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Integrative omics analysis reveals differentially distributed proteins in dimorphic euspermatozoa of the squid, *Loligo bleekeri*

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ABSTRACT

In the coastal squid *Loligo bleekeri*, each male produces one of two types of fertilization-competent spermatozoa (eusperm) that exhibit morphological and behavioral differences. Large "consort" males produce short-tailed spermatozoa that display free-swimming behavior when ejaculated into seawater. Small "sneaker" males, on the other hand, produce long-tailed spermatozoa that exhibit a self-swarming trait after ejaculation. To understand the molecular basis for adaptive traits employed by alternative male mating tactics, we performed the transcriptome deep sequencing (RNA-seq) and proteome analyses to search for differences in testicular mRNAs and sperm proteins, respectively. From mature male testes we identified a total of 236,455 contigs (FPKM ≧1) where 3789 and 2789 were preferentially (≧10-fold) expressed in consort and sneaker testes, respectively. A proteomic analysis detected 4302 proteins in the mature sperm as post-translational products. A strongly biased (≧10-fold) distribution occurred in 55 consort proteins and 61 sneaker proteins. There was no clear mRNA–protein correlation, making a ballpark estimate impossible for not only overall protein abundance but also the degree of biased sperm type expressed in the spermatozoa. A family encoding dynein heavy chain gene, however, was found to be biased towards sneakers, whereas many enzymes involving energy metabolism were heavily biased towards consort spermatozoa. The difference in flagellar length matched exactly the different amount of tubulins. From these results we hypothesize that discrete differential traits in dimorphic eusperm arose from a series of innovative alterations in the intracellular components of spermatozoa.

1. Introduction

In essence, most cells can respond and adapt to their environments by means of transcriptional, post-transcriptional and post-translational events [1]. In particular, a specific set of gene expression facilitates a cell's optimal reactions in favorable or unfavorable conditions. Spermatozoa are highly specialized, differentiated haploid cells that largely lack transcriptional and translational activities, although some exceptional cases have been reported [2]. Basically, sperm design and their behavioral traits are endowed during spermatogenesis and spermiogenesis. Therefore, in the same species, each sperm cell is expected to behave equally in response to the same environmental cue. In this context, the squid *Loligo bleekeri* offers a very unique feature regarding two separate sperm traits in two separate male types; (1) large males called "consort" produce small spermatozoa and small "sneaker" males produce ~50% much larger sperm [3], (2) for the sneakers only, spermatozoa form a cluster in response to carbon dioxide [4,5]. Such sperm dimorphism is tightly linked to male mating behaviors. Large males have an advantage in copulation, placing sperm inside the oviduct. Small males, however, avoid male-male competition and undertake "sneaky copulation" placing their

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spermatozoa on the buccal membrane of the female [6,3,7]. Fitness consequences of these different sperm phenotypes remain unknown. Moreover, the mode of inheritance as well as the ontogenetic question of how this male dimorphism arose remains unanswered.

Apart from trying to gather some insight into these intriguing evolutionary questions, we attempted to understand the developmental processes that drive spermatogenesis into different types of sperm cells. Because sperm are in general translationally silent, the differences should be evident at the levels of transcription and/or translation in the testis. Using this assumption, we used a comparative "omics" approach to identify differentially expressed transcripts and proteins that can potentially contribute to consort- or sneaker-specific traits.

2. Materials and methods

2.1. Handling of animals and spermatozoa

Mature *L. bleekeri* were collected at Miura (Sagami-bay, Kanagawa) or Matsumae (southeast Hokkaido Island), Japan, and transported in chilled containers (4 °C) to the laboratory within two days. Adult males produce spermatophores, which are cylindrical capsules containing mature sperm. Consort males and sneaker males were distinguished by spermatophore lengths and spermatangium morphology [6,3]. Spermatozoa were released from spermatophores by mechanically stimulating the spermatophoric reaction [8] in a 1.5 mL tube containing 200 μL of artificial seawater (ASW), followed by 1 h incubation on ice to recover the concentrated sperm suspension. For proteomic analysis, spermatozoa were homogenized in phosphate-buffered saline (PBS) on ice. The homogenates were centrifuged (14,000*g*, 10 min) twice to remove debris, thereafter ultra-centrifuged (100,000*g*, 1 h). The supernatant (water-soluble fraction) was collected and stored at 4 °C until use. The debris recovered from the first 2 centrifugations was suspended in PBS containing 1% SDS for 1 h on ice, followed by ultracentrifugation (a detergent-soluble fraction). Composition of ASW: 480 mM NaCl, 10 mM KCl, 27 mM $MgCl_2$, 29 mM $MgSO_4$, 10 mM $CaCl_2$ and 2 mM $NaHCO_3$ (pH 8.0).

For transcriptome analysis, the testes were removed and homogenized in TRIzol reagent (Invitrogen) immediately after the animals were killed. Total RNAs were isolated according to the manufacture's protocol (Invitrogen), followed by on-column DNase treatment using QIAGEN RNeasy kit. To minimize variation among individuals, we mixed the same amount of total RNAs from 3 specimens into one stored RNA sample, stored for later analysis. RNAs were tested for their purity using a UV spectrophotometer (Agilent Nanodrop and Agilent 2100 bioanalyzer). Only samples that met the company's criteria (BGI: http://bgiamericas.com/service-solutions/transcriptomics/rnaseq-quantification/) were then processed to the RNA-seq analysis. Short read sequences were obtained by illumina Hiseq2000 according to the company's procedures.

2.2. Transcriptome assembly and functional analysis

Sequences of consort and sneaker testes were pooled into one dataset and were assembled using the Trinity platform [9]. To obtain normalized gene expression intensities (fragments per kilobase per million reads, FPKM), reads from the two samples were mapped on to the Trinity assembly with bowtie [10] and analyzed with RSEM [11] and edgeR [12]. In the assembly procedure, putative alternative splicing variants (sub-components of the Trinity output) were estimated as different contigs, but we merged variants from one sub-component based on the "%comp_fpkm" values of edgeR output. Analytical pipelines on a NIG Cell Innovation program (http://cell-innovation.nig.ac.jp/) were used with the annotation steps to the assembled contigs. Gene ontology (GO) terms were assigned to the set of protein database of UniprotKB that showed hits with a threshold *E* value of 10^{-10}. Assembled contigs were then translated into protein sequences and used as a database in proteome analysis. Raw data is available on DRA: TBA (data submitted to DDBJ Sequence Read Archive (DRA)).

2.3. Electrophoresis and in-gel digestion

The protocols followed were identical to previous studies [13–15]. Electrophoresis was conducted using 10–20% gradient polyacrylamide gel (ATTO). The gel was stained with coomassie brilliant blue and sliced into 32 segments. Each gel piece was destained with 25 mM ammonium bicarbonate (AB) in 30% acetonitrile (MeCN), and then incubated in 100% MeCN at room temperature for 5 min. The gel pieces were dehydrated at 45 °C for 15 min using SpeedVac (SPD1010, Thermo Fisher Scientific). The gel strips were then re-hydrated with 10 mM DTT in 25 mM AB and incubated at 56 °C for 1 h. The proteins in the gels were subsequently alkylated by incubating 55 mM iodoacetamide (IAA) in 25 mM AB under the light shielding for 45 min. The gels were then washed twice in 25 mM AB in 50% MeCN, dehydrated with MeCN and dried by Speed Vac. Each gel strip was incubated with 50 μL of trypsin-buffer (50 mM AB containing 10 μg/mL trypsin) on ice for 30 min. After removing superfluous trypsin buffer, the gel strip was incubated at 37 °C overnight. The gel strips were then incubated at room temperature in 50% MeCN containing 3% formic acid in 25 mM AB for 30–60 min. After the latter step was repeated twice, 70% MeCN was added to the gels and incubated at room temperature for 1 h. Extracts were collected into new tubes and concentrated by Speed Vac at room temperature. Finally, the samples were adjusted to 20 μL by adding 1% formic acid (FA), and stored at −80 °C until the LC/MS/MS analysis was undertaken.

2.4. LC/MS/MS

Digested peptides with trypsin were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a capillary liquid chromatography system (Ultima3000, Dionex) connected online to a mass spectrometer (LTQ-XL, Thermo Fisher Scientific) equipped with a nanoelectrospray source and operated in the positive ion mode. Extracted peptides were injected onto a trapping column (μ-Precolumn cartridge filled with PepMap C18, 5 μm, 100 Å, 300 μm in inner diameter, 5 mm in length (LC Pakings)) and washed with aqueous 0.1% FA at a flow rate of 25 μL/min for 10 min. The trap column was then connected to a C18 reversed-phase analytical column integrated with an ESI needle (Nikkyo Technos) by valve switching. The analytical column was pre-equilibrated with 5% solvent B (see below) and eluted at a flow rate of 300 nL/min with a 66-min MeCN gradient (see below). For gradient elution, the 3 μL/min combined from pumps A (solvent A: 5% MeCN, 0.1% FA) and B (solvent B: 95% MeCN, 0.1% FA) was reduced through splitting to ~300 nL/min. Gradients (starting with sample injection) were the following: 5% B from 0 to 4 min, linearly increased to 45% B over the next 56 min, increased to 95% B over the next 1 min, maintained at 95% B for the next 5 min, and decreased to 5% B over the next 1 min.

Acquisition of MS/MS spectra was performed using the following parameters. Electrospray ionization was performed with the spray voltage set at 1500 V, the capillary voltage set at 40 V, and the capillary temperature at 200 °C. The collision cell was set as a default setting, and the multichannel plate detector was set at 1900–2200 V. The mass spectrometer was operated in the survey

scan mode, with up to two of the most intense precursor ions automatically selected. Doubly or triply charged ions were targeted for collision-induced dissociation fragmentation. Survey scans were recorded from 400 to 1800 *m/z*. Zoom scans and MS/MS scans, performed sequentially for the selected precursor ions, were recorded with a default setting. Target ions already selected for MS/MS were dynamically excluded during the subsequent period of 60 s. Raw spectrum data were processed using SEQUEST software to extract peak lists. The obtained peak lists were analyzed using the MASCOT program (version 2.3; Matrix Science, London, UK) against *L. bleekeri* testis RNA-seq sequences combined with sequences of common contaminants, such as human keratins. The false discovery rates (FDR) were set to 0.05. Proteins with the peptide counts of more than 2 in either consort or sneaker samples were considered significant in this study.

3. Results and discussion

The lifespan of *L. bleekeri* is estimated to be ~1 year [16] and during the last few months (Dec–Apr) of their lifetime individuals conduct the mass spawning in shallow water. Sneaker and consort males were collected simultaneously at the same place to obtain comparative gene/protein expression profiles (Fig. 1). We undertook this in the middle of the spawning season (Feb in Sagami-bay) and relatively smaller mature individuals for both male types were chosen to collect testicular mRNAs, because (1) both types co-exist more frequently and (2) a greater chance of the testes having a similar developmental stage. Total RNAs were isolated from 6 individuals for each male type and 3 samples were selected and mixed after a RNA quality test, thus validating the requirements for quantitative RNA sequencing (BGI China; $OD_{260/280}$ = 1.8–2.2; $OD_{260/230} \geqq 1.8$; for animal RIN $\geqq 7.0$). From 18 Gb RNA-seq reads per run, we obtained 334,899 contigs (FPKM > 0) from both male types, and 233,600 contigs or 236,455 contigs (FPKM $\geqq 1$) from consort or sneaker testes, respectively. Comparative gene expression profiles revealed that 3,789 contigs were strongly consort-biased (≧10-fold in the FPKM ratio), whereas sneaker-biased contigs were much less so (2789 contigs with ⩾10-fold in the FPKM ratio). Even if the stringency of the expression bias was lowered (≧5 or ≧2), this tendency was still observed.

To compare the functional gene set of the two male types, the contigs were grouped into 57,298 "subcomponents". Although the read assembly is still incomplete, these subcomponents can be interpreted as distinct genetic loci. The subcomponents were compared against UniprotKB with BLASTX and 15,722 subcomponents were matched to known functional genes. All unique matches were annotated (Supplemental dataset), thereafter abstracted to gene ontology (GO) terms corresponding to a total of 73,926 GO annotation counts. The GO:0006355 (regulation of transcription, DNA-templated) category of the biological process class contributed to the largest proportion (3.0%) of all annotations, followed by the GO:0006351 (transcription, DNA-templated, 3.0%) and GO:0006810 (transport, 2.4%) categories (Fig. 2A). Among the gene set, 8650 subcomponents (including 12,153 contigs) were assigned only in consorts (≧1 FPKM in consort <1 in counterpart, Fig. 2B). On the other hand, 8883 subcomponents (including 12,246 contigs) were sneaker specific (Fig. 2B).

Next, we performed comparative proteomic analysis using mature spermatozoa obtained from the spermatophore. Sperm proteins were separated into aqueous and 1% SDS-soluble phases, subjected to LC/MS followed by an algorithmic comparison with a MASCOT search engine [17] against RNA-seq datasets.

A total of 2191 and 2883 subcomponents were assigned from aqueous and detergent phases, respectively (≧2 peptide counts), and they were abstracted to 4102 GO terms. In comparison with the transcriptome dataset, very similar GO terms were top-ranked except for GO:0055114 (oxidation–reduction process) and GO:0007165 (signal transduction, Fig. 2A). They were ranked 14th (oxidation–reduction process) and 13th (signal transduction) in the transcriptome dataset. These results suggest that both transcriptome and proteome analyses covered similar functional genes.

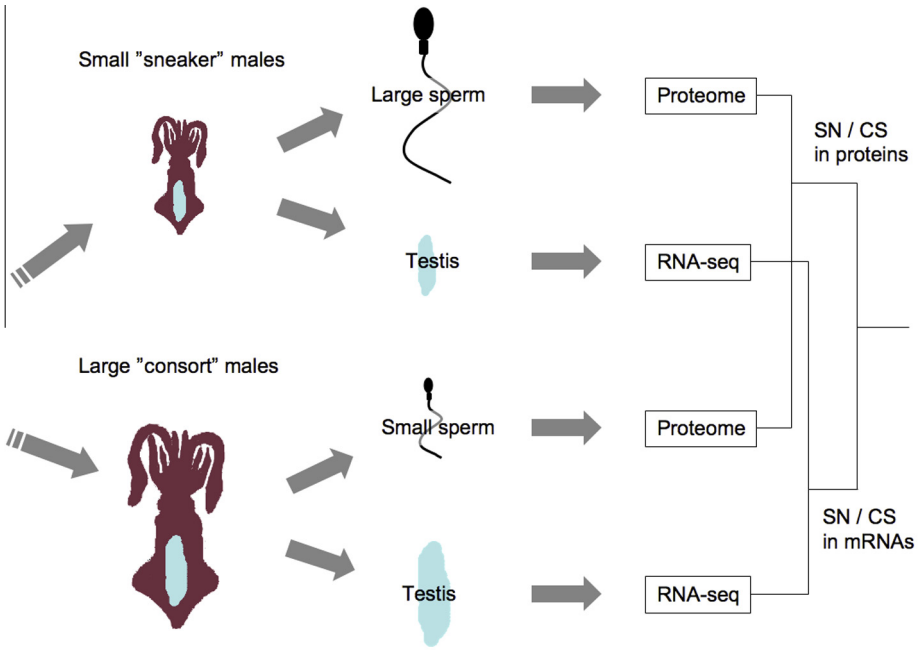

Fig. 1. A scheme of comparative omics-approach for dimorphic spermatozoa in the squid. Spermatozoa and testes were collected from sneaker and consort males of the squid, *L. bleekeri*. Proteins were extracted from spermatozoa and subjected to SDS polyacrylamide gel electrophoresis followed by in-gel digestion. These samples were then subjected to proteomic analysis. mRNAs were isolated from testes of both types of males and subjected to quantitative transcriptome (RNA-seq) analysis. Digital gene expression profiling (using FPKM value) of each subcomponents was calculated, which was used to compare counts of each testicular transcript between sneaker and consort. Similarly, in proteomic analysis, peptide counts for each identified subcomponent were compared between sneaker sperm and consort sperm. Finally, the intensity of expression bias (relative abundance between sneaker and consort) was compared between testis mRNAs and corresponding sperm proteins.

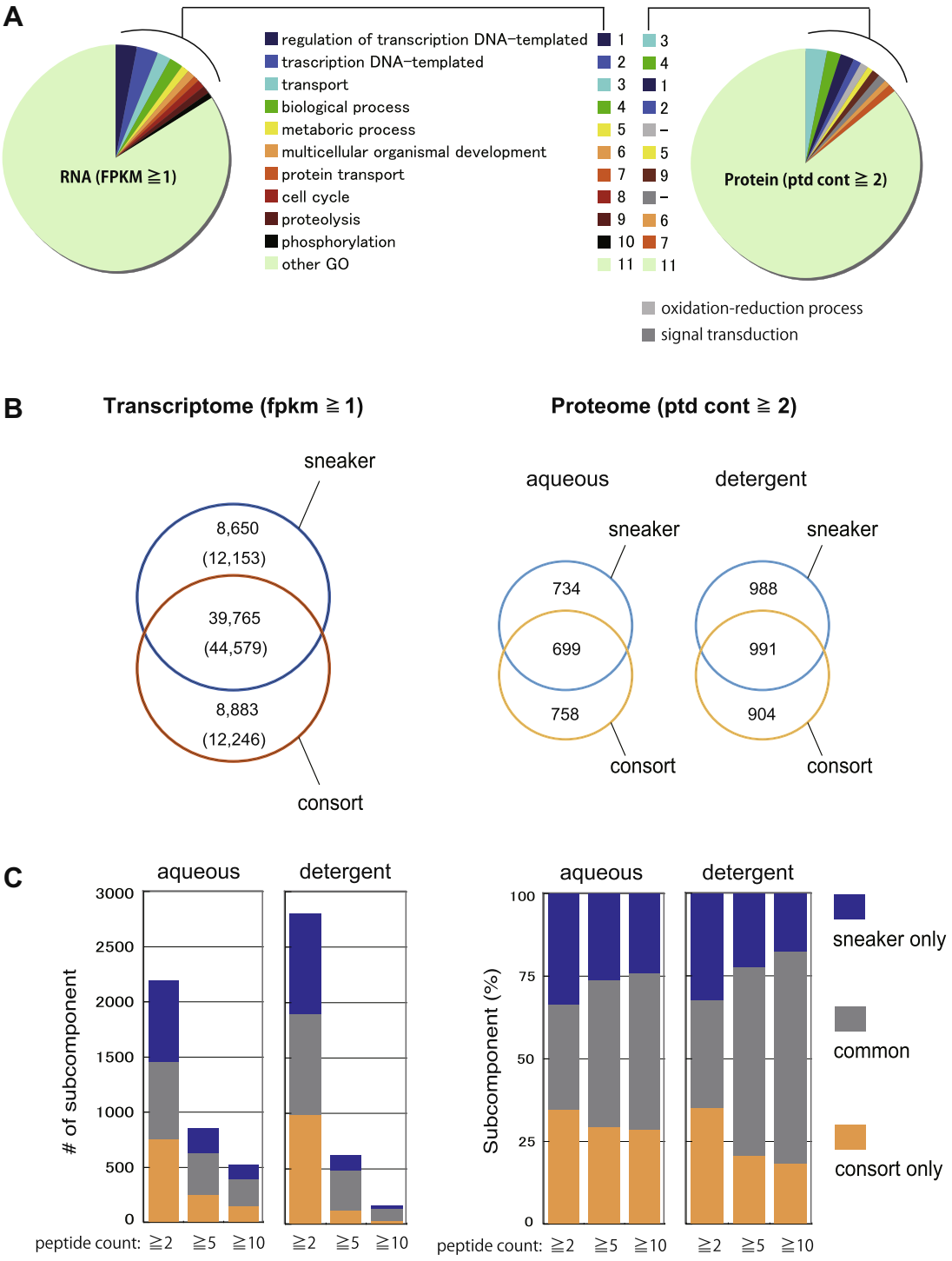

Fig. 2. Differential gene expression profiles in testis and sperm. (A) Pie charts with the top 10 GO terms (biological process) in genes annotated from transcriptome (testis, *left*) and proteome (sperm, *right*) analyses. (B) Venn diagrams showing overlap of differentially expressed genes (the number of subcomponents) between two subgroups defined herein (transcripts in sneaker vs. consort and proteins in sneaker vs. consort). The number of contigs was shown in the perentacy. (C) The number (*left*) and the percentage (*right*) of subcomponents distributed in sneaker only (*blue*), consort only (*orange*) and both (*gray*) with the different peptide counts thresholds.

Because sneaker spermatozoa exhibit a unique feature: chemotaxis to CO_2 [4], we next analyzed genes that preferentially express in sneaker spermatozoa. Of 48,415 subcomponents that were found in sneaker testis (RNA), 8650 (5.6%) were sneaker-specific. In contrast, sperm proteomic analysis identified a total of 4302 subcomponents among which 51.2% and 49.9% were specifically distributed to sneaker sperm in aqueous and detergent-soluble phases, respectively. We found that most subcomponents that express only sneaker (or only consort) spermatozoa (<2 in counterpart) are less abundant (2–4 peptide counts) and those with greater peptide counts (such as ≧10) are largely present in both types of spermatozoa (Fig. 2C). These data provided a platform for identifying genes that involve differences in sperm structure and function.

We next investigated dose correlation between testicular mRNAs and their translational products in sperm. First, we

performed a pair-wise comparison between FPKM and peptide count for each subcomponent (Fig. 3A and B). Next, we set the thresholds for both FPKM (≧1) [18] and peptide count (≧2) [19] to eliminate fluctuations or background noise (color-corded). The criteria (FPKM ≧1 and peptide counts ≧2) in sneaker spermatozoa covered 1.7% (821) and 2.4% (1,173) of subcomponents identified in our testicular transcriptome analysis in the aqueous and detergent phases, respectively (Fig. 2B). In the consorts, 1.7% (822) and 2.3% (1,110) were covered in the aqueous and detergent phases, respectively (Fig. 2B). There appeared to be a trend that the greater the peptide counts, the greater in the FPKM values, regardless of the male types. This is, however, not true for the reverse situation, i.e., greater FPKM subcomponents do not always show larger peptide counts. These results suggest that the mRNA level in the testis does not necessary reflect protein abundance in sperm. This might be due to technical and biological problems. Because spermatogenesis is a relatively long-term process, RNA sampling at a particular developmental point in time fails to recover dynamically changing mRNAs. Also, a large part of the transcripts in spermatogenic cells are shared with other types of cells in the testis, which obscures a germ cell-specific dose–response. Moreover, there are well-known post-transcriptional regulation events [20,21], and during spermiogenesis, the excess cytoplasmic contents are known to be removed from maturing spermatozoa. All of these factors can cause the discrepancy between mRNA and protein levels [1,22].

We then investigated whether the bias of distribution in sperm proteins (either sneaker-biased or consort-biased) originates as a bias of expression in testicular mRNA. We chose subcomponents (proteins) with a distribution bias of greater than 10, and plotted this against the mRNA expression bias (Fig. 3B). Protein preparations obtained by detergent-based phase partitioning were analyzed separately. In all four cases, there were no significant correlations suggesting that quantification of testicular mRNAs does not provide useful information for discovering genes involved in dimorphic sperm generation.

We therefore focused on the protein distribution bias in sneakers and consorts (Fig. 4A and B). Scatter-plots of the sneaker/consort ratio in peptide counts combined with that in FPKM revealed two-dimensional normal distributions with low correlation coefficients of 0.003 (aqueous) and 0.12 (detergent), supporting the previous results that the distribution bias in sperm proteins is not attributable to the expression bias in the testis mRNAs (Fig. 3). Within sneaker-biased subcomponents encoding detergent-soluble proteins (Fig. 4B), we found major axoneme components, dynein heavy chain (dynein HC) and tubulin (Fig. 4C upper panels). The flagellum length of sneaker spermatozoa (90.5 ± 7.20 μm) is longer (×1.39) than that of consort spermatozoa (64.9 ± 3.22 μm) [3], which can explain the rate of tubulin abundance (×1.65) which is biased to sneakers. However, dynein HC revealed a 4.28-times increase in abundance, whereas dynein light chain showed a non-biased expression (×0.95), suggesting an alternative explanation may exist (see below). In contrast, a relatively large number of consort-biased subcomponents were found in the aqueous fraction (Fig. 4A). Notably, enzymes involving glycolysis and Krebs cycle are considerably consort-biased (Fig. 4C, lower panels). Even more strikingly, phosphoprotein phosphatase 1 (PP1) was found exclusively in consort spermatozoa. PP1 plays a crucial role in glycogen metabolism (both glycogen breakdown and glycogen synthesis) [23] through interaction with glycogen phosphorylase [24], a major sperm component found in both sneakers (peptide count, 2211) and consorts (2642). Another key glycogen-related enzyme is phosphoglucomutase (PGM) that catalyzes the inter-conversion of glucose 1-phosphate and glucose 6-phosphate [25]. PGM-1 is also abundant in sperm but more enriched in consorts (peptide count, 1518) than sneakers (590). Together, comparative proteomic analysis revealed a remarkable difference in glucose metabolic pathways.

Unlike most marine organisms, spermatozoa of Cephalopod species are capable of utilizing extracellular hexoses to generate ATP for flagellar movement [8]. Under an anaerobic environment, octopus spermatozoa release D-lactate, an end product of glycolysis, if extracellular glucose is available [26]. It will be therefore fascinating to determine the metabolic pathway(s) which each type of spermatozoa use for energy production under aerobic and

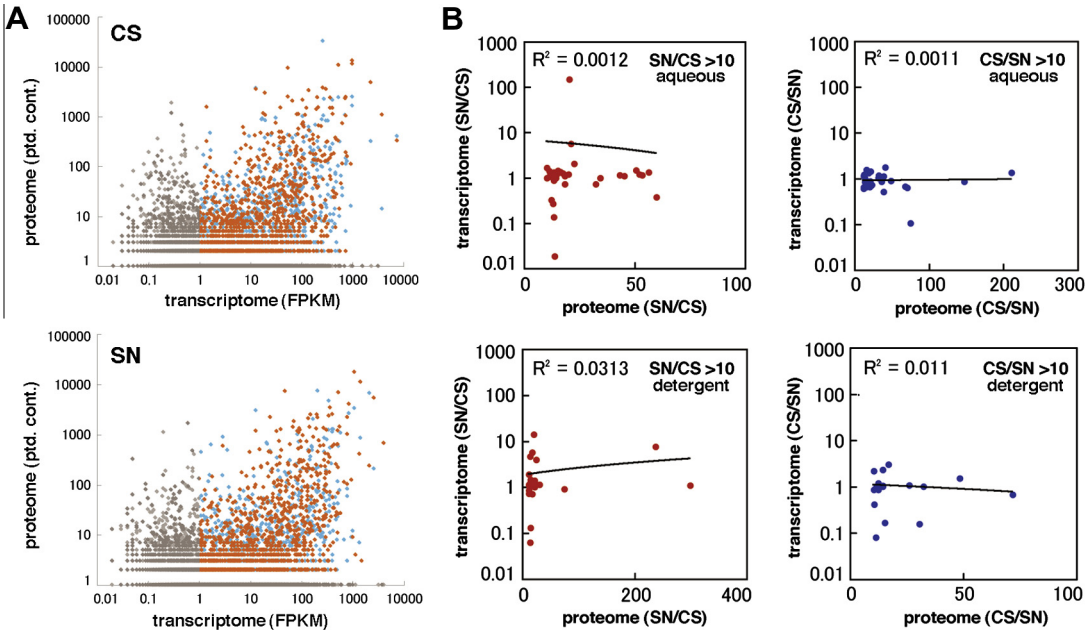

Fig. 3. A biased translation/distribution of the testicular transcripts into sperm cells. (A) Scattered-plots showing the dose correlation between testis mRNAs (FPKM) and sperm proteins (ptd. cont.) in consort (CS) and sneaker (SN). *Cyan* and *magenta* dots indicate aqueous and detergent-soluble fractions, respectively. Unreliable counts of RNA (FPKM < 1) and protein (ptd. cont. < 2) are labeled in *gray*. (B) The subcomponents with the greater (≧10) bias rate (CS/SN or SN/CS) in aqueous or detergent-soluble sperm proteins were selected. These bias rates are plotted against the bias rates in FPKM. The approximation lines are shown.

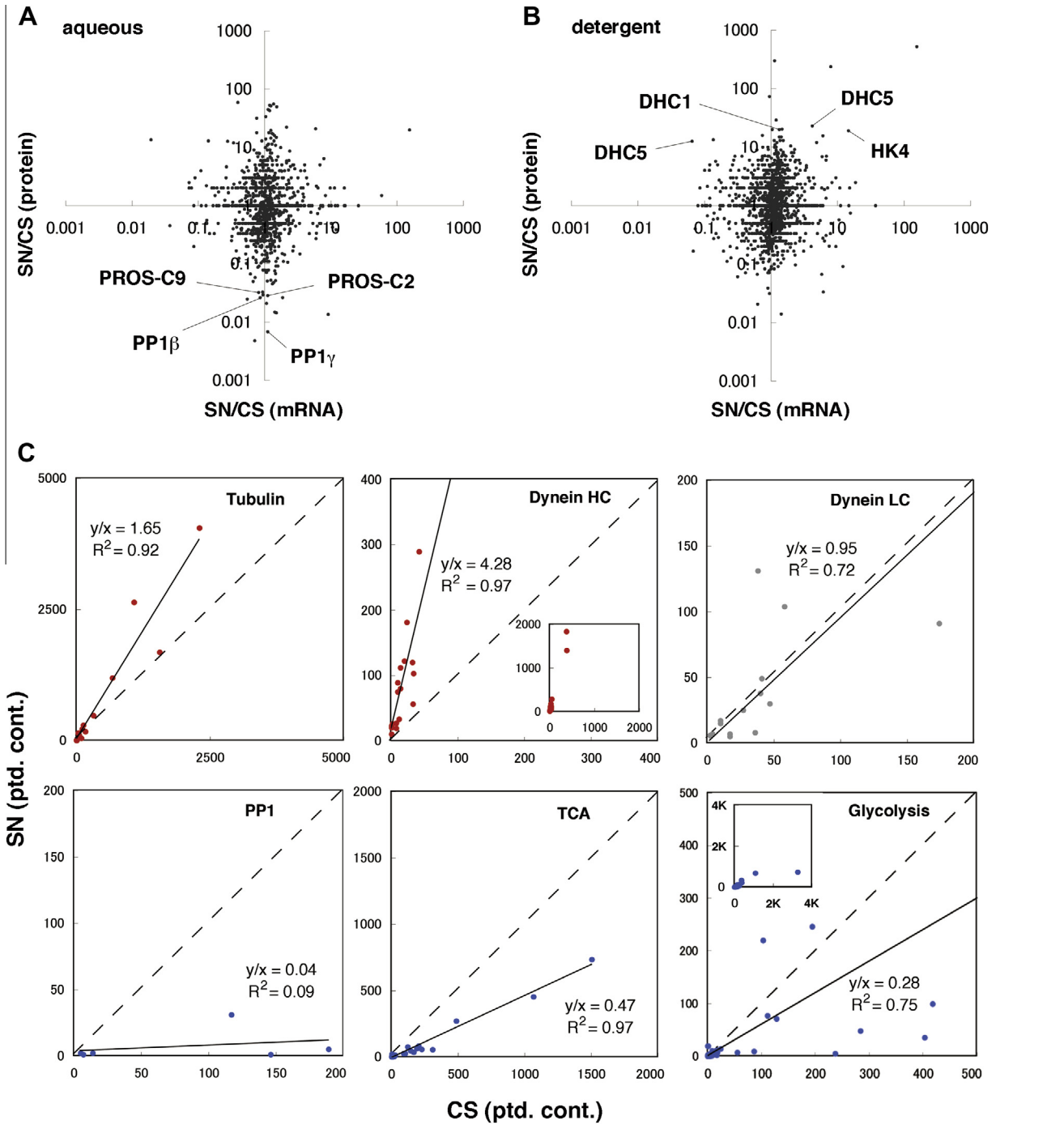

Fig. 4. Proteins that are preferentially distributed in one of two types of spermatozoa. (A, B) Global correlation between transcriptome and proteome (A, aqueous; B, detergent-soluble) in the bias rate (SN/CS). PROS; proteasome, PP1, phosphoprotein phosphatase 1; DHC, dynein heavy chain; HK, hexokinase. (C) Shown are the representative protein families (subtypes or enzymes within the same metabolic pathway) biased to sneaker (*red*), consort (*blue*) or neither (*gray*). Dynein HC, dynein heavy chain; Dynein LC, dynein light chain; PP1, phosphoprotein phosphatase 1; TCA, tricarboxylic acid cycle.

anaerobic conditions [27], as oxygen concentrations as well as glucose utility inside the female's body (experienced by consort spermatozoa) might be different from those outside the female's body (experienced by sneaker spermatozoa). Compositional and quantitative differences in energy metabolism between sneaker and consort spermatozoa might be related to the CO_2-sensing trait that only sneaker spermatozoa have acquired. In the current proteomic analysis, however, we have not found candidate molecules that might be involved in downstream signaling of CO_2 sensing, specifically after CO_2 hydration by a membrane-bound carbonic anhydrase, except for a sodium-proton exchanger (NHE; peptide count in sneaker, 1; consort, 2). Because, in general, ion channels and pumps are least abundant in the sperm plasma membranes, the enrichment process for membrane proteins is a requirement for detection in the LC–MS analysis. Nonetheless, substantial differences in major intracellular components such as dynein HC suggests the adaptive consequence in the molecular machinery that wires energy production to power generation. Sneaker spermatozoa are ~50% larger than consort sperm, yet both spermatozoa swim at the same speed [3]. Only sneaker spermatozoa acidify

their intracellular pH (pH_i) as the result of CO_2-sensing, yet flagellar motility remains high [4]. Thus, sneaker-biased distribution of dynein HC may explain how sneaker spermatozoa can overcome unfavorable situations (larger size and lower pH_i) for swimming.

The results of this study provide a glimpse into the complexity of the different fertilization tactics exhibited by *Loliginid* squid. The research methodology undertaken allows us, in the context of cell biology and sperm evolution, to explore links between the markedly different molecular composition and observed phenotypic variation in the reproductive biology of a single species of squid.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.076.

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