Reviews

Molecular signature of the ontogenic development of the prawn *Macrobrachium tenellum* (#88313)

29 The prawn *Macrobrachium tenellum* shows promise for aquaculture due to its well-defined

Comentado [f1]: Your introduction needs more detail. I suggest that you improve the description at lines 29 change wording for aquaculture potential

35 in M. tenellum eggs. Of these, 27 were exclusive to early-stage development and three to late-

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48 development in Macrobrachium tenellum, with implications for controlled reproduction

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72 (glutamine, asparagine, glycine, cysteine, and alanine) [18]. In M. occidentale, embryonic

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103 (PBS: NaCl 137 mM/L, KH2PO4 1.8 mM/L, KCl 2.7 mM/L, Na2HPO4 10 mM/L, pH 7.4)

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159 M.S. files were analyzed and searched against the Macrobrachium (Crustacea, Decapoda) protein

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169 G.O. term name and I.D. were obtained for each protein (separated as early and late stages of egg

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180 3.1 Quantitative proteomic analysis

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182 After the search of M.S. data on Macrobrachium databases, we identified 89 different proteins in

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The meaning of the initials G.O. It is mentioned in the abstract, however it is not described in this paragraph or before it in introduction and methodology, its meaning is only understood in paragraph 225 3.2 Gene ontology analysis of proteins.

it is important to mention in advance what the acronyms mean, since the reader can get lost in looking for its meaning in the content

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183 M. tenellum eggs (Table 1). The most abundant protein in eggs (emPAI>15) was Vitellogenin

193 Hemocyanin (Prot. Id: F5CEX2) was the 5th more abundant protein found in M. tenellum eggs,

290 The prawn M. tenellum is a species with regional commercial importance. Although

315 synthesis and egg development via the juvenile hormone biosynthesis [35]; in M. tenellum we

360 to the hemocyanin and tyrosine kinases family. M. nipponense is distributed in most tissues

368 polarity and probably at later developmental stages for an innate immune response in Drosophila.

391 we identified a total of 89 proteins within M. tenellum eggs, none of which have been previously

403 **References**

404 [1] L. D. Espinosa-Chaurand et al., .Biología y cultivo de Macrobrachium tenellum: Estado del
405 arte,. Hidrobiologica, vol. 21, no. 2, pp. 99.117, 2011, doi:10.1016/j.biocon.2006.03.014.

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Molecular signature of the ontogenic development of the prawn *Macrobrachium tenellum*

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The prawn *Macrobrachium tenellum* shows promise for aquaculture due to its well-defined reproductive cycle linked to female nutritional requirements. Significant changes occur in egg composition during the 16 to 17-day embryo development. Understanding the ontogenic proteins is crucial for developmental insights and controlled reproduction. We employed free-label quantitative proteomics to analyze egg peptides at wild females' initial and final stages. Using the emPAI protocol and Proteome Discoverer 2.0, we identified 89 differentially expressed proteins in M. tenellum eggs. Of these, 27 were exclusive to early-stage development and three to late-stage. Abundant proteins included Vitellogenin, glyceraldehyde-3-phosphate dehydrogenase, histone 4, beta-actin, and hemocyanin. Gene Ontology analysis revealed 518 terms across molecular functions, biological processes, and cellular components using the GoRetriever tool of AgBase and CateGOrizer tool of Animal Genome Research Program. Carbohydrate metabolism was significant in early-stage development, with glyceraldehyde-3-phosphate dehydrogenase being the second most abundant protein. Proteins involved in ATP synthesis and cytoplasmic proteins associated with catalytic and binding activities related to primary metabolism were also detected. Our study elucidates the role of Vitellogenin in lipid transport activity and its potential involvement in the juvenile hormone feedback pathway. This pathway includes farnesoic acid O-methyltransferase and juvenile hormone epoxide oxidase, regulating protein biosynthesis, molt cycles (including chitinase activity), and potentially influencing controlled reproduction. Our proteomic analysis provides insights into the molecular mechanisms driving ontogenic development in Macrobrachium

tenellum, with implications for controlled reproduction strategies and advancements in aquaculture practices.

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Abstract

The prawn *Macrobrachium tenellum* shows promise for aquaculture due to its well-defined reproductive cycle linked to female nutritional requirements. Significant changes occur in egg composition during the 16 to 17-day embryo development. Understanding the ontogenic proteins is crucial for developmental insights and controlled reproduction. We employed free-label quantitative proteomics to analyze egg peptides at wild females' initial and final stages. Using the emPAI protocol and Proteome Discoverer 2.0, we identified 89 differentially expressed proteins in M. tenellum eggs. Of these, 27 were exclusive to early-stage development and three to late-stage. Abundant proteins included Vitellogenin, glyceraldehyde-3-phosphate dehydrogenase, histone 4, beta-actin, and hemocyanin. Gene Ontology analysis revealed 518 terms across molecular functions, biological processes, and cellular components using the GoRetriever tool of AgBase and CateGOrizer tool of Animal Genome Research Program. Carbohydrate metabolism

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Introduction

Macrobrachium is one of the most diverse, abundant, and widely distributed genres of Decapoda [1]–[5]. The river prawn *Macrobrachium tenellum* is an endemic species of the Pacific Ocean coast [3], [6], [7] with a strong potential for aquaculture since it has fast growth in a broad range of temperature, salinity, altitude, density, and biochemical parameters; besides its adaptive capacity on polyculture with Tilapia in rustic ponds and lagoons [8], [9].

As in Penaeidae, *M. tenellum* larval development is closely related to the nutritional requirements of females during maturation [10], [11]; four phases of courtship, mating, and impregnation have been reported, after mating, females lay the eggs under the abdomen, attached to the pleopods by secretions produced by the cementing glands. *M. tenellum* eggs are elliptical with an average diameter of 0.55 mm [10]. Embryo development lasts 16 to 17 days, during which eggs exhibit color changes [10].

As a palaemonid, the principal energetic metabolites in eggs are carbohydrates and lipids [12], [13], while proteins are mainly structural components [14]–[16]. Some reports demonstrated that protein and lipid content in yolk varies according to the maturation stage of the eggs [12]. In *Macrobrachium japonicus* eggs, protein content increases during early embryonic development, apparently as a source for tissue differentiation for early structures, while lipid content decreases since it is used as an energy source; In late embryonic development, protein decreases after being used as an energy source (after all structures are formed), while lipid turns into anatomical structures [17]. This very same pattern was observed in *M. idella* idella, where total amino acids in eggs increase during the I-IV developmental stages, particularly in 5 essential amino acids (glutamine, asparagine, glycine, cysteine, and alanine) [18]. In M. occidentale, embryonic development correlates with proteins on day 3, simultaneously with gastrulation [19].

Proteins seem to play a central role in in the ontogeny of *Macrobrachium*. Therefore, information about its proteome would give a better understanding of the development process for possible controlled reproduction. Quantitative Proteomics identifies and quantifies proteins from complex samples using liquid chromatography, resulting in large datasets related to the proteome. Free Label Quantitative Proteomics is an automated, label-free methodology to identify and quantify changes in the proteome of complex mixtures using liquid

chromatography-mass spectrometry [20], [21]; its applications on aquatic organisms are limited, despite its relatively low-cost and comprehensive output.
In this study, we used Free Label Quantitative Proteomics to identify and quantify the proteins related to metabolism in two different stages of egg development and to provide the necessary information for a better understanding of the successful transition from yolk-dependent nutrition (lecithotrophy) to life as a free-feeding organism.

Materials & Methods

Animals

Adult females of *M. tenellum* were purchased from the local market in Santa Maria Colotepec, Oaxaca, Mexico. Organisms were transported in darkness and with oxygen supplementation into aquaculture facilities. Organisms were kept in 1200 L tanks (T=25-28ºC, 12:12 h of light/darkness, constant aeration, in a closed carbon-UV filtered water system) until use. Prawns were fed a commercial diet twice daily (08:00 and 18:00 h) at 5% of body weight daily. Ovigerous females were moved to unique aquariums individually and continuously monitored from day 1 to 16; egg mass was extensively washed using distilled water and carefully removed in a biosafety cabinet; afterward, eggs were classified and measured according to sub-stages of stage III of reproduction by using an optical microscope with an eyepiece graticule (40×). Only eggs from stages A (n=8) and E (n=11) were used.

Egg Lysate and Protein Quantification

On the obtention of protein lysate from eggs, we used RIPA buffer (Sigma Chemicals, USA) and mechanical mortar grinding at -20°C. Frozen eggs in 1x filtered Phosphate-Buffered Saline (PBS: NaCl 137 mM/L, KH2PO4 1.8 mM/L, KCl 2.7 mM/L, Na2HPO4 10 mM/L, pH 7.4) placed in a sterile mortar previously cooled to -20°C, 300 µl of protease inhibitor RIPA (-4°C) were added, ground to a homogeneous suspension of lysate and later centrifuged at 12,000 g (10 min, 0°C); the supernatant transferred to a sterile microtube, washed twice using sterile PBS by centrifugation (12,000 g, 10 min, 0ºC) and frozen at -80°C until use.
Soluble protein from eggs lysates was measured using a Pierce BCA protein micro assay kit (Thermo Fisher Scientific, USA) according to the manufacturer, using Bovine Seric Albumin as standard; absorbance was measured at 570 nm in an ELISA reader (Thermo Scientific, USA). Values were calculated using a cubic equation of the BSA standard curve.

Label-Free Based Quantitative Proteomic Analysis

To verify the integrity of the sample, the protein from the eggs lysate (50 µg) we separated electrophoretically using a 12.5% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE), with the Laemmli (1970) [22] buffer system. Gels were stained using Coomassie blue brilliant G-250 (Sigma, USA).

Lysate samples (A and E, early and late stage of development, respectively) were lyophilized and shipped to Creative Proteomics (Shirley, NY, EUA), where samples were digested by trypsin and quantified using a nanoLC-MS/M.S. platform, as follows:

Chemicals and Instrumentation

DL-dithiothreitol (DTT), iodoacetamide (IAA), formic acid (F.A.), acetonitrile (ACN), and methanol were purchased from Sigma (St. Louis, MO, USA); trypsin from bovine pancreas from Promega (Madison, WI, USA). Ultrapure water was obtained from a Millipore purification system (Billerica, MA, USA). The essays were done in a Dionex Ultimate 3000 Nano LC system coupled with a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA) with an ESI nano-spray source.

Sample Preparation

We transferred 5 mg of protein dissolved in 200 µl of 50 mM ammonium bicarbonate into Microcon devices YM-10 (Millipore) and centrifuged at 12,000 g at four °C for 10 min, subsequently added 200 µl of ammonium bicarbonate (50 mM), followed by centrifugation and repeated once. After, it was reduced with ten mM DTT at 56°C for 1 h and alkylated with 20 mM IAA at room temperature in the dark for 1 h; the device was centrifuged at 12,000 g at 4°C for 10 min and washed once with 50 mM ammonium bicarbonate. Then we added 100 µl of 50 mM ammonium bicarbonate and free trypsin into the protein solution at a ratio of 1:50 and incubated at 37°C overnight. Finally, the device was centrifuged at 12,000 g at 4°C for 10 min. Next, 100 µl of 50 mM ammonium bicarbonate was added, centrifuged, and repeated once. Extracted peptides were lyophilized. All peptides were resuspended in 2-20 µl of 0.1% formic acid before LC-MS/MS analysis.

Nanoflow UPLC

Liquid chromatography was carried on an easy-nLC1000 (Thermo Fisher Scientific, USA); using a Nanocolumn (100 µm×10 cm in-house made column packed with a reversed-phase ReproSil-Pur C18-AQ resin, 3 µm, 120 Å, Dr. Maisch GmbH, Germany); loaded sample volume was 5 µl; mobile phase was A: 0.1 % formic acid in water; B: 0.1 % formic acid in acetonitrile. The total flow rate was adjusted to 600 nL/min. L.C. linear gradient ranged from 6 % to 9 % B for 8 min, from 9 % to 14 % B for 16 min, from 14 % to 30 % B for 36 min, from 30 % to 40 % B for 15 min, and from 40 % to 95 % B for 3 min, eluting with 95% B for 7 min.

Q Exactive mass spectrometry

For mass spectrometry, we used a spray voltage of 2.2 kV; the capillary temperature was 270°C; M.S. parameters were resolution: 60000 at 400 m/z, precursor m/z range: 300.0-1650.0; MS/MS parameters were product ion scan range, start from m/z 100. Data-dependent MS/MS was up to the top 5 most intense peptide ions from the preview scan in the Orbitrap.

Data analysis

M.S. files were analyzed and searched against the Macrobrachium (Crustacea, Decapoda) protein databases using Proteome Discoverer 2.0 (Thermo Fisher Scientific). Parameters were: protein modifications: carbamidomethylation (C) (fixed), oxidation (M) (variable); enzyme specificity: trypsin; maximum missed cleavages: 2; precursor ion mass tolerance: 10 ppm; MS/MS tolerance: 0.6 Da. PSM, q-Value and Score of Sequest HT were used to determine and choose only highly confident identified peptides for downstream protein identification analysis. We used the Exponentially Modified Protein Abundance Index (emPAI protocol, Ishihama et al., 2005) as a component of the abundance of proteins; we calculated the ratio of abundance (A.R.) compared with protein abundance in late/early stages. If R>1, the protein is considered relatively more abundant in the late stage, and if R<1: the protein is relatively more abundant in the early stage. G.O. term name and I.D. were obtained for each protein (separated as early and late stages of egg development) using the respective UniProt Id number on the GoRetriever tool from AgBase [23], [24]. Afterward, G.O. Terms were classified (Classification: GoSlim2) using the CateGOrizer tool of the Animal Genome Research Program (www.animalgenome.org/tools/catego) [25], [26] on cellular components, molecular function, and biological process. Finally, the REVIGO web-based tool visualized the G.O. categorization (http://revigo.irb.hr/) [27].

Results

Eggs in the early stage of development had 4.23 mg/mL (sd=0.49, n=8) protein content; late-stage egg protein content was 1.17 mg/mL (sd=0.52, n=11). Electrophoresis showed a broad-spectrum pattern of proteins (10-250 kDa).

3.1 Quantitative proteomic analysis

3.1.1 Abundance of proteins in eggs

After the search of M.S. data on Macrobrachium databases, we identified 89 different proteins in M. tenellum eggs (Table 1). The most abundant protein in eggs (emPAI>15) was Vitellogenin (Prot. Id: Q95P34 and A0A0E3JBN6), identified from those in *M. nipponense* and from a translated genetic sequence from *M. rosenbergii*; it is a 285 kDa protein (pI 8.9), in which 173 and 157 peptides covered 49.7 and 49.0% of the reference proteins, respectively. Gliceraldehyde-3-phosphate dehydrogenase (Prot. Id: A0A088BEN6) was the 2nd most abundant protein found, with 82% homologous to those reported in *M. rosenbergii*; this is a 13.6 kDa protein, with a 6.9 calculated isoelectric point. Histone H4 (Prot. Id: A0A0D6DQV4) was the 3rd most abundant protein found in *M. tenellum* eggs, being 51.4% homologous to the 11.4 kDa translated sequence from *M. rosenbergii*. Beta-actin (Prot. Id: Q58ZF3) was the 4th most abundant protein found in *M. tenellum* eggs, and it showed 56.1% homology to the 41.84 kDa protein from *M. rosenbergii*. Hemocyanin (Prot. Id: F5CEX2) was the 5th more abundant protein found in M. tenellum eggs, being 34.1% homologous to the 76 kDa translated sequence from *M.* nipponense. On the contrary, the less scarce proteins found were Retinoid X receptor (Prot. Id: V9PNQ4), Dorsal protein (Prot. Id: A0A173G7X5), Prophenoloxidase (Prot. Id: Q58HZ8), the Very large

inducible GTPase 1 (VLG1; Prot. Id: A0A173GTS1) and the Very large inducible GTPase 2 (VLG2; Prot. Id: A0A173GTR2). (Table 1)
From the 89 proteins identified, 30 proteins were found in only one stage of egg development: being 27 proteins in the early stage (Table 1, proteins in blue) and 3 in the late stage (Table 1, proteins in red).
From those proteins exclusively found in the early stage, the most abundant (emPAI>1) was actin (Prot. Id: A0A0A6Z676), which is a globular, multi-functional protein; likewise, in the late stage, only three proteins were exclusively found: glutathione peroxidase (Prot. Id. B9W2R6), hyperglycemic hormone (Prot. Id. G0YP40) and chitinase 4 (Prot. Id. W8PC51). Additionally, in the late stage, the proteins exclusively found were the ribosomal L-10 (Prot. Id: G8H336), male-reproductive related LIM protein (Prot. Id: B8LG57), farnesoic acid O-Methyl transferase (Prot. Id: H6UXP2), HSP-60 (Prot. Id: A0A0A7AD33), cytochrome-c oxidase (Prot. Id: I7BBT8) and ATP synthase (Prot. Id: A0A0U2DW78).

3.1.2 Relative abundance of proteins by the developmental stage of eggs
During the embryonic development of the organism, we found 59 proteins, classified according to their ratio as relatively more abundant in the late stage (R.A.>1) and relatively more abundant in the early stage (R.A. <1). Most of these proteins were abundant in the early stage, with only 10 % being most abundant in the late stage. For the early stage, we identified 53 proteins; the most importantly downregulated were a putative clotting protein (Prot. Id. A0A0U1W4T3), cathepsin-D (Prot. Id. A0A142G5I9), heat shock protein-70 (Prot. Id. A0A0A6ZEG8), pyruvate kinase (Prot. Id. A0A140AZ38) and hemocyanin (Prot. Id. A0A0A0PM26). For proteins relatively more abundant in the late stage, our data suggest that catalase (Prot. Id. H8XYP6) is the most upregulated protein since its relative abundance is 26-fold greater at the late than at the early stage; Elongation factor 1 alpha (Prot. Id. A0A26TKR7), glutamate dehydrogenase (Prot. Id. D2KK94), DEAD box polypeptide 39 (Prot. Id. K9J9V3) and Vitellogenin (Prot. Id. A0A0E3JBN6 and Q95P34) were also found upregulated.

3.2 Gene ontology analysis of proteins.
To determine the protein's biological meaning, we identified 518 GO terms from 84 proteins (5 proteins have no G.O. term associated); 73 G.O. terms classified in 3 main categories: 135 in 6 main molecular functions (Fig 1A), 116 cellular components (Fig 1B), and 120 in 8 main biological processes (Fig 1C).
3.2.1 Molecular function
We found six molecular functions at each stage: catalytic (GO:0003824), binding (GO:0005488), structural molecule activity GO:0005198), transporter (GO:0005215), antioxidant (GO:0016209) and receptor (GO:0004872) (Fig 1A).
For the molecular function, a significant number of G.O. terms were found for the catalytic and binding activity in both stages. Still, these functions were more abundant in the early than the late stage. Binding activity was 1.45-fold more active earlier than in the late stage. Binding to

nucleic acids, RNA, and DNA (GO:0003676, GO:0003723, GO:0003677) and proteins (GO:0005515) were the primary function found in the early stage; meanwhile, nucleic acid, nucleotide, and protein binding showed the same activity in the late stage. Transcription factor (GO:003700) and signal transducer (GO:0004871) activities were only present in the early stage. (Fig 2A). G.O. terms classified as catalytic were 1.34-fold more abundant in the early stage than late stage (74/55). We found more transferases (12/8) and kinases (6/3) during egg development. (Fig 2B)

For the catalytic activity-related proteins, in the early stage, transferases (GO:0016740) had the most relevant function, followed by hydrolases (GO:0016787), kinases (GO:0016301), peptidases (GO:0008233) and enzyme regulation (GO:00302234); meanwhile in the late stage, hydrolases were more active than kinases.

3.2.2 Cellular component

According to the GO-Slim2 analysis, the cellular components related to the cell were more abundant in the early stage (1.92-fold); meanwhile, the extracellular region (GO:0005576) remained without changes in both developmental stages of the eggs. (Fig 1B). The main components of the cell were in the cytoplasm (GO:0005737) in both stages of development. Mitochondrion (GO:0005739) had the most considerable number of G.O. terms associated, followed by cytosol (GO:0005829), ribosome (GO:0005840), nucleus (GO:0005634) and chromosome (GO:0005654); E.R. (GO:0005783), nucleoplasm (0005654), endosome (00057768) and Golgi (GO:0005794) were only present in the early stage (Fig 3).

3.2.3 Biological process

The biological process was the most complex classification in *M. tenellum* eggs; according to the GO-slim2 analysis, the scatter plot of the main biological processes during ontogeny showed a significant complexity during the early stage (Fig 4A) than during the late stage (Fig 4B). We found eight primary generic biological processes; Metabolism (GO:008152) was the most active on the eggs, followed by Transport (GO:0006810), Response to Stress (GO:0006950), Cell Organization, and Biogenesis (GO:0016043), with the same activity were Cell Communication (GO:0007987), Cell Homeostasis (GO:0018725), Viral Cell Life (GO:0016032), and Signal Transduction (GO:0007165). (Fig 1C)

For the main biological process during ontogeny, we found more G.O. terms (1.32-fold more) in the early stage than in the late stage (Fig 1C). Metabolism-related terms were more abundant, but the response to stress and transport was 2.33 and 1.71-fold more active, respectively, in the early than in the late stage. The metabolic processes were more active in the early than in the late stage; the most important metabolic processes in the early stage were nucleotide-related metabolism (GO:0006139), followed by biosynthesis (GO:0009058), metabolite and energy precursor and protein metabolism (GO:0006091 and GO:0019538), carbohydrate metabolism and catabolism (GO:0005975 and 0009056) and protein modifications and protein biosynthesis (GO:0006464 and 0006412); in late stages, protein metabolism was more active than precursor

metabolites and energy metabolism, and protein biosynthesis was more involved than protein modifications.

Discussion

The study of the ontogeny of crustaceans has been an essential factor in the development of the aquaculture of the principal commercial species. Molecular-level studies on marine shrimps have provided necessary information about their energetic metabolism and embryonic development for breeding and reproduction. There are limited studies on the ontogeny of aquatic prawns, mainly concerning the proximate composition of metabolites. For those species, the primary metabolites during the ontogeny are carbohydrates and lipids as a source of energy and precursors [13]. At the same time, proteins are the main structural components of embryonic development. Some reports suggest a higher protein concentration in the early development stage than in the late stage because of their use as structural materials [13], [17]–[19].

The prawn M. tenellum is a species with regional commercial importance. Although comprehensive studies of embryonic development, there is no information at the molecular level. Therefore, we quantified the protein from extracts of fertilized eggs and found a similar pattern to those previously reported for crustaceans; protein content was 3.6-fold higher in the early stage than in the late stage of embryonic development, mainly related to the presence of a more significant concentration of the protein vitellogenin in the early stage of embryogenesis. Eighty-nine proteins were identified and quantified; 27 were exclusive during the beginning of the embryogenesis, and three were found only in the late stage.

The low quantity of proteins identified compared to the amount present in electrophoretic gels may be related to the limited information contained in the Macrobrachium databases associated with only seven species (*M. rosenbergii, M. nipponense, M. amazonicum, M. aff. Pilimanus, M. acanthurus, M. lanchesteri, and M. bullatum*); this problem is broader, since in the only similar report -to our knowledge- in *Pacifastacus leniusculus* (crayfish) eggs and spermatophore, were 41 proteins identified; the most diverse being respiratory and cytoskeleton related, while no proteins were related to DNA or cell defense [28]

The most abundant protein found in eggs, according to our data was Vitellogeninn (Vg), the most studied and well-known protein among crustacean eggs that disappear during embryogenesis after cleavage as vitellin, which explains the lower concentration of total protein in the late-stage of development [29]–[31]. Vg is the yolk protein precursor from eggs synthesized in the hepatopancreas and released to the hemolymph, where yield several subunits [32], [33]. In *M. nipponense*, Bai, et al., (2015) [34] reported a fluctuation of Vg expression during embryonic development, with a peak at the beginning of the larval development; Vg seems to be an essential protein during embryogenesis, especially during the first stages of life of the organism, providing critical metabolites and energy; any deficiency on its synthesis or accumulation could carry developmental defects. In insects, the amino acids from diet induce Vg synthesis and egg development via the juvenile hormone biosynthesis [35]; in M. tenellum we found 1.54 fold more Vg concentration during the initial stage of embryogenesis, and according

to the G.O. Term (GO:0005319), and as reported by Wyatt and Davey (1996) [36], it enables and participates in the lipid transport activity; moreover, this suggests that it could be related to juvenile hormone feedback, where farnesoic acid O-methyltransferase (Prot. Id. H6UXP2) and juvenile hormone epoxide oxidase (Prot. Id. A0A0H3WF09) catalyze the conversion of farnesoic acid to juvenile hormone III [37]–[39]. This hormone is related to general protein synthesis and the molt cycle control [40]; both processes are more active during the initial stages of embryonic development.

According to the relative content of protein, Vitellogenin is associated with lipid mobilization in the initial developmental stages; meanwhile, the use of carbohydrates as energy supply is more active during the late stages of embryonic development since the relative content of proteins related to glycolysis is lower >1; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the second more abundant protein; this is a cytosolic enzyme of eukaryotic cells, and is involved in the regulation of the energetic metabolism of the eggs; it plays a central role on glycolysis and gluconeogenesis and catalyzes the conversion of D-glyceraldehyde-3-phosphate (G3P) to 1,3 diphosphoglycerate [41], [42], it has also reported having a role in cytoskeleton and apoptosis [43]–[45]. From the glycolytic pathway, we also identified phosphofructokinase, which converts fructose-6-phosphate to fructose-1,6-biphosphate; enolase, which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP); and the pyruvate kinase, which converts PEP to pyruvate. For gluconeogenesis, besides GAPH and enolase, we found pyruvate carboxylase, an enzyme needed to convert pyruvate to oxaloacetate, and malate dehydrogenase, an enzyme that catalyzes the interconversion of oxaloacetate to malate (an intermediary of the citric acid cycle); downstream the metabolic process we found proteins related to the electron transport chain and the oxidative-phosphorylation for ATP synthesis (Cytochrome B, Cytochrome C, and ATP-synthase).

H4 histone is a protein involved in eukaryotic cells' chromatin structure and functions, as a component of the nucleosome, in a central role in regulating transcription, DNA repair, DNA replication, and chromosomal stability, is mainly recognized as a DNA binding and a hetero dimerization activity protein implicated in the biological process of DNA-template transcription, initiation, and nucleosome assembly due to its ability to undergo several post-translational modifications, is expressed in all tissues, mainly in gills, and it is upregulated after viral and bacterial injection; In addition, peptides derived from its N- and C-terminus present antibacterial activity [46].

Beta-actin is a ubiquitous, conserved protein with multi-diverse functions, which participates in several processes such as cell motility, cell division, vesicle movement, apoptosis, gene expression, and cell signaling [47]. Its molecular function is ATP binding. In *Macrobrachium rosenbergi*i it is a cytoplasmic protein distributed widely in all tissues: it is highly expressed in muscle, being upregulated during embryonic development; interestingly, beta-actin is downregulated in *M. tenellum* in the same period [48].

Hemocyanin is a conserved extracellular, multi-subunit respiratory protein among arthropods and mollusks [49], [50]. It is a copper-containing protein that typically constitutes up to 85 % of

the total proteins in the hemolymph. Other functions reported for this protein are mainly in the defense mechanism as phenol oxidase, antiviral and antibacterial protein [51]–[55]. Its molecular functions related to *M. nipponense* are metal ion binding and oxidoreductase activity; it belongs to the hemocyanin and tyrosine kinases family. M. nipponense is distributed in most tissues, more expressed in hepatopancreas. After bacterial infection, hemocytes' expression is downregulated at 3 h and upregulated after 6 h [56].

From the less abundant proteins identified, we identified two embryonic development-related proteins and two immune-related functions; the retinoid X receptor (Prot. Id: V9PNQ4), a nuclear receptor related to retinoic acid (vitamin A metabolite) and an essential protein that guides the development of the posterior side of the vertebrate embryo; and the dorsal protein (Prot. Id: A0A173G7X5), which is required for the generation of embryonic dorsoventral polarity and probably at later developmental stages for an innate immune response in Drosophila. In addition, Prophenoloxidase (Prot. Id: Q58HZ8), a crucial inactive protein of melanization in crustaceans, the very large inducible GTPase 1 (VLG1; Prot. Id: A0A173GTS1) and VLG2 (Prot. Id: A0A173GTR2) are members of a protein family related to multiple biological processes.

The study of gene ontology is crucial for an integrated vision of the identified proteins. Our data suggest that during the ontogeny of this species, proteins are more abundant and participate more actively in cellular and biological processes and molecular functions (according to the G.O. terms found) in the early stage, revealing a more active metabolism and decreasing at the final stage.

Our data shows that the majority and more complex relation of the G.O. terms identified in M. tenellum eggs were related to metabolic processes (Fig 5); during ontogeny, the primary molecular functions are catalytic and binding proteins (Fig 1A) associated with the intracellular space in the cytoplasm (Fig 1B). The metabolism of the embryo was focused on nucleotides (Fig 5), and it encompasses a complex interaction between biosynthesis and the generation of metabolites and energy amino acids; protein and carbohydrate metabolism decrease in the late stage; moreover, the generation of precursor metabolites and energy decreases their abundance, as protein metabolism becomes more critical.

Conclusions

Until our knowledge, this is the first report on quantitative proteomic profiling of ontogeny of crustacea. Our findings highlight that protein concentration is more pronounced during the early stage of embryonic development in *Macrobrachium tenellum*. Through our proteomic analysis, we identified a total of 89 proteins within M. tenellum eggs, none of which have been previously reported for this species. Intriguingly, 27 proteins were found exclusively in the early stages of development, while three proteins were exclusively present in the late stages. Our research sheds light on the diverse functions of these proteins, encompassing metabolic processes, molecular functions, and cellular components. These insights contribute to our understanding of the intricate molecular mechanisms driving he ontogenic development of M. tenellum.

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Figure 1

Molecular GO Slim2 analysis.

It is presented as the number of Gene Ontology I.D.s identified on the significant categories Molecular Function (A), Cellular Component (B), and Biological Process (C) for proteins from *M. tenellum* eggs. Analysis was performed using the Slim2 classification system. All proteins identified in the late and early stages are red and blue, respectively

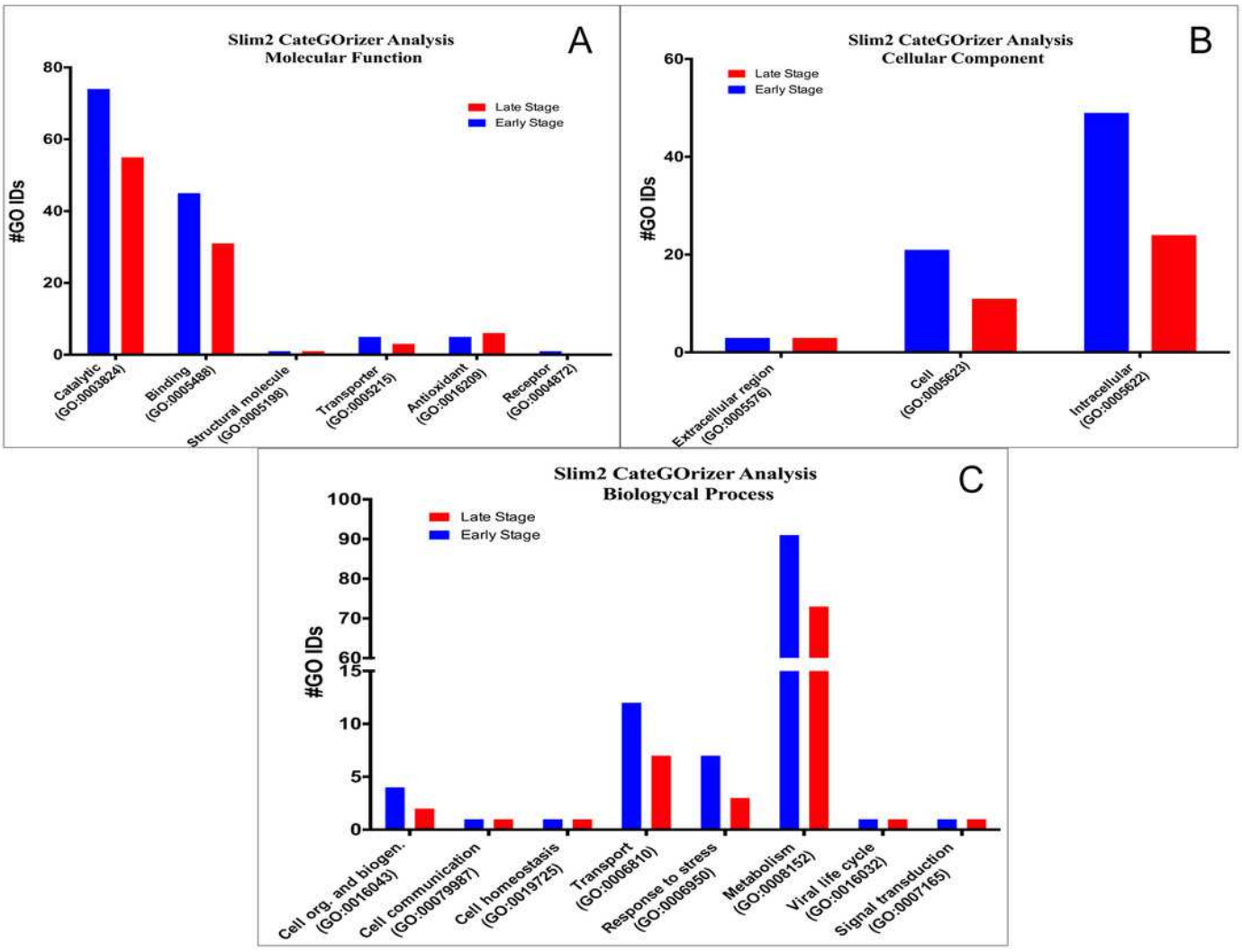

Figure 2

Molecular G.O. terms for the most critical molecular functions: binding and catalytic activities

Presented as the number of Gene Ontology Ids identified on the significant classes of A. Binding, B. Catalytic, for proteins from *M. tenellum eggs*. Analysis was done using the Slim2 classification system on February 17th, 2018. All proteins identified in the late and early stages are red and blue, respectively.

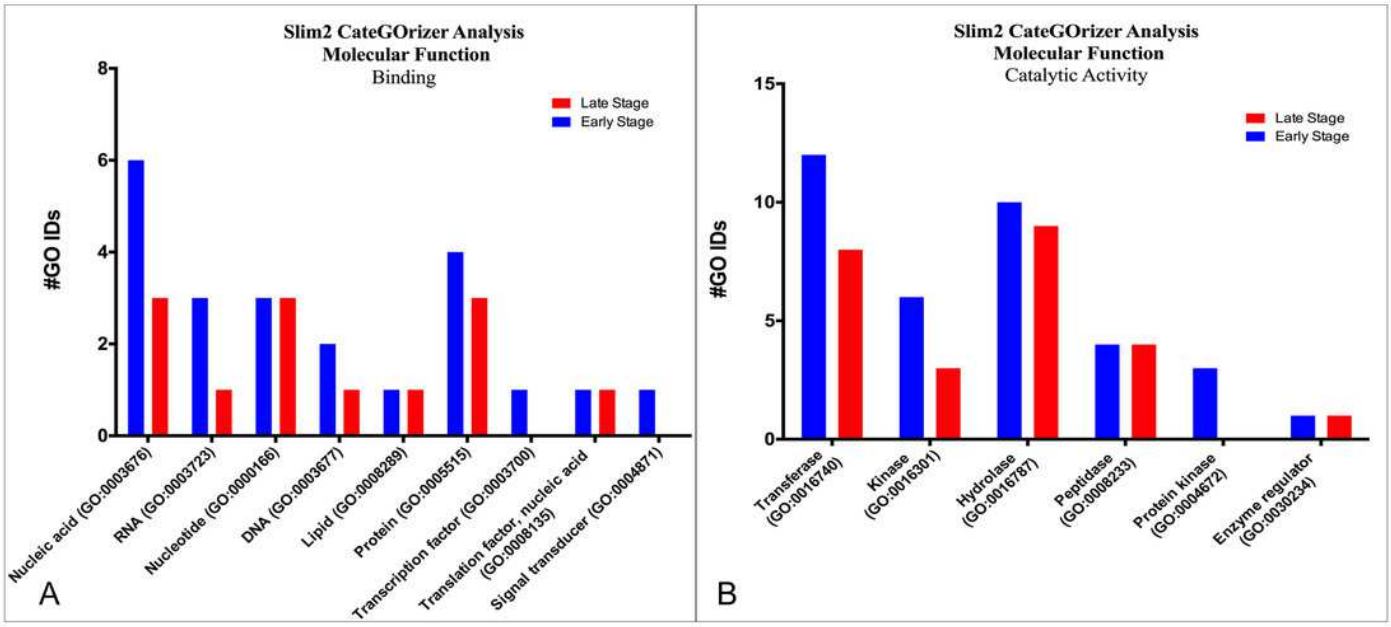

Figure 3

Molecular G.O. terms for the Intracellular Component protein classification from *M. tenellum* eggs.

Analysis was performed using the Slim2 classification system on February 17th, 2018. All proteins identified in the late and early stages are red and blue, respectively.

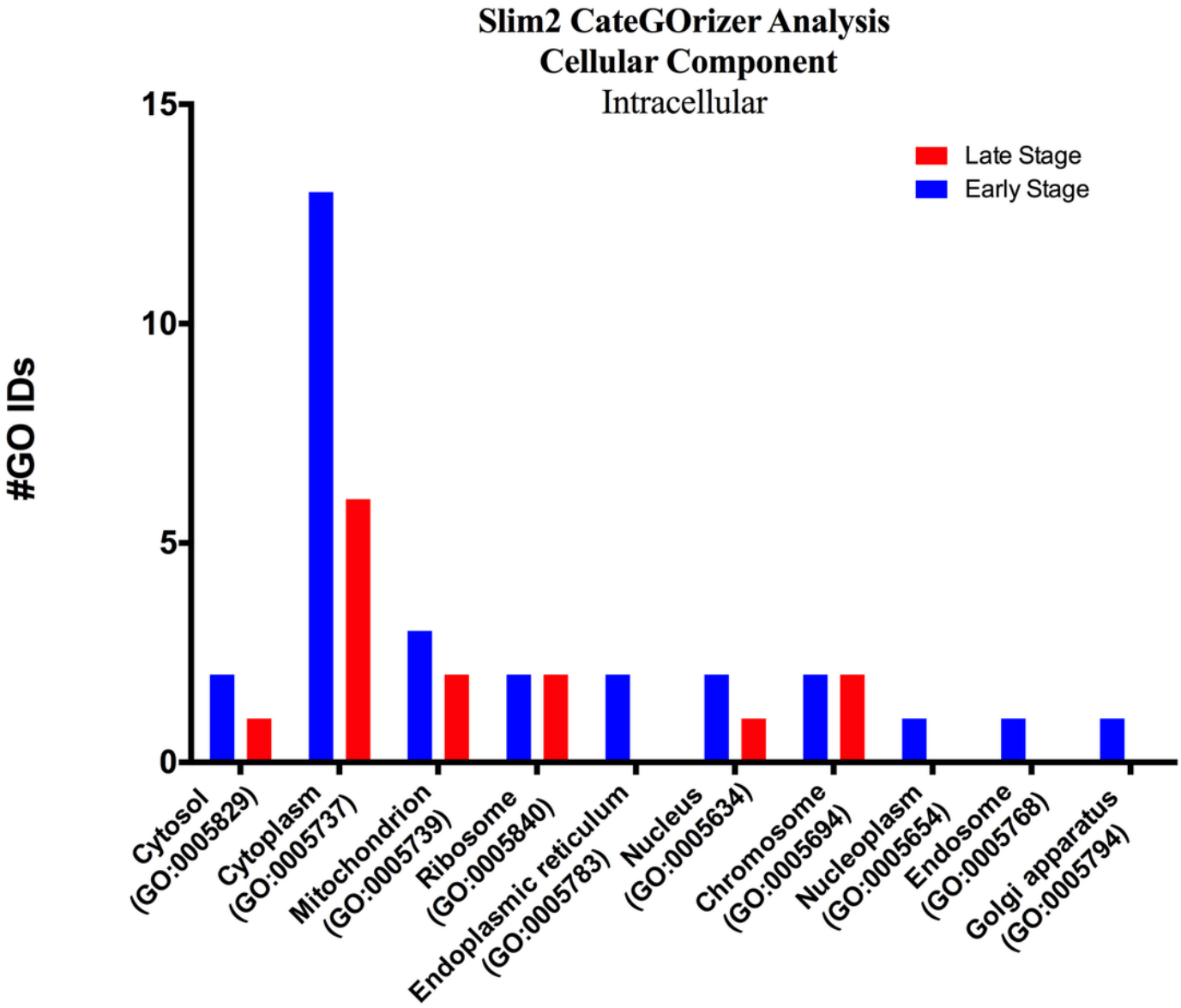

Figure 4

Scatter plot of G.O. Terms of biological process for proteins from *M. tenellum* eggs.

A. Early and B. Late stage of development. Analysis was performed by using the REVIGO web-based software.

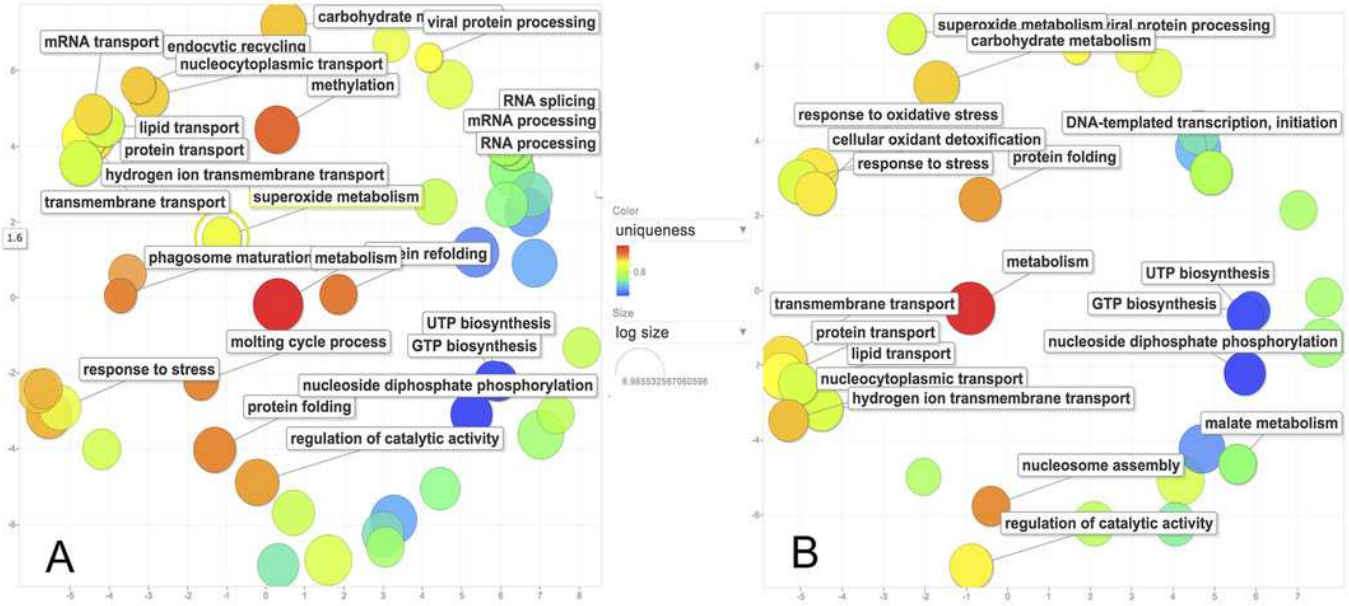

Figure 5

Interactive graph of G.O. biological processes from *M. tenellum* egg proteins.

A. Early stage of development, B. Late stage of development. Analysis was performed by the use of the REVIGO web-based software.

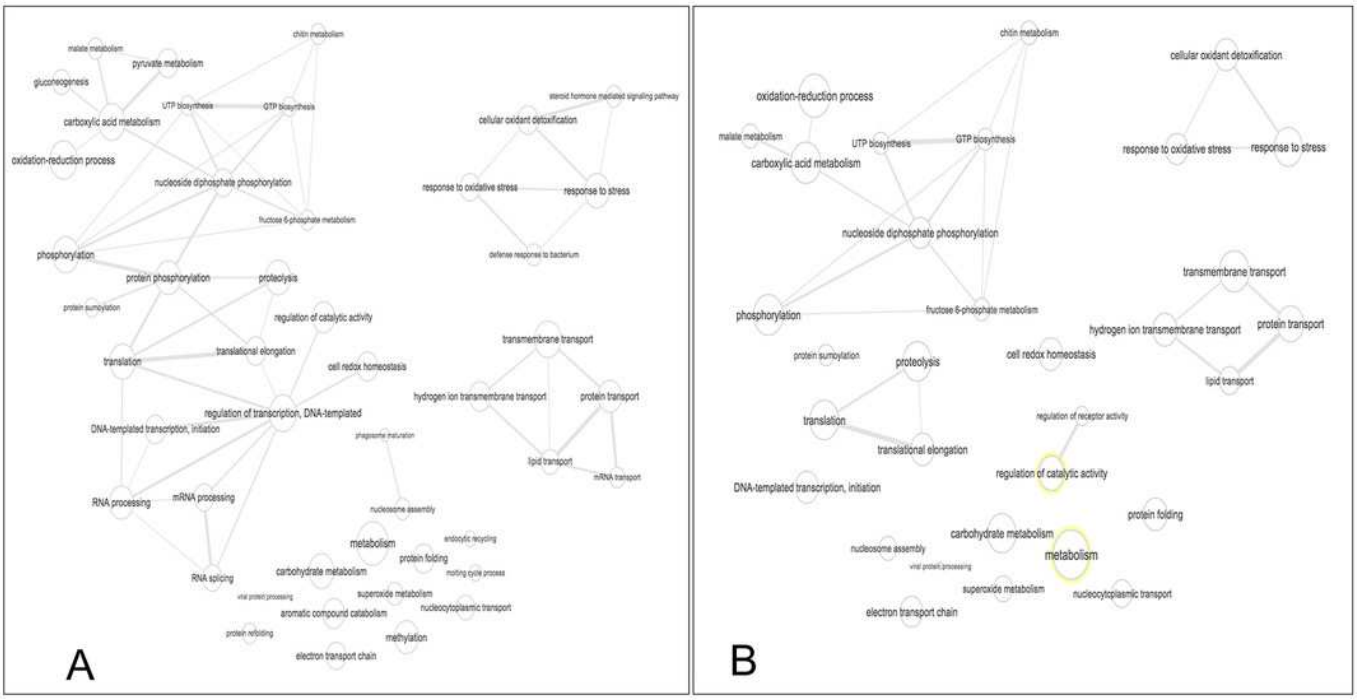

Table 1(on next page)

Proteins identified in *M. tenellum* eggs by label-free quantitative proteomics

Proteins are listed by the abundance of proteins using the emPAI protocol. Accession numbers are in UniProt format. Repeated proteins were identified separately according to accession numbers. Species refers to the species where the protein (P.E.) or gene (G.N.) was reported. Proteins in blue are those exclusively found in the early stage, while red ones are those found exclusively in the late stage. We used the SEQUEST HT score to control the quality of the peptides and proteins identified. Relative Abundance Ratio E/A compares the average area of late-stage and those of early-stage.

Accession	Description	Species	Origin	emPAI	S Sequest HT	A. Ratio
Q95P34	Vitellogenin	M. rosenbergii	GN	89,852	14270,07	1,54
A0A0E3JBN6	Vitellogenin	M. nipponense	PE	76,668	14829,93	1,76
A0A088BEN6	Glyceraldehyde-3-phosphate dehydrogenase	M. rosenbergii	PE	55,234	122,00	0,50
A0A0D6DQV4	Histone H4	M. rosenbergii	GN	45,416	158,31	0,20
Q58ZF3	Beta-actin	M. rosenbergii	PE	29,079	510,07	0,26
F5CEX2	Hemocyanin	M. nipponense	PE	25,367	1357,99	0,19
W6E8R7	Slow-tonic S2 tropomyosin	M. nipponense	PE	16,783	189,02	0,51
A0A0A0PM26	Hemocyanin	M. nipponense	PE	16,783	1068,84	0,30
E2JE77	Arginine kinase 1	M. rosenbergii	GN	15,876	272,61	0,26
Q6S4R6	Heat shock protein 70	M. rosenbergii	PE	15,037	416,18	0,46
D3XNR9	Tropomyosin	M. rosenbergii	PE	12,895	207,14	0,10
A0A0A6Z676	Actin (Fragment)	M. rosenbergii	PE	12,335	248,17	0,00
A0A142G5I8	Adenine nucleotide translocase	M. rosenbergii	GN	9	126,05	1,96
A0A142G5I9	Cathepsin-D	M. rosenbergii	GN	9	191,15	0,64
K9J9V3	DEAD(Asp-Glu-Ala-Asp) box polypeptide 39	M. nipponense	GN	9	289,82	0,16
A0A126TKR7	Elongation factor 1 alpha (Fragment)	M. amazonicum	PE	9	86,57	7,80
A0A0A6ZEG8	Heat shock protein 70	M. nipponense	PE	7,886	365,20	0,62
A0A088BES7	Elongation factor 1-alpha (Fragment)	M. rosenbergii	PE	7,577	198,97	0,41
A0A168SIW1	Nucleoside diphosphate kinase	M. nipponense	PE	7,111	168,15	0,33
M4IQR3	Hemocyanin subunit 1	M. nipponense	PE	6,263	771,93	0,21
A0A0B4L994	Peroxiredoxin	M. nipponense	PE	6,197	105,20	0,32
W6FEW7	Acyl-CoA-binding protein	M. nipponense	GN	6,197	40,29	0,41
A0A172Q3Z0	GTP-binding nuclear protein	M. rosenbergii	GN	5,579	68,14	0,12
B8ZXC6	Histone 3 (Fragment)	M. aff. pilimanus	GN	5,31	44,17	0,24
R4JXM8	Enolase (Fragment)	M. nipponense	PE	5,31	49,54	0,16
G0Z048	Cytosolic manganese superoxide dismutase	M. nipponense	PE	5,105	94,30	0,49
D2KK94	Glutamate dehydrogenase (Fragment)	M. rosenbergii	GN	3,642	30,89	3,01
Q58HZ6	Actin (Fragment)	M. rosenbergii	PE	3,642	19,95	0,00
G8H336	Ribosomal protein L10 (Fragment)	M. acanthurus	PE	3,642	8,93	0,00
A0A140AZ38	Pyruvate kinase	M. nipponense	PE	3,549	163,62	0,53
A0A0B4UBV8	Rab1A	M. rosenbergii	PE	2,875	61,12	0,34
A0A0F7RQ78	Heat shock protein 90	M. rosenbergii	GN	2,728	78,82	0,31
B5A8X6	Putative uncharacterized protein	M. rosenbergii	PE	2,162	13,46	1,41
B8LG57	Male reproductive-related LIM protein	M. rosenbergii	PE	2,162	6,80	0,00
J9PNK3	Chaperonin	M. rosenbergii	PE	2,065	81,21	0,11
E5DHS2	Heat shock protein 90	M. nipponense	PE	1,907	136,98	0,70
H6UXP2	Farnesoic acid O-methyltransferase	M. nipponense	PE	1,848	13,43	0,00
C0M152	Cytochrome c oxidase subunit 2	M. lanchesteri	GN	1,783	11,07	0,26
A0A0B4UBB1	Rab11A	M. rosenbergii	PE	1,683	26,35	0,46
I1SSS7	Cathepsin L	M. nipponense	PE	1,637	92,35	0,12
I7BBT8	Cytochrome c oxidase subunit 3 (Fragment)	M. rosenbergii	PE	1,512	8,58	0,00
A0A0A7AD33	HSP60	M. nipponense	PE	1,477	58,15	0,00
A0A0B4UBA7	Rab5C	M. rosenbergii	PE	1,424	41,44	0,31
H9BC94	Fatty acid binding protein 10	M. nipponense	PE	1,371	21,24	0,14
A0A0D6DQG1	Histone H2A	M. rosenbergii	GN	1,371	26,86	0,79
A0A0M5DID3	Histone 1	M. rosenbergii	GN	1,154	11,76	0,08
A0A0U2DW78	ATP synthase subunit a	M. bullatum	GN	1,154	2,03	0,00
G9BRM2	Apoptosis inhibitor	M. rosenbergii	PE	1,054	31,84	0,49
M9UTQ1	Glutathione S-transferases	M. nipponense	PE	1,054	38,00	0,29
Q6J1F2	Peroxinectin (Fragment)	M. rosenbergii	PE	0,931	3,40	0,00
I0B8W2	Alpha2-macroglobulin	M. nipponense	GN	0,905	16,63	0,00
A0A0B4UCH1	Rab7A	M. rosenbergii	PE	0,896	14,41	0,45
Q3Y596	Superoxide dismutase	M. rosenbergii	PE	0,874	12,12	0,17
I1SSS8	Cathepsin B	M. nipponense	PE	0,778	31,42	0,25
A7UL77	Superoxide dismutase [Cu-Zn]	M. rosenbergii	PE	0,778	2,62	0,00
A4Z4V4	Crustacyanin-like lipocalin	M. rosenbergii	PE	0,668	15,78	0,23
A0A168SIY7	Ribosomal protein L24	M. nipponense	PE	0,668	6,33	0,00
I6LTQ0	Mitochondrial prohibitin	M. rosenbergii	PE	0,624	18,39	0,11
A0A193CGZ5	ATP-dependent 6-phosphofructokinase	M. nipponense	PE	0,613	29,78	0,33
I1VWN9	Ribosomal protein L26	M. rosenbergii	GN	0,585	7,36	0,50
G4XX74	PL10-like protein	M. nipponense	PE	0,48	37,51	0,11
A0A0B4UC48	Rab6A	M. rosenbergii	PE	0,468	20,30	0,35
H8XYP6	Catalase	M. rosenbergii	PE	0,45	10,89	26,36
A0A140AZ39	Pyruvate carboxylase subunit B	M. nipponense	PE	0,448	28,82	0,00
A0T1M1	Alpha-2-macroglobulin	M. rosenbergii	PE	0,434	28,23	0,22
M9MSS8	Mago nashi 1	M. nipponense	PE	0,425	9,44	0,65
A0A0U1W4T3	Putative clotting protein	M. rosenbergii	PE	0,425	63,33	0,74
K9JA10	DEAD(Asp-Glu-Ala-Asp) box polypeptide 5	M. nipponense	GN	0,417	13,84	0,13
C7EYW3	Vacuolar H+-ATPase (Fragment)	M. amazonicum	PE	0,389	1,73	0,38
A0A140AZ37	Malate dehydrogenase	M. nipponense	PE	0,389	16,59	0,37
A0A0U2DYD0	Cytochrome b	M. bullatum	GN	0,389	2,18	0,00
A0A0B4UC52	Rab14	M. rosenbergii	PE	0,359	24,13	0,00
A0A0B4UCH7	Rab18	M. rosenbergii	PE	0,359	1,74	0,00
P00031	Cytochrome c	M. malcolmsonii	PE	0,334	4,25	0,00
M9MSS7	RNA-binding protein 8A	M. nipponense	PE	0,334	2,35	0,00
G0YP40	M. nipponense hyperglycemic hormone protein	M. nipponense	GN	0,334	0,00	*
D4N5G6	SUMO-conjugating enzyme	M. nipponense	GN	0,259	6,34	0,32
B9W2R6	Glutathione peroxidase	M. rosenbergii	PE	0,212	6,09	*

A0A140AZ43	Glycogen synthase kinase 3 beta	M. nipponense	PE	0,202	5,13	0,00
W0LYS5	Calcium-calmodulin-dependent protein kinase I	M. nipponense	GN	0,145	2,35	0,00
W8PC51	Chitinase 4	M. nipponense	PE	0,129	0,00	*
W8PFL4	Chitinase 3C	M. nipponense	PE	0,122	0,00	0,00
I3UJK1	Cyclin-dependent kinases 2	M. rosenbergii	GN	0,116	3,80	0,00
A0A0H3WF09	Juvenile hormone epoxide hydrolase	M. rosenbergii	GN	0,110	1,68	0,00
V9PNQ4	Retinoid X receptor	M. nipponense	GN	0,105	1,73	0,00
A0A173G7X5	Dorsal	M. rosenbergii	PE	0,075	2,56	0,00
Q58HZ8	Prophenoloxidase (Fragment)	M. rosenbergii	PE	0,053	2,69	0,00
A0A173GTS1	VLIG2	M. rosenbergii	PE	0,020	1,71	0,00
A0A173GTR2	VLIG1	M. rosenbergii	PE	0,019	7,05	0,24

1