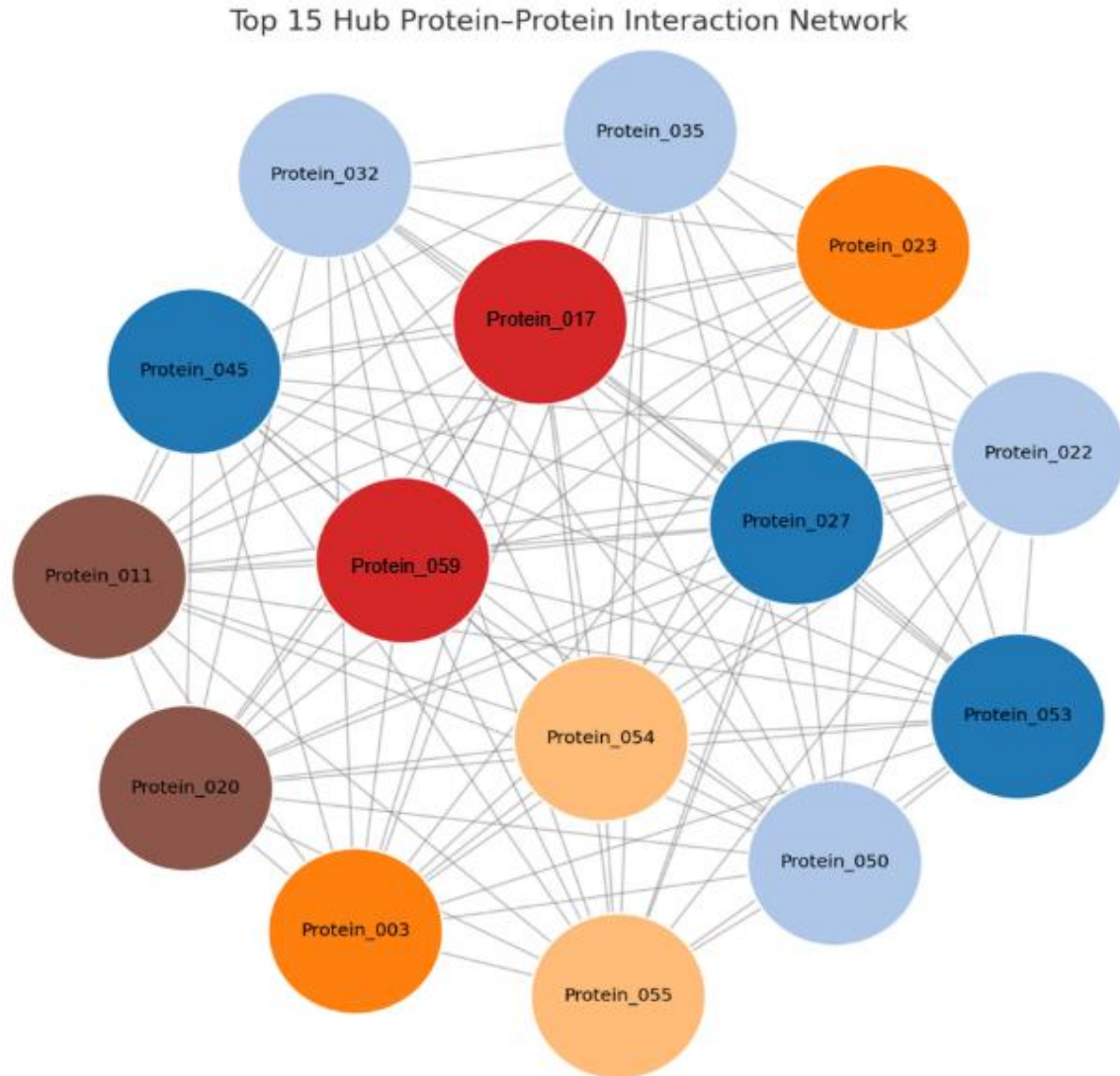


Fig. 10 Top 15 hub proteins in the lactylated PPI network of the studied species. Node size reflects hub strength (number of connections), and edges denote high-confidence similarity interactions (cosine ≥ 0.8). Prominent hubs include HSP70 (Protein_059) and histones (Protein_017), which anchor major interaction clusters. These hubs are candidate regulatory nodes, linking lactylation with stress adaptation, proteostasis, and chromatin regulation

Our data on predicted lactylation patterns (Figs 11 - 14) also provide evidence for interspecific variation in lactylation which may reflect variation in ecology or physiology. However, these differences should be interpreted with caution, as they may also reflect developmental stage-specific regulation or regulation in response to local environmental conditions. Because myxozoans infect mainly fish, it is relevant to consider that aquatic environments are often subject to hypoxia through eutrophication, thermal fluctuations, or anthropogenic impacts such as organic pollution and global warming [50]. Such

conditions impose selective pressures on both hosts and parasites, often driving metabolic adjustments such as anaerobic glycolysis, which allows ATP production with limited oxygen but also results in lactate accumulation [51].

Notably, white muscle of fish is shown to be a major site of lactate production under hypoxia [52, 53, 54, 55]. The consistently higher lactylation levels of *H. salminicola* (Fig. 13) are of particular interest in this context because the species has been suggested to lack a mitochondrial genome and thus relies primarily on anaerobic respiration [56]. If the elevated lactylation in *H. salmonica* reflects exposure to both endogenous lactate from its own metabolism and exogenous lactate from host muscle tissue it would provide an example of lactylation that integrates parasite and host physiology.

Clustering and hub analyses suggest that species such as *C. shasta* display elevated targeting of regulatory proteins. This may indicate that lactylation is involved with modulating host cellular responses, including immune regulation, to support infection and persistence. We also note the relatively higher overall lactylation levels revealed by the average and distributional profiles (Figs 11 and 12) for *H. salminicola*, *M. squamalis*, and *M. macroplasmoidal* which infect muscle, skin, and abdominal cavities, respectively. Other species display lower or more variable patterns. These findings highlight interspecific heterogeneity but do not, on their own, establish functional enrichment in specific protein classes.

Together, the observed species-specific and protein-specific differences (Figs 13 and 14) provide a comparative view of lactylation across the myxozoan proteome. These analyses also provide evidence that lactylation may contribute to the fine-tuning of host–parasite interactions and metabolic adaptation.

Overall, our comparative analyses are consistent with evolutionary divergence in lactylation signatures. Although causality cannot be established from our data, the variation in lactylation signatures revealed here highlights the importance of addressing the role of environmental variation when evaluating lactylation in myxozoans. Parasitic organisms can be expected to impact by complex environmental effects imposed by both the external environment and the host environment, offering interconnected avenues for future investigation.

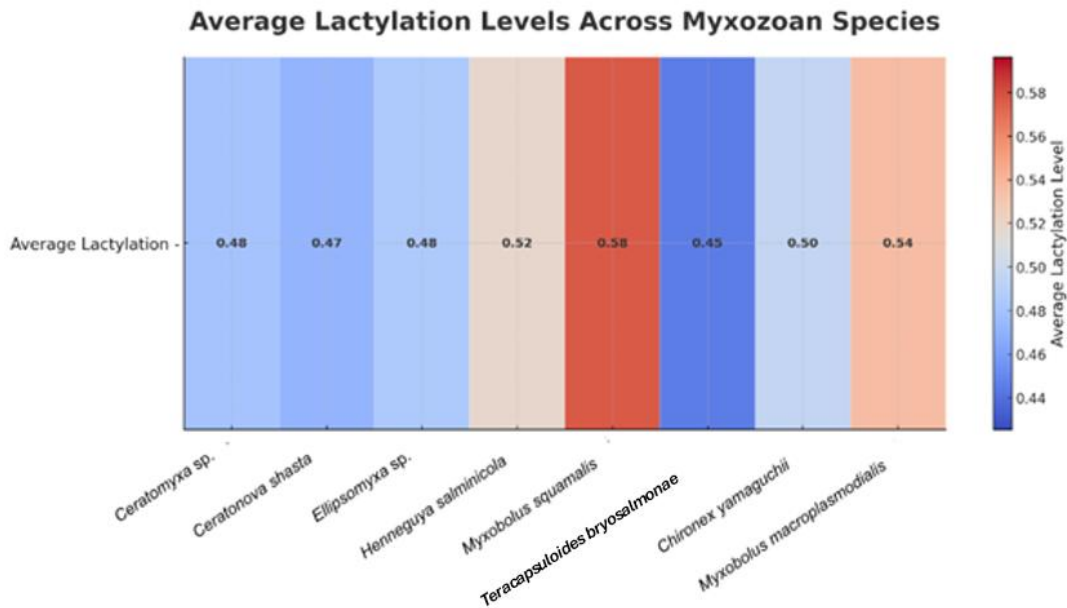


Fig. 11 Heatmap illustrates the comparative analysis of the average lactylation levels across the selected myxozoan, and free-living species. The mean lactylation scores show significant interspecies variation, with *Myxobolus squamalis* displaying higher lactylation scores

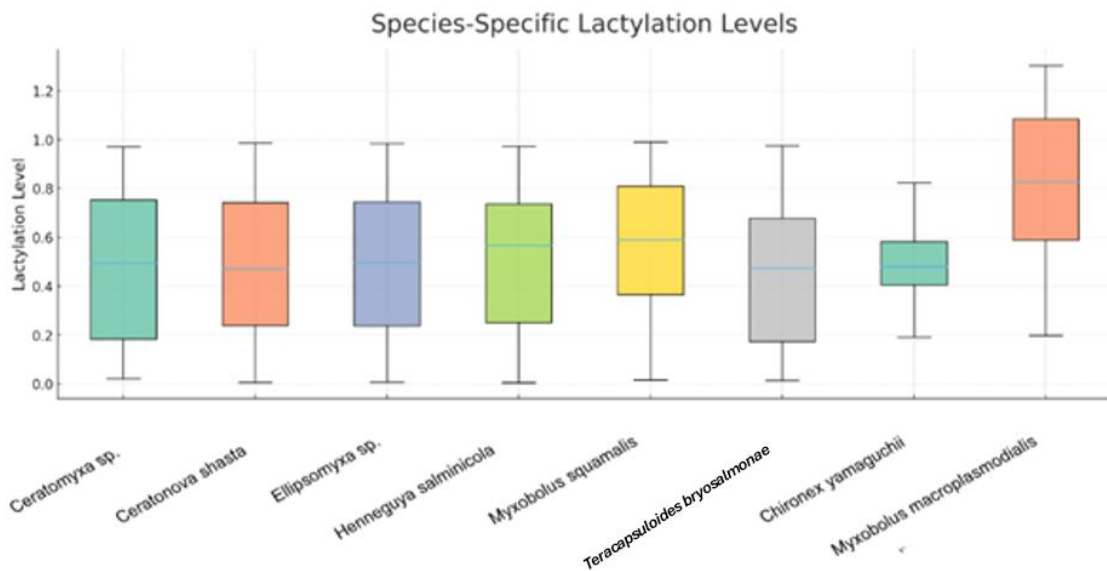


Fig. 12 Boxplots illustrate interspecies variability in lactylation profiles among the seven myxozoan, and the free-living species, without distinguishing functional categories (metabolic vs regulatory proteins). Note that *Henneguya salminicola*, *Myxobolus squamalis* and *Myxobolus macroplasmoidalis* exhibit relatively higher median levels

3.7 Enrichment and functional analyses support lactylation in core regulatory networks

Functional classification revealed 59 lactylated proteins across myxozoans species, encompassing both conserved and species-specific targets (Table 1; Supplementary Table S2). A considerable number of proteins remain uncharacterized. A substantial subset of the 59 lactylated proteins could be assigned to categories such as metabolism, immune-related signaling, ribosomal/translation processes, and chromatin organization (Fig. 15). These findings suggest that lactylation does not occur randomly but preferentially targets proteins with potential regulatory significance. Gene Ontology enrichment analysis showed a pronounced overrepresentation of proteins linked to immune system processes, signal transduction, ribosomal function, DNA replication and repair, protein processing, and oxidative phosphorylation (Fig. 16). The enrichment of immune-related terms was particularly robust ($\text{FDR} < 0.05$), strongly supporting the hypothesis that lactylation is involved in parasite–host immune interactions (Fig. 16) and that it plays a regulatory role in host–parasite interactions. This is consistent with recent findings in *Plasmodium*, where histone lactylation was shown to mediate transcriptional regulation of genes important for immune evasion and developmental plasticity [4].

The distribution of enriched GO terms is summarized in Fig. 17, which combines complementary visualization approaches. Bar plots highlight the most strongly enriched pathways, bubble charts integrate fold enrichment, adjusted significance, and protein counts, and pie charts provide a global overview of the distribution across biological processes, molecular function, and cellular component categories. This integrative view underscores that lactylation is a conserved mechanistic feature across myxozoan taxa, targeting processes that are critical. The GO categories related to ribosomal and chromatin-associated proteins further highlight lactylation in modulating translation and gene expression. Notably, the enrichment of DNA repair and replication pathways suggests that lactylation may contribute to genome stability under environmental or host-induced stress. Together, these findings indicate that lactylation functions at multiple levels, such as controlling transcriptional activity, stabilizing genome integrity, and integrating metabolic and immune responses. The evidence from both functional

classification and GO enrichment aligns with the growing recognition that lactylation acts as a dynamic regulator of cellular processes in eukaryotic parasites [4, 5], expanding its relevance beyond *Plasmodium* to early-diverging metazoans – the Cnidaria.

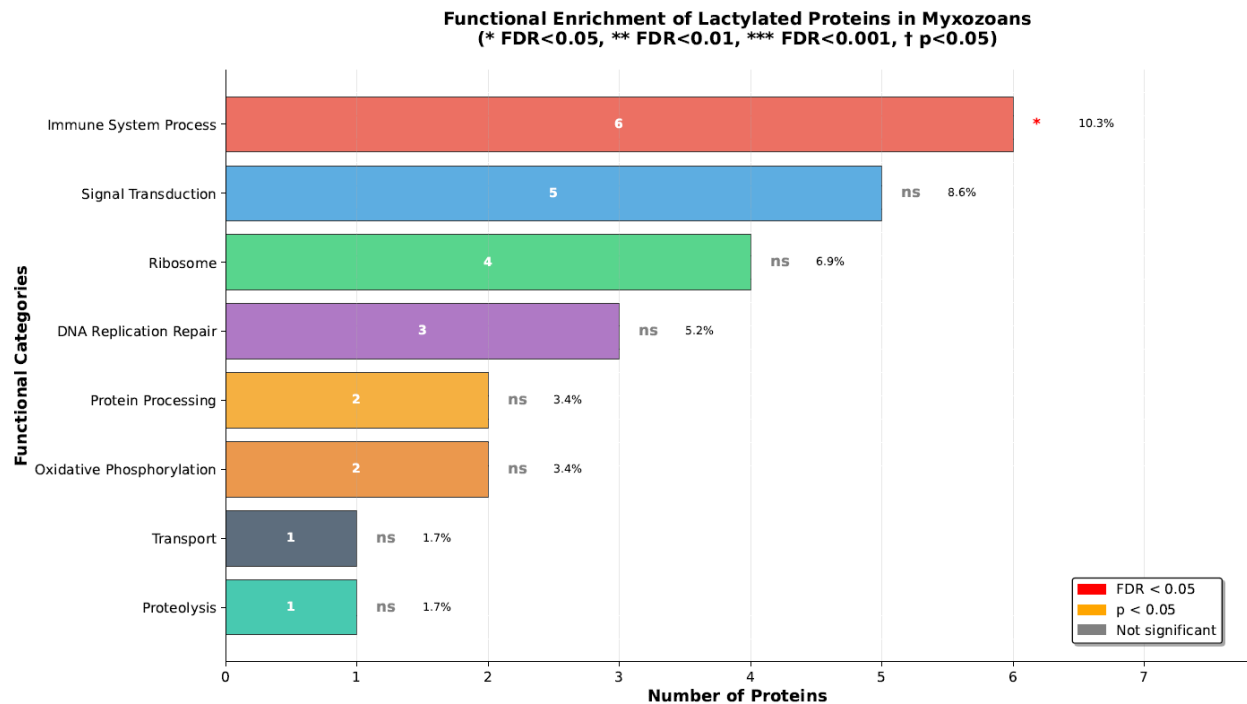


Fig. 15 Functional enrichment analysis of lactylated proteins. Bar plot shows the distribution of 59 lactylated proteins across major functional categories, based on KEGG and GO annotations. Categories include immune system processes, signal transduction, ribosomal function, DNA replication and repair, protein processing, oxidative phosphorylation, transport, and proteolysis. The number of lactylated proteins assigned to each category is indicated within the bars, with corresponding percentages shown on the right. Statistical significance of enrichment was evaluated, with thresholds denoted as *FDR < 0.05; **FDR < 0.01; ***FDR < 0.001; †p < 0.05 (uncorrected); ns, not significant. Enrichment was most pronounced in immune system-related processes

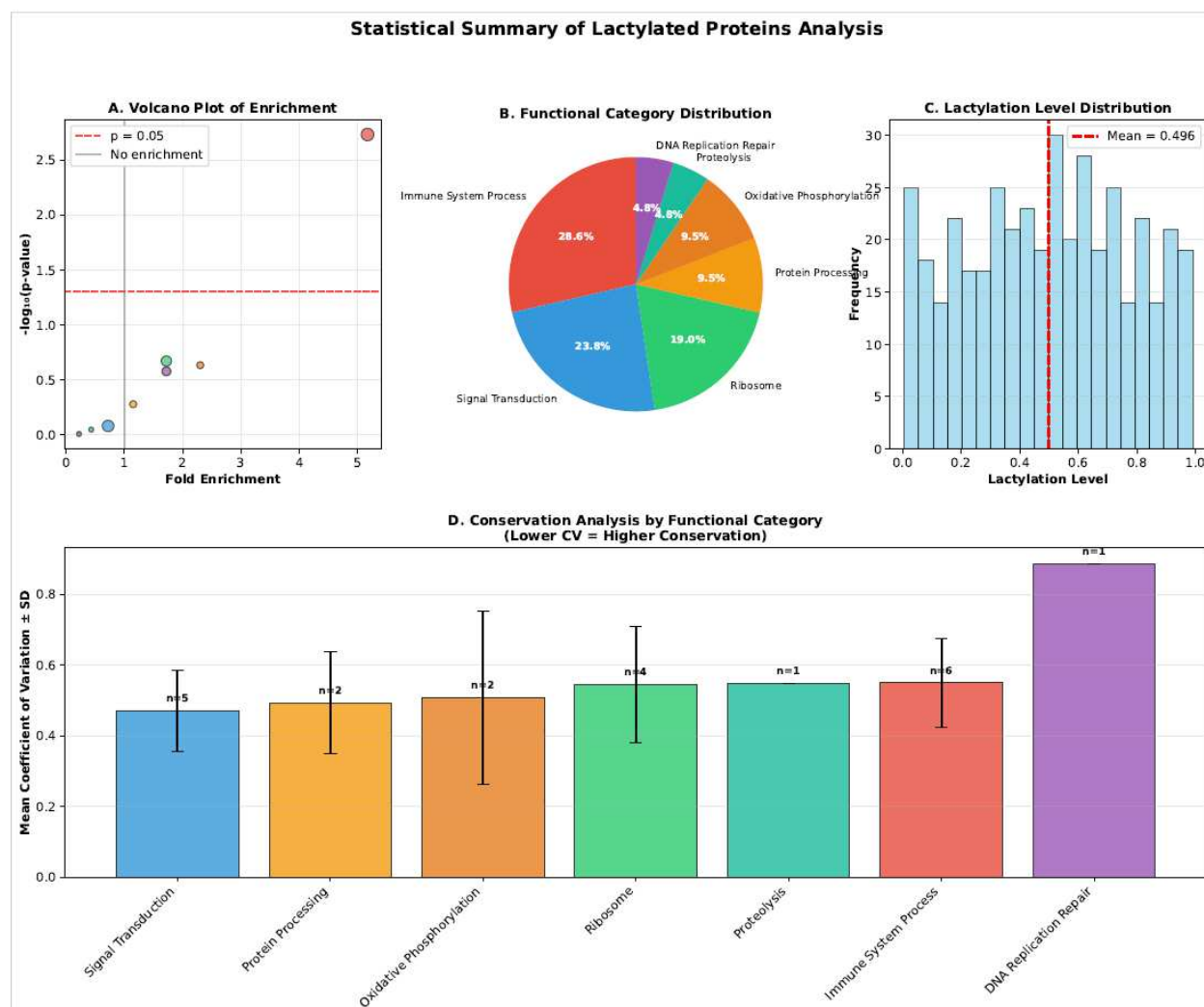


Fig. 16 Statistical summary of lactylated protein analysis in myxozoans. (A) Volcano plot of enrichment showing fold enrichment versus significance (dashed line: $p = 0.05$). (B) Functional category distribution of 59 lactylated proteins, highlighting enrichment in immune system processes, signal transduction, and ribosomal proteins. (C) Histogram of lactylation level distribution across proteins, with a mean level of 0.496 (red dashed line). (D) Conservation analysis of lactylated proteins grouped by functional category, shown as mean coefficient of variation ($CV \pm SD$); lower CV indicates greater conservation

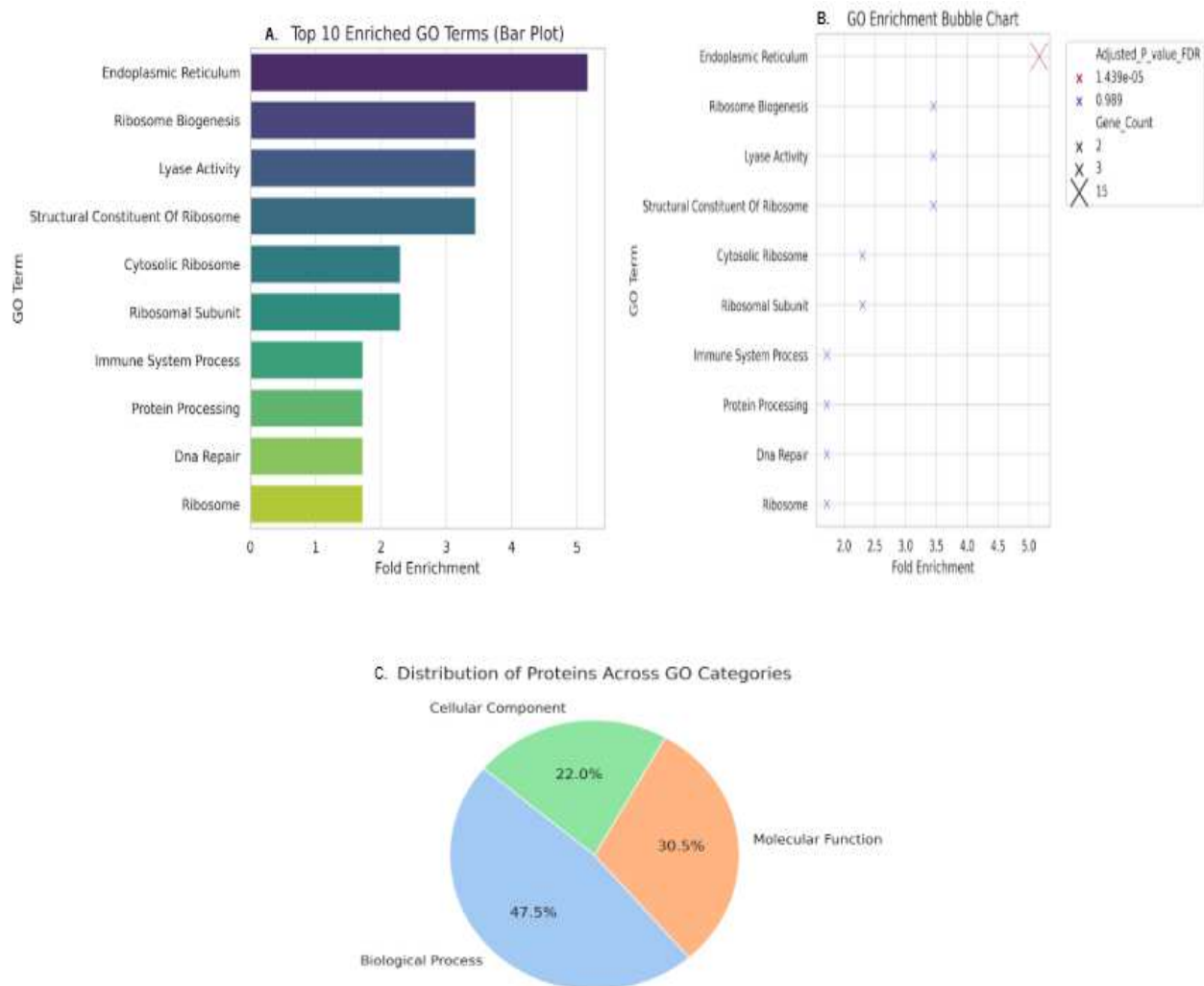


Fig. 17 Gene Ontology enrichment analysis of lactylated proteins in myxozoans. (A) Bar plot of the top 10 enriched GO terms ranked by fold enrichment. (B) Bubble chart of the same GO terms, with bubble size representing the number of annotated proteins and bubble color indicating FDR-adjusted p-values. (C) Pie chart showing the overall distribution of annotated proteins across the three primary GO categories: Biological Process, Molecular Function, and Cellular Component

Table 1 List of the top 10 conserved proteins modified by lactylation with functional annotations

Lactylated protein	Similar protein names	Length	Sequence similarities	Protein families
Protein_001	DNA-(apurinic or apyrimidinic site) lyase (EC 4.2.99.18) (40S ribosomal protein S3)	245	SIMILARITY: Belongs to the universal ribosomal protein uS3 family. {ECO:0000256 ARBA:ARB A00010761, ECO:0000256 RuleBase:R U003624}.	Universal ribosomal protein uS3 family
Protein_002	RanBD1 domain-containing protein	514		
Protein_003	creatine kinase (EC 2.7.3.2)	381	SIMILARITY: Belongs to the ATP:guanido phosphotransferase family. {ECO:0000256 ARBA:ARB A00006798, ECO:0000256 PROSITE-ProRule:PRU00842, ECO:0000256 RuleBase:R U000505}.	ATP:guanido phosphotransferase family
Protein_004	Laminin EGF-like domain-containing protein	330		
Protein_005	V-set and immunoglobulin domain-containing protein 1	324		
Protein_006	RRM domain-containing protein	652		
Protein_007	Peptidase S1 domain-containing protein	331		
Protein_008	Ig-like domain-containing protein	481		
Protein_009	Suppressor of forked domain-containing protein	726		
Protein_010	GB1/RHD3-type G domain-containing protein	497	SIMILARITY: Belongs to the TRAFAC class dynamin-like GTPase superfamily. GB1/RHD3 GTPase family. {ECO:0000256 PROSITE-ProRule:PRU01052}.	TRAFAC class dynamin-like GTPase superfamily, GB1/RHD3 GTPase family

3.8 HSP70 protein family lactylation

Our analyses revealed that Heat Shock Protein 70 (HSP70), a central stress-response chaperone, is potentially modified by lactylation at several lysine residues (supplementary File S6). Lactylation within the ATPase domain could modulate ATP binding and hydrolysis, thereby altering the energy regulation of the chaperone cycle. Similarly, predicted modifications in the peptide-binding domain suggest that lysine lactylation may influence substrate recognition and the stabilization of unfolded proteins [48, 49]. In addition, lactylation in the linker region may impair communication between domains, potentially disrupting HSP70's coordinated folding activity. While these predictions require experimental validation, such as mass spectrometry-based proteomics, they raise the hypothesis that lactylation represents a regulatory switch that enables myxozoans to fine-tune proteostasis under host-induced stress. This aligns with broader evidence that post-translational modifications (PTMs) dynamically regulate chaperone activity and stress adaptation in parasites [57].

3.9 Histone lactylation as epigenetic regulator in myxozoans

We identified histone H3 as a candidate target of lactylation, with predicted modifications at residues such as H3K9, H3K18, and H3K27 (Supplementary File S6). These sites overlap with known regulatory positions implicated in transcriptional activation and chromatin remodeling. Lactylation at these positions may facilitate transcription by loosening chromatin structure and promoting accessibility of DNA regulatory regions, as previously reported for lactylation in mammalian systems [5]. In myxozoans, elevated lactylation of H3 under hypoxic or nutrient-limited conditions could represent an adaptive strategy, linking lactate metabolism to the regulation of gene expression. This model parallels recent studies suggesting that H3 lactylation integrates environmental signals with transcriptional control in other eukaryotes [58]. Taken together, these observations point to histone lactylation as an epigenetic regulator of host–parasite interactions, modulating stress responses and potentially influencing virulence gene expression in *C. shasta* and related species.

3.10 AARS1 as a putative lactyltransferase

AARS1 (alanyl-tRNA synthetase 1) was recently proposed as a candidate enzyme mediating lysine lactylation, functioning as a “moonlighting” lactyltransferase by using lactate and ATP as substrates [12]. Our comparative analysis identified conserved features of AARS1 in myxozoans, including the HIGH and KMSKS motifs and the CP1 insertion domain (Table 2). These structural features support the possibility that myxozoan AARS1 may catalyze lysine lactylation, paralleling its hypothesized function in other metazoans. Notably, this ortholog showed ~55% identity to the human AARS1 sequence, while retaining conserved catalytic residues that could enable substrate flexibility. Although its enzymatic role in lactylation has not been experimentally confirmed in cnidarians, these findings suggest that AARS1 may represent an evolutionarily conserved link between glycolytic byproducts and epigenetic regulation, potentially coupling parasite metabolism with host environmental conditions.

Table 2 Comparative features of myxozoan AARS1 orthologs and human AARS1

Feature	Myxozoan AARS1 Ortholog	Human AARS1
Sequence identity to human	55 % over 850 aa (Ju et al. 2024)	100 % (reference sequence)
Overall length	~850 amino acids	~968 amino acids
Conserved AARS motifs	Present (HIGH, KMSKS, and CP1 motifs)	Present (canonical alanyl-tRNA synthetase motifs)
Key catalytic residues	Strictly conserved	Identical residues required for aminoacylation
Lactyltransferase activity	Predicted (alignment of lactyl-donor binding pocket)	Demonstrated (Ju et al. 2024: AARS1 “moonlights” as a lactyltransferase)
Known functional role	Putative regulator of histone lactylation in Myxozoa	Promotes YAP signaling via histone lactylation in cancer cells
Implication	Suggests parasite-specific epigenetic control of host interactions	Validated epigenetic “writer” linking metabolism to gene expression

3.11 Lactylation and disease outbreaks

The ecological significance of lactylation in myxozoans is underscored by its potential role in disease outbreaks. Salmonids, which serve as vertebrate hosts for a number of myxozoans, are frequently exposed to hypoxia and rising water temperatures associated with eutrophication and climate change [50]. Under such conditions, anaerobic glycolysis is enhanced, leading to increased lactate accumulation [59, 51]. High lactate levels and histone lactylation have been linked to the activation of virulence gene expression and immune modulation in the apicomplexan, *P. falciparum*, which infects humans [4]. A comparable mechanism may operate in myxozoans. We speculate that lactylation could contribute to the upregulation of stress- and virulence-related genes in myxozoans, thereby exacerbating fish disease outbreaks that are often linked with hypoxic or warming conditions [21, 60]. While further experimental work is required to test this hypothesis, the parallels with other pathogens highlight lactylation as a candidate epigenetic mechanism enabling parasite adaptation and promoting disease severity in aquatic ecosystems.

3.12 Lactylation in a free-living cnidaria

To place myxozoan lactylation patterns within an evolutionary framework, we extended our analyses to the free-living cubozoan *C. yamaguchii*, a model species with a complex life cycle and potent venom. In particular, this approach may provide insights on whether lactylation represents a parasitic innovation or a trait shared between free-living and parasitic cnidarians.

Our analyses revealed that *C. yamaguchii* exhibited elevated lactylation levels across conserved proteins, underscoring the fundamental role of this post-translational modification in transcriptional regulation and stress adaptation in free-living cnidarians. Among these, AARS1 (Protein_023) displayed particularly high lactylation scores (Fig. 18), paralleling the enrichment observed in myxozoan parasites. This finding is consistent with recent reports that AARS1 can act as a lactyltransferase across diverse metazoans, directly linking lactate metabolism to post-translational regulation [12].

To evaluate whether these similarities extend beyond single proteins, we conducted a phylogenetic analysis of lactylated protein profiles across myxozoans and *C. yamaguchii*. The correlation matrix, mean-level comparisons, and hierarchical

clustering consistently grouped conserved hubs such as AARS1 and histones across taxa (Fig. 19; Table 3), which is aligned with previous work on histone H3 lacylation as an epigenetic regulator of stress responses and gene expression [5, 58]. Together, this indicates that the functional roles of lacylation are deeply conserved within Cnidaria.

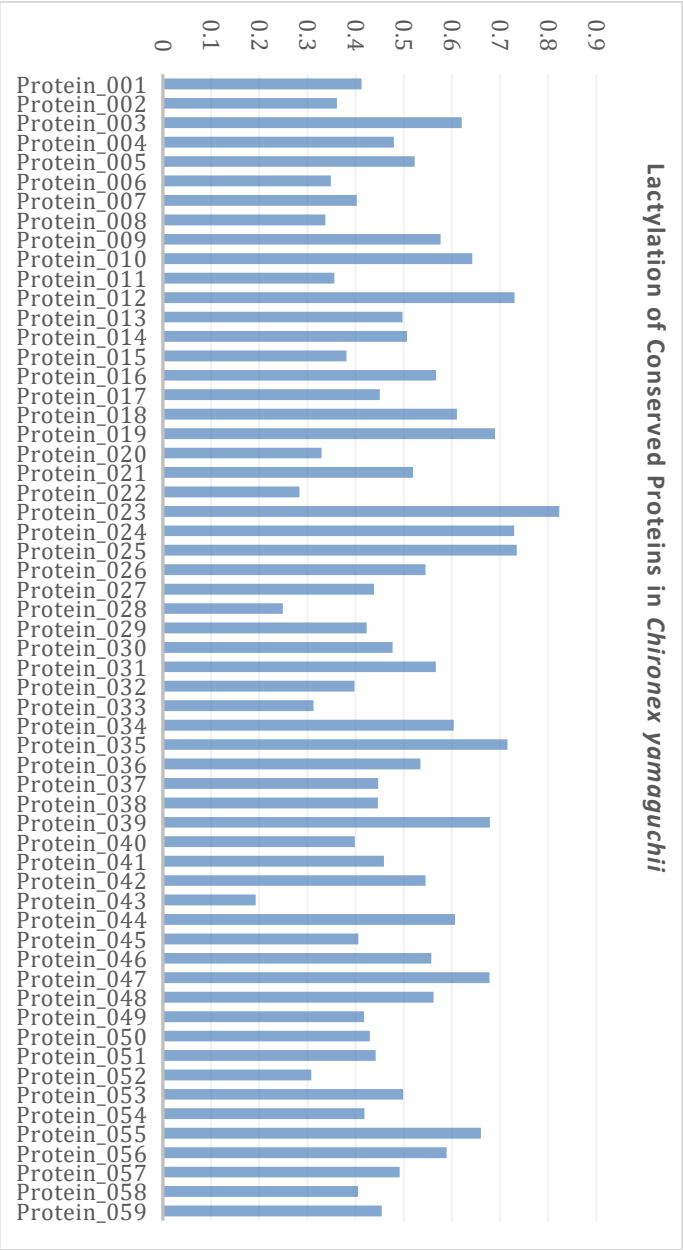


Fig. 18 Graphical showing lacylation levels of conserved proteins in cubozoan *Chironex yamaguchii*. Note the Protein_023 (AARS1) with high modification levels

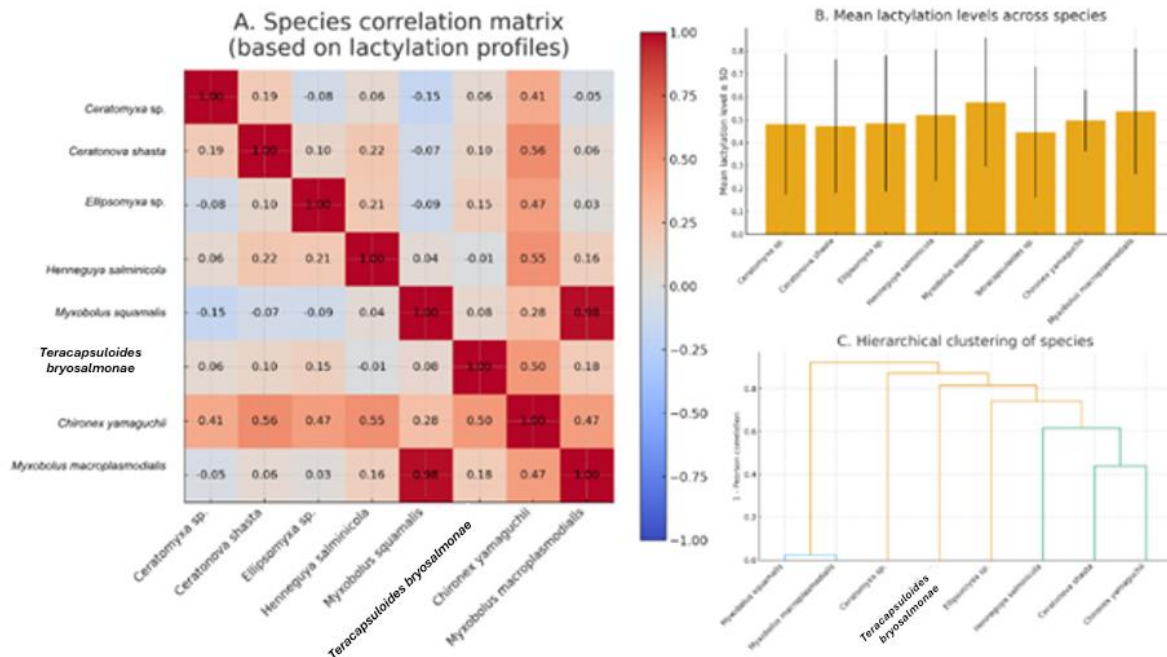


Fig. 19 Phylogenetic analysis of lactylation profiles across myxozoan parasites and the free-living cnidarian *Chironex yamaguchii*. (a) Species correlation matrix based on lactylation profiles of 59 conserved proteins, showing Pearson correlation coefficients across taxa. (b) Mean lactylation levels ($\pm$ SD) for each species, illustrating interspecific variation and highlighting elevated levels in myxozoans and *C. yamaguchii*. (c) Hierarchical clustering of species based on lactylation similarity, using correlation distance and average linkage, revealing grouping patterns that separate parasite-specific lineages from the free-living cnidarian reference

Table 3 Summary statistics of lactylation levels derived from 59 conserved proteins with mean lactylation levels ($\pm$ SD) across myxozoan species and the free-living cnidarian *Chironex yamaguchii*. Values represent species-specific means with associated standard deviations. These data provide the quantitative basis for the correlation, bar plot, and clustering analyses shown in Fig. 21

Species	Mean	SD
<i>Ceratomyxa</i> sp.	0.480351014	0.306935704
<i>Ceratonova shasta</i>	0.471680379	0.291442452
<i>Ellipsomyxa</i> sp.	0.484288109	0.296954729
<i>Henneguya salminicola</i>	0.518825352	0.287573934
<i>Myxobolus squamalis</i>	0.576336705	0.28058658
<i>Tetracapsuloides bryosalmonae</i>	0.445486866	0.286108024
<i>Chironex yamaguchii</i>	0.496161404	0.134535203
<i>Myxobolus macroplasmoidalis</i>	0.536249	0.274874

4 Conclusion

This study provides the first in-silico predictive and experimental demonstration of lysine-lactylation in myxozoans. Our analyses suggesting lactylation plays a central role in regulating parasite biology and host–parasite interactions, providing a versatile means of regulating genes that are linked to transcription, control, metabolic adaptation. Proteins, including histones and HSP70, emerged as major lactylation hubs, thus implying their key roles in maintaining parasite fitness under host-induced stress. Meanwhile, comparative analyses across species demonstrated the predicted conserved regulatory roles and species-specific variation in processes controlled by lactylation. Functional enrichment analyses further implicated lactylation in critical pathways such as glycolysis, oxidative phosphorylation, and immune signaling, supporting its integration into core regulatory networks that underly the functional biology of parasitism.

Taken together, our findings suggest that lactylation has been modified to support parasitic lifestyles but is also a conserved regulatory strategy shared with free-living cnidarians. Future studies focusing on drivers of variation in lactylation within and between species over space and time will help to clarify how patterns reflect myxozoan responses to e.g. environmental variation and host physiology. In turn, such insights might lead to therapeutic approaches for fish disease outbreaks.

Supplementary Information

The online version contains supplementary material available at (DOI inserted by the Journal)

Data Availability

The datasets generated and/or analyzed during the current study are available in the following repositories:

Genomic and proteomic datasets for myxozoan species were retrieved from the NCBI under the following accession numbers: *Ceratomyxa* sp. (GCA_040939075.1); *Ceratonova shasta* (GCA_918559645.1); *Ellipsomyxa* sp. (GCA_040938985.1); *Henneguya salminicola* (GCA_009887335.1); *Myxobolus squamalis* (GCA_010108815.2); *Tetracapsuloides bryosalmonae* (GSE198859).

The *Myxobolus macroplasmodialis* genome, sequenced using Oxford Nanopore technology, has been deposited in the NCBI Sequence Read Archive (SRA) under accession number (PRJNA1449577).

Protein sequences, their predictions, and predicted lactylation datasets generated during this study are available on the Zenodo platform at <https://doi.org/10.5281/zenodo.19460040>.

Author contributions.

Juliana Naldoni, Amr G.A. Ibrahim, and Edson A. Adriano conceived the study. , Amr G.A. Ibrahim performed the bioinformatic analyses. Caroline M. Meira, Antônio A.M. Maia, and Edson A. Adriano conducted fieldwork and sampling for experimental analyses. Rayline T.A. Figueredo and Edson A. Adriano obtained molecular samples for ONT sequencing. Juliano R. Sangalli performed western blot analysis. Amr G.A. Ibrahim, Juliana Naldoni, and Edson A. Adriano interpreted the data, wrote and revised the manuscript. Beth Okamura, Paul F. Long, Antônio A.M. Maia, and Edson A. Adriano revised the manuscript., Edson A. Adriano and Antônio A.M. Maia were responsible for project administration and funding acquisition. All authors reviewed and approved the final version of the manuscript.

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Data availability

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

Declarations

Animal Ethics Committee

This study involved **parasite** samples obtained from fish hosts. All procedures involving animals were conducted in accordance with institutional and national ethical guidelines and were approved by the ethics committee of the Universidade Federal de São Paulo-UNIFESP (CEUA #7344040422).

Clinical trial number

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish declarations

Not applicable.

Competing interests

The authors declare no competing interests.

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