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Proteom układu rozrodczego jesiotra syberyjskiego *(Acipenser baerii)*: charakterystyka profili białkowych plemników, plazmy nasienia, ikry i płynu owaryjnego oraz zmian w wyniku kriokonserwacji nasienia

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Spis treści

Streszczenie

Jesiotry to jedne z najstarszych ryb, często określane jako „żywe skamieniałości". Jesiotr syberyjski (*Acipenser baerii*) jest uważany za najbardziej wartościowy gatunek w akwakulturze, ponieważ jest źródłem kawioru i wysokiej jakości mięsa. Biologia rozrodu samic i samców ryb jesiotrowatych znacznie różni się od ryb doskonałokostnych. Wyjątkową cechą plemników jesiotrów jest obecność funkcjonalnego akrosomu oraz występowanie reakcji akrosomalnej, która nie występuje u ryb doskonałokostnych. Ikra jesiotra wyróżnia się natomiast osłonką złożoną z trzech lub czterech warstw, w której znajdują się liczne mikropyla. Unikalność układu rozrodczego jesiotrów podkreśla potrzebę badania jego składu na poziomie białek. Ogólnym celem niniejszej pracy była charakterystyka proteomu gamet (plemników i ikry) jesiotra syberyjskiego i otaczającego je środowiska (plazmy nasienia i płynu owaryjnego (OF)) oraz zmian proteomu plemników w wyniku kriokonserwacji nasienia w celu zrozumienia biochemicznych i fizjologicznych podstaw funkcjonowania układu rozrodczego jesiotrów oraz poznania mechanizmów kriouszkodzeń plemników w zależności od zastosowanego krioprotektanta.

Przeprowadzone badania są pierwszymi, które szczegółowo scharakteryzowały profile białkowe plemników, plazmy nasienia, ikry i OF oraz zmiany zachodzące w wyniku kriokonserwacji nasienia jesiotra syberyjskiego. Nasze wyniki wskazują, że białka plazmy nasienia odgrywają kluczową rolę w procesach immunologicznych, metabolizmie, transporcie za pośrednictwem pęcherzyków zewnątrzkomórkowych oraz proteolizie. Ponadto, zidentyfikowano białka zaangażowane w procesy rozrodcze, w tym spermatogenezę, zapłodnienie, reakcję akrosomalną i ruchliwość plemników. Co więcej, po raz pierwszy zidentyfikowane zostały cztery kompleksy białkowe w plazmie nasienia jesiotra. Analiza proteomiczna OF wykazała znaczący udział białek w procesach odpornościowych, tj. aktywacja kaskady dopełniacza i koagulacji, odporność mediowana przez neutrofile i leukocyty, a także metabolizmie cholesterolu oraz regulacji cytoszkieletu aktynowego. Nasze badania wykazały także zaangażowanie białek ikry w szlaki metaboliczne, takie jak fosforylacja oksydacyjna i metabolizm kwasów tłuszczowych, a także ubikwitynację białek i translację. Wykazano także, że pomimo podobnych wartości parametrów jakości nasienia kriokonserwowanego z użyciem dimetylosulfotlenku (DMSO) i metanolu (MeOH), jako krioprotektantów, plemniki kriokonserwowane w DMSO charakteryzowały się znacząco niższym procentem wylęgu oraz wykazywały odmienny profil białkowy w porównaniu z plemnikami kriokonserwowanymi z MeOH. Analiza bioinformatyczna wykazała, że białka o

zmienionej intensywności w wyniku mrożenia z MeOH były powiązane ze strukturą chromosomów i funkcjonalnością mitochondriów. Z kolei białka, których poziom zmieniał się wyłącznie pod wpływem mrożenia z DMSO były głównie zaangażowane w reakcję akrosomalną, wiązanie osłonki przejrzystej, strukturę wici i organizację porów jądrowych. Białka te są potencjalnie odpowiedzialne za zdolność plemników jesiotra do zapłodnienia oraz wczesny rozwój embrionalny.

Uzyskane wyniki w ramach realizacji pracy doktorskiej znacząco przyczyniają się do poszerzenia wiedzy na temat molekularnych mechanizmów zachodzących w układzie rozrodczym jesiotra syberyjskiego w warunkach fizjologicznych jak i zaburzeń podczas kriokonserwacji. W przyszłości uzyskana wiedza może zostać wykorzystana w celu wytypowania biomarkerów jakości nasienia i ikry oraz udoskonalenia metod długookresowego przechowywania, co ma kluczowe znaczenie dla zachowania różnorodności genetycznej oraz efektywności programów restytucji i hodowli jesiotrów.

Summary

Sturgeons, the oldest fish species, are recognized as “living fossils”. The Siberian sturgeon *(Acipenser baerii)* is considered as the most valuable species in aquaculture due to its production of caviar and high-quality meat. Their reproduction differ substantially from teleost fish. A unique feature of sturgeon sperm is the presence of a functional acrosome and the occurrence of acrosomal reaction, which does not appear in teleost fish. Sturgeon eggs are distinguished by an envelope composed of three or four layers, which contains numerous micropyles. The uniqueness of the sturgeon reproductive system highlights the need to study semen composition at the protein level. The aim of this study was to characterize the proteome of the reproductive system of the Siberian sturgeon, specifically the gametes (spermatozoa and eggs) and their surrounding environments (seminal plasma and ovarian fluid), as well as the changes in the sperm proteome resulting from semen cryopreservation, to understand the biochemical and physiological changes in sturgeon reproductive system and to explore the mechanisms of sperm cryodamage depending on the cryoprotectant used.

Our research is the first to describe the protein profiles of sperm, seminal plasma, eggs, and ovarian fluid, as well as the changes occurring due to semen cryopreservation. Our results indicate that proteins in seminal plasma play a key role in immune processes, metabolism, transport via extracellular vesicles, and proteolysis. Additionally, proteins involved in reproductive processes such as spermatogenesis, fertilization, the acrosome reaction, and sperm motility were identified. For the first time, four protein complexes were identified in sturgeon seminal plasma. The proteomic analysis of ovarian fluid revealed a significant involvement of proteins in immune processes, such as the activation of the complement and coagulation cascades, neutrophil- and leukocyte-mediated immunity, cholesterol metabolism, and regulation of the actin cytoskeleton. Our research also uncovered the involvement of egg proteins in metabolic pathways, including oxidative phosphorylation and fatty acid metabolism, as well as protein ubiquitination and translation. Moreover, it was shown that despite similar semen quality parameters in cryopreserved sperm using dimethyl sulfoxide (DMSO) and methanol (MeOH) as cryoprotectants, sperm cryopreserved in DMSO had a significantly lower hatching rate and exhibited a different protein profile compared to sperm cryopreserved with MeOH. The bioinformatics analysis revealed that the proteins with altered intensity as a result of cryopreservation with MeOH were associated with chromosome structure and mitochondrial functionality, whereas the proteins affected by cryopreservation with DMSO were mainly involved in the acrosomal reaction, binding to the zona pellucida, flagellar structure, and

nuclear pore organization. These proteins are potentially involved in the fertilization ability of sturgeon sperm.

The results obtained in this doctoral thesis significantly contribute to expanding knowledge about the molecular mechanisms in the reproductive system of the Siberian sturgeon under physiological conditions and disturbances during cryopreservation. In the future, this knowledge may be used to identify biomarkers of sperm and egg quality and to improve long-term storage methods, which are crucial for preserving genetic diversity and the effectiveness of sturgeon restoration and breeding programs.

Wykaz najczęściej stosowanych skrótów

1D/SDS-PAGE; elektroforeza jednokierunkowa

2DE; elektroforeza dwukierunkowa

2D SDS-PAGE; elektroforeza dwukierunkowa w warunkach denaturujących

2D BN/SDS–PAGE; elektroforeza dwukierunkowa Blue Native

2D-DIGE; dwukierunkowa fluorescencyjna elektroforeza różnicowa, *ang. Two-Dimensional Difference Gel Electrophoresis*

DMSO; dimetylosulfotlenek

EIFs; czynniki inicjacji translacji, *ang. eukaryotic initiation factors*

EM; płyn zewnątrzkomórkowy; *ang. extracellular medium*

IPA; *ang. Ingenuity Pathway Analysis*

LC-MS/MS; chromatografia cieczowa połączona ze spektrometrią mas

MeOH; metanol

OF; płyn owaryjny; *ang. ovarian fluid*

ROS+; reaktywne formy tlenu

TCA; cykl kwasu cytrynowego

Publikacje stanowiące rozprawę doktorską

Wyniki uzyskane w ramach zadań badawczych zostały opublikowane w czasopismach indeksowanych przez ISI Web of Science:

1. **Kodzik N**, Ciereszko A, Szczepkowski M, Karol H, Judycka S, Malinowska A, Świderska B, Dietrich MA. Comprehensive proteomic characterization and functional annotation of Siberian sturgeon seminal plasma proteins. *Aquaculture*. 2023;568:739326. https://doi.org/10.1016/j.aquaculture.2023.739326.

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2. **Kodzik N**, Ciereszko A, Szczepkowska B, Malinowska A, Dietrich MA. Comparative proteomic analysis of the ovarian fluid and eggs of Siberian sturgeon. *BMC Genomics*. 2024;25(1):451. https://doi.org/10.1186/s12864-0240309-y.

   (IF$_{2024}$: 3,5; MEiN2024: 140 pkt.)

3. **Kodzik N**, Ciereszko A, Judycka S, Słowińska M, Szczepkowska B, Świderska B, Dietrich MA. Cryoprotectant-specific alterations in the proteome of Siberian sturgeon spermatozoa induced by cryopreservation. *Scientific Reports*. 2024; 14, 17707. https://doi.org/10.1038/s41598-024-68395-7.

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1. Wstęp

Jesiotr syberyjski (*Acipenser baeri* Brandt) należy do rodzaju jesiotry (*Acipenser*) rodziny jesiotrowatych (*Acipenseridae*), rzędu jesiotrokształtnych (*Acipenseriformes*). Jesiotry należą do podgromady *Chondrostei*, co oznacza, że ich szkielet pozostaje częściowo chrzęstny, co jest cechą przypominającą bardziej prymitywne formy ryb. Są one powszechnie określane jako „żywe skamieliny", zwierzęta reliktowe i najstarsze żyjące kręgowce, pochodzące z okresu jury, co oznacza, że ich linia ewolucyjna rozdzieliła się od głównego pnia ryb kostnoszkieletowych ponad 200 milionów lat temu. (Billard i Lecointre, 2000). Populacja jesiotrów dramatycznie spadła z powodu nadmiernego odłowu, zanieczyszczenia wód oraz ograniczania naturalnych siedlisk dla wzrostu ryb młodocianych (Dettlaff i in. 1993, Bronzi i in. 2019). W rezultacie większość jesiotrów uznawana jest obecnie za gatunki krytycznie zagrożone lub narażone na wyginięcie (27 gatunków jesiotrów znajduje się na Czerwonej liście (IUCN, 2024). Jesiotr syberyjski uznawany jest za najcenniejszy gatunek w akwakulturze, gdyż jest źródłem kawioru i wysokiej jakości mięsa. Charakteryzuje się szybkim tempem wzrostu, odpornością na patogeny, tolerancją na niekorzystne warunki środowiskowe oraz stosunkowo krótkim cyklem rozrodczym (7–8 lat). Obecnie prowadzi się intensywne prace nad sztucznym rozrodem i hodowlą materiału w warunkach kontrolowanych w celu zarybienia wód otwartych i produkcji (Ciereszko i Psenicka, 2018). Ponadto, tworzone są banki genów mlecza ryb jesiotrowatych, co pozwala na zachowanie różnorodności genotypów i odtworzenie populacji w przyszłości (Judycka i in. 2024). Dlatego też badania gamet i otaczającego je płynu są istotne zarówno dla wiedzy podstawowej jak i kontroli rozrodu w warunkach hodowlanych. Zarówno jakość ikry jak i nasienia mają kluczowy wpływ na skuteczność zapłodnienia. Z tego powodu uzasadnione są badania nad pogłębioną charakterystyką nasienia i ikry oraz nad doskonaleniem technik stosowanych w rozrodzie ryb jesiotrowatych, w szczególności kriokonserwacji.

1.1. Specyfika biologii rozrodu ryb jesiotrowatych

Jesiotry charakteryzują się unikalnymi mechanizmami rozrodu, które różnią się od powszechnie występujących ryb doskonałokostnych (*Teleostei*). Samce jesiotra syberyjskiego wcześniej osiągają dojrzałość płciową w wieku ok 8-9 lat (masa ciała 6,2-8,2 kg), a samice w wieku 14-15 lat (masa ciała 9,5-10,5 kg) (Szczepkowski i in. 2020). Dojrzewanie plemników u jesiotra zachodzi poza jądrem po wcześniejszym rozcieńczeniu nasienia z moczem podczas przejścia ejakulatu przez nerki, przewód nasienny aż do przewodów Wolffa (Dzyuba i in. 2014), co jest unikatowym mechanizmem wśród ryb. Ponadto, u jesiotrów występuje bezpośrednie połączenie kanalików nasiennych z nerkami. Jedną z kluczowych cech plemników ryb jesiotrowatych jest obecność akrosomu, co jest rzadkością wśród ryb. Akrosom zawiera enzymy takie jak akrozyna (ACR) czy arylsulfataza (ARSA) (Ciereszko i in. 1996), niezbędne do reakcji akrosomalnej podczas procesu zapłodnienia (Cherr i Clarck, 1984; Ginzburg i in. 1968; Jamieson i in. 1991). Reakcja akrosomalna polega na tworzeniu aktynowego filamentu, który odgrywa kluczową rolę w procesie zapłodnienia (Psenicka i in. 2010). Plemniki jesiotrów charakteryzują się wydłużoną, cylindryczną główką (długość ok. 5 µm). Ponadto, charakterystyczną cechą plemników jest wydłużony czas ich ruchliwości po aktywacji, od kilku minut do kilku godzin (Linhart i in. 1995, Toth i in. 1997), co jest znacząco dłuższym czasem niż obserwowany u ryb doskonałokostnych (20 s do 2 min). Jesiotry produkują znaczne ilości nasienia (>200 ml, Glogowski i in. 2002), jednakże koncentracja plemników jest niższa (zwykle około 1-2 x 10^9 plemników/ml), w porównaniu do karpia *(Cyprinus carpio),* gdzie koncentracja plemników jest około 20 razy wyższa (Piros i in. 2002; Alavi i in. 2012a,b).

Samice posiadają parzyste jajniki zawierające skupiska pojedynczych jaj, które stanowią od 10 do 30% ich całkowitej masy ciała. Dojrzała ikra uwolniona podczas owulacji dostaje się do jamy ciała, a następnie trafia do rozszerzonych, lejkowatych zakończeń jajowodów, skąd jest uwalniana porcjami na zewnątrz. Proces ten umożliwia rozmieszczenie ikry na dużej przestrzeni tarliska, co jest przystosowaniem anatomicznym do tarła porcyjnego. Jednakże w warunkach hodowlanych pozyskanie ikry stanowi poważne trudności. U większości gatunków jesiotrów, samice nie przechodzą owulacji co roku. Proces ten zachodzi w dłuższych odstępach czasu, sięgając nawet 2 – 5 lat (Szczepkowski i in. 2020).

1.1.1. Charakterystyka plazmy nasienia jesiotra

Główną funkcją plazmy nasienia ryb jest zapewnienie optymalnego środowiska dla przechowywanych plemników przed tarłem. Składniki plazmy nasienia utrzymują plemniki w stanie nieruchomym, dostarczają substancji odżywczych, wspomagają ich dojrzewanie i nabycie zdolności zapładniającej oraz chronią plemniki przed atakiem drobnoustrojów i stresem oksydacyjnym (Ciereszko i in. 2008). Plazma nasienia jesiotrów charakteryzuje się obniżoną osmolarnością (50–80 mOsm/kg (Dzyuba i in. 2014) w przeciwieństwie do ryb doskonałokostnych (~300 mOsm/kg), przy czym osmolarność ich osocza krwi jest bliska 300 mOsm/kg. Zawiera głównie składniki mineralne (sód, potas, wapń, magnez) oraz niskie stężenia składników organicznych, tj. hormony i feromony, cholesterol, witaminy, wolne aminokwasy, cukry, lipidy oraz białka (Psenicka i Ciereszko, 2018). Co więcej, skład plazmy nasienia jest bardzo podobny do moczu (Dzyuba i in. 2014). Wykazano, że składniki plazmy nasienia takie jak enzymy trypsynopodobne, antyproteazy, substancje o wysokiej masie cząsteczkowej oraz antyoksydanty (dysmutaza ponadtlenkowa, katalaza) są zaangażowane w mechanizm dojrzewania plemników sterleta oraz jesiotra syberyjskiego (Dzyuba i in. 2014, 2016). Natomiast aktywność antytrypsynowa zidentyfikowana z plazmie nasienia jesiotrów może odgrywać rolę w regulacji aktywności akrosyny oraz w utrzymaniu integralności plemników (Ciereszko i in., 1994). Dotychczas zidentyfikowano tylko kilka białek w plazmie nasienia jesiotra, które zostały omówione poniżej w podrozdziale „Analiza proteomiczna nasienia ryb jesiotrowatych”

1.1.2. Charakterystyka ikry i płynu owaryjnego jesiotra

Dojrzała ikra jesiotra (średnica ok. 2,3–2,7 mm dla jesiotra syberyjskiego) ma owalny kształt i kolor brązowo-szary, z wyraźnie widocznymi biegunami: animalnym, w którym położone jest jądro oraz cytoplazma uboga w składniki odżywcze oraz biegunem wegetatywnym bogatym w białka żółtka i krople lipidowe. Ikra ryb chrzęstnokostnych (jesiotrów i łopatonosów) jest otoczona pięciowarstwową glikoproteinową osłonką jajową (50 µm) (Cherr i Clark, 1984; Siddique i in. 2014), w przeciwieństwie do dwuwarstwowej osłonki u innych ryb kostnoszkieletowych. Co ciekawe, w osłonce znajduje się wiele mikropyl zlokalizowanych na biegunie animalnym (3–16 u jesiotra syberyjskiego) (Cherr i Clark, 1984; Siddique i in. 2014). Obecność licznych mikropyle jest cechą charakterystyczną dla ryb jesiotrowatych, gdyż ikra ryb doskonałokostnych posiada tylko jedno mikropyle. Osłonka odgrywa kluczową rolę podczas zapłodnienia tj. bierze udział w przyciąganiu plemników, indukcji reakcji akrosomalnej, bloku przeciwko polispermii oraz chroni rozwijający się zarodek przed czynnikami środowiskowymi i mechanicznymi aż do momentu wylęgu. Głównym składnikiem odżywczym żółtka są produkowane w wątrobie witelogeniny, które dostarczają zarodkowi niezbędnych składników odżywczych, tj. białka, lipidy i fosfolipidy, które są kluczowe dla jego wzrostu i rozwoju (Sun i in. 2015). Ponadto, ryby chrzęstnokostne wyróżniają się bruzdkowaniem holoblastycznym w przeciwieństwie do bruzdkowania częściowego tarczkowego, które występuje u ryb doskonałokostnych (Conte i in. 1988).

Płyn matczyny tzw. płyn owaryjny (OF; *ang. ovarian fluid*), który otacza ikrę w jamie ciała i jest wydalany podczas tarła wraz z ikrą, odgrywa ważną rolę w zapewnieniu optymalnych warunków dla dojrzewania ikry, działa jako „powłoka ochronna” dla żeńskich gamet, co jest istotne w kontekście zewnętrznego zapłodnienia i tworzy środowisko sprzyjające zapłodnieniu. Wykazano wpływ płynu owaryjnego na ruchliwość plemników jesiotra, w tym ich prędkość oraz trajektorię ruchu, co prowadzi do zwiększenia sukcesu zapłodnienia (Kholodnyy i in. 2021). Skład biochemiczny OF jesiotra, obejmujący jony, białka, aminokwasy i cukry, wspomaga i chroni gamety ryb przed szkodliwym działaniem niskiej osmolarności słodkiej wody (Kholodnyy i in. 2021). Zawartość jonów potasu, sodu i wapnia są podobne do innych gatunków ryb, natomiast osmolarność różni się w zależności od gatunku (u ryb jesiotrowatych około 200 mOsm/kg, natomiast u ryb łososiowatych około 250 mOsm/kg (Kholodnyy i in. 2019). OF jesiotra syberyjskiego charakteryzuje się również wyższym stężeniem białka ok. 2,41 ± 0,30 mg/ml (Siddique i in. 2016) w porównaniu do ryb karpiowatych gdzie wartości te wynoszą średnio 1,58 mg/ml dla uklei *(Alburnus alburnus)* (Lahnsteiner i in. 1997). Dotychczas większość badań koncentrowała się na biochemicznych i biofizycznych właściwościach OF jesiotra. Wiedza na temat identyfikacji poszczególnych białek w OF i ikrze jesiotra syberyjskiego jest niezbędna do lepszego zrozumienia specyfiki rozrodu ryb jesiotrowatych.

Unikalne cechy gamet jesiotrów, takie jak obecność akrosomu w plemnikach oraz liczne mikropyle w oocytach, dostarczają także cennych informacji na temat ewolucji mechanizmów rozrodczych (Ginzburg, 1968; Cherr i Clark, 1984). Mogą one stanowić ważne ogniwo ewolucyjne między prostszymi formami rozrodu, charakterystycznymi dla większości ryb doskonałokostnych (plemniki bez akrosomu, oocyty z jedną mikropyle), a bardziej złożonymi mechanizmami obserwowanymi u ssaków (Jamieson i in. 1991).

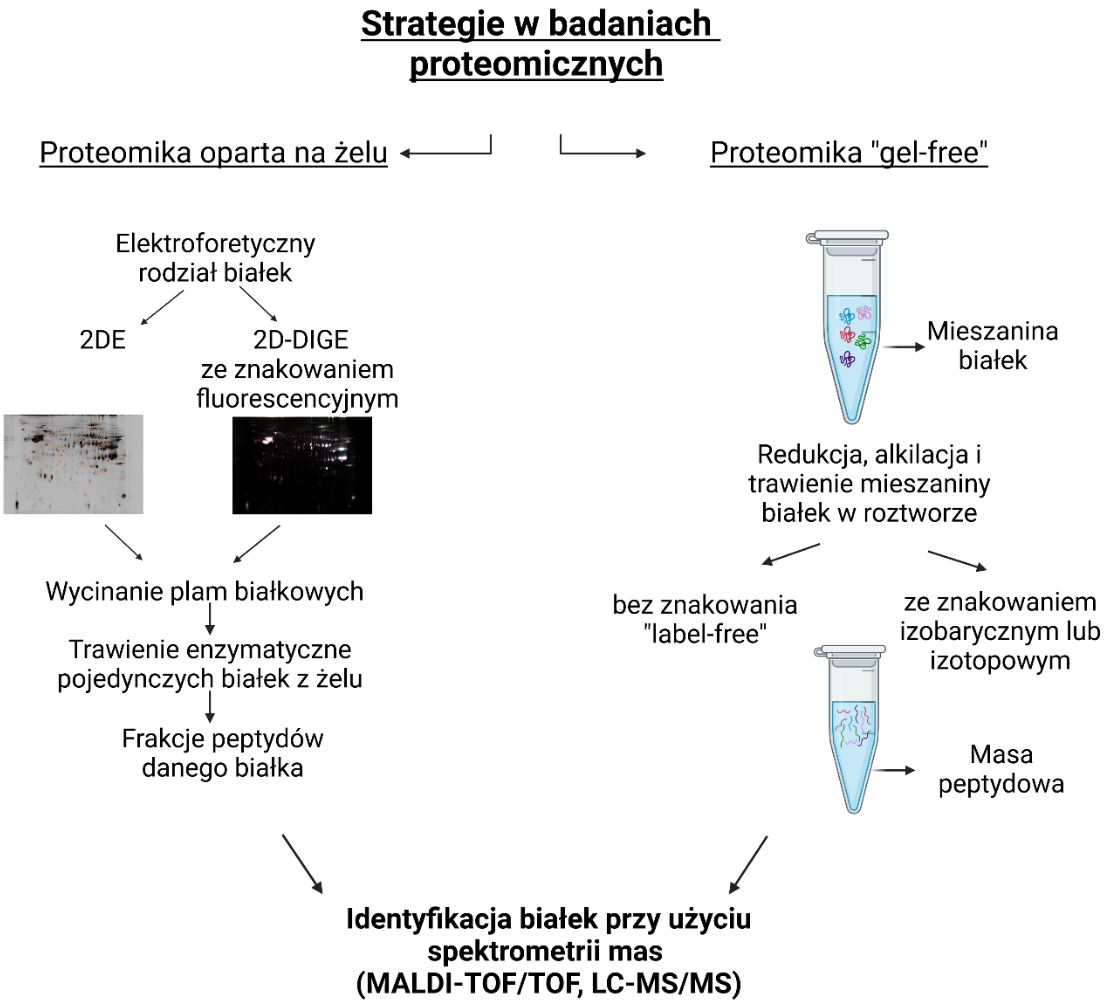
1.2. Kriokonserwacja nasienia ryb jesiotrowatych

Kriokonserwacja komórek polega na ich zamrożeniu i przechowywaniu w niskich temperaturach (-196°C), z zapewnieniem żywotności nawet po długookresowym przechowywaniu (do 3000 lat). Kriokonserwacja nasienia umożliwia także utworzenie banku genów w celu zabezpieczenia cennego materiału genetycznego populacji hodowlanych, co umożliwia utrzymanie zmienności genetycznej populacji i gatunków (Judycka i in. 2024). Kriokonserwacja nasienia jest również niezbędna dla zachowania dziko żyjących populacji na wypadek katastrof ekologicznych, zarówno naturalnych, jak i antropogenicznych. Taki materiał jest używany do „rewitalizacji" populacji pod względem optymalnych proporcji poszczególnych genotypów i wykorzystywany w programach ochrony oraz restytucji gatunków dziko żyjących oraz hodowlanych. Opracowano wiele metod kriokonserwacji nasienia jesiotra syberyjskiego, z użyciem odpowiednich krioprotektantów, które zapewniają ochronę komórek zarówno od zewnątrz jak i od wewnątrz. Do pierwszej grupy należą cukry w tym sacharoza, trehaloza, glukoza, białka oraz tłuszcze: albumina, żółtko jaja kurzego, mleko w proszku, natomiast do drugiej grupy należy metanol (MeOH), dimetylosulfotlenek (DMSO), dimetyloacetamid (DMA) oraz glicerol (Glogowski i in. 2002; Bozkurt i in. 2005; Mansour i in. 2006). Niestety, mimo doboru najlepszych warunków kriokonserwacji, zamrażanie/rozmrażanie indukuje liczne uszkodzenia kriogeniczne, które prowadzą do uszkodzeń struktur i zaburzeń funkcjonowania plemników, m.in. ruchliwości, zmian w obrębie membran oraz DNA i w konsekwencji do obniżenia jakości nasienia i zdolności zapładniającej (Shaliutina- Kolešová i in. 2015; Ciereszko i Psenicka, 2018). Dlatego też niezmiernie istotne jest poznanie mechanizmu powstawania tych uszkodzeń na poziomie molekularnym, co w rezultacie może przyczynić się do optymalizacji metodyki zamrażania nasienia jesiotrów.

Chociaż kriokonserwacja nasienia z użyciem MeOH i DMSO zapewnia bardzo dobrą ruchliwość plemników po rozmrożeniu, nasienie kriokonserwowane z użyciem DMSO, charakteryzowało się bardzo niskim procentem zapłodnienia oraz wylęgu (Lahnsteiner i in. 2004; Psenicka i in. 2008). Ponadto wykazano, że kriokonserwacja z użyciem DMSO indukuje przedwczesną reakcję akrosomalną plemników sterleta *(Acipenser ruthenus)*, co może skutkować obniżoną zdolnością zapładniającą. Jednakże Lahnseiner i in. (2004) nie wykazali znaczących różnic w uszkodzeniu akrosomu plemników sterleta pomiędzy próbami mrożonymi z MeOH i DMSO w porównaniu do nasienia świeżego. Wykazano także, że obecność enzymów akrosomalnych, tj. dehydrogenaza L-mleczanowa (LDH), arylosulfataza (ARS), kwaśna fosfataza (ACP) i β-N-acetyloglukozaminidaza (BetaGlcNAc) w plazmie kriokonserwowanego nasienia jesiotra syberyjskiego, może być dowodem kriouszkodzeń akrosomu (Piros i in. 2002). Dokładny mechanizm uszkodzeń kriogenicznych skutkujący obniżoną zdolnością zapładniająca nasienia kriokonserwowanego z użyciem DMSO, nie został poznany.

1.3. Proteomiczne podejścia w badaniach białek

Proteomika to dynamicznie rozwijająca się dziedzina nauki zajmująca się badaniem proteomu (ang. *proteome*, PROTEin complement of the genOME) czyli zbioru wszystkich białek obecnych w komórce, tkance lub organizmie kodowanych przez genom. Celem proteomiki jest identyfikacja białek, ich modyfikacji posttranslacyjnych oraz zrozumienie interakcji zachodzących między nimi, które są wyjątkowo zmienne w zależności od stanu rozwojowego organizmu i warunków środowiskowych. Wyróżniamy dwie podstawowe strategie w analizach jakościowych (w celu uzyskania katalogu białek obecnych w próbie) i ilościowych proteomu: 1) metoda oparta na rozdziale białek w żelu z wykorzystaniem technik elektroforetycznych, tj. elektroforeza dwukierunkowa w warunkach denaturujących (2D/SDS-PAGE), elektroforeza Blue Native (2D BN/SDS–PAGE) oraz dwukierunkowa fluorescencyjna elektroforeza różnicowa (2D-DIGE, ang. *Two-Dimensional Difference Gel Electrophoresis*) oraz 2) metoda z pominięciem żelu oparta na rozdziale peptydów z użyciem chromatografii cieczowej połączonej ze spektrometrią mas (LC-MS/MS) (Rys. 1). Elektroforeza dwukierunkowa jest techniką rozdzielania białek na podstawie punktu izoelektrycznego (pI) w procesie izoelektroogniskowania (pierwszy kierunek) oraz na podstawie ich masy cząsteczkowej (drugi kierunek). Dzięki temu możliwe jest stworzenie mapy białek (2D/SDS-PAGE) liczącej nawet kilkaset spotów białkowych oraz detekcja modyfikacji posttranslacyjnych białek (Brewis i Brennan, 2010) lub identyfikacja kompleksów białkowych (2D BN/SDS-PAGE). Modyfikacją metody 2DE jest technika 2D-DIGE, wykorzystywana do analiz ilościowych, w której próby przed rozdziałem są znakowanie fluorescencyjnymi barwnikami cyjaninowymi (Cy2, Cy3, Cy5), co zwiększa czułość metody i pozwala na rozdział dwóch prób oraz standardu wewnętrznego (mieszanina wszystkich prób w eksperymencie) w jednym żelu. Zastosowanie standardu wewnętrznego pozwala na wyeliminowanie różnic pomiędzy żelami, które wynikają z niedoskonałości 2DE (Alban i in., 2003). Zaletą 2D-DIGE jest wykazanie subtelnych różnic w ilości białek oraz podobnie jak w tradycyjnej 2DE wizualizacja ich proteoform i modyfikacji posttranslacyjnych. Niestety 2DE oraz 2D-DIGE nie pozwala na detekcję białek o bardzo dużych i małych masach cząsteczkowych (>150 kDa i <10 kDa) lub niskich lub wysokich wartościach pI (10 < pI <3). Alternatywą dla 2D-DIGE jest zastosowanie ilościowej analizy z pominięciem żelu opartej na LC-MS/MS bez znakowania prób (ang. *label-free*) lub ze znakowaniem izobarycznym lub izotopowym prób. Strategia bez znakowania polega na trawieniu mieszaniny białek, najczęściej trypsyną, a następnie rozdzieleniu mieszaniny peptydów na podstawie ich hydrofobowości z wykorzystaniem wysokosprawnej chromatografii cieczowej i analizie z wykorzystaniem spektrometru mas. Podejście oparte na LC-MS/MS jest bardziej czułe, jednak charakteryzuje się trudnościami w rozróżnianiu proteoform i modyfikacji posttranslacyjnych bez dodatkowych ukierunkowanych analiz. Dlatego też w celu zwiększenia liczby zidentyfikowanych białek, możliwe jest zastosowanie dwóch uzupełniających się strategii analiz proteomicznych (Jankowska i in. 2021).



Rysunek 1. Schematyczne przedstawienie strategii analiz proteomicznych.

1.4. Analiza kompleksów białkowych

Interakcje białko-białko odgrywają kluczową rolę w funkcjonowaniu większości procesów biologicznych. Większość występujących białek w komórce można znaleźć w kompleksach z innymi białkami. Odgrywają one istotną rolę w procesach regulacyjnych, funkcjach komórkowych i szlakach sygnalizacyjnych (Sali i in. 2003). Do analizy kompleksów białko-białko najczęściej stosuje się metodę opartą na elektroforezie natywnej- 2D BN/SDS–PAGE, która to umożliwia identyfikacje pojedynczych składników kompleksów. Białka często występują w formie oligomerycznej, składającej się z wielu podjednostek, które mogą składać się z identycznych (homooligomery) lub różnych (heterooligomery) łańcuchów polipeptydowych (Danielli i in. 2020). Według dostępnej wiedzy, obecność i funkcja kompleksów białkowych w plazmie nasienia ryb nie została dotychczas zbadana.

1.5. Analiza proteomiczna nasienia ryb jesiotrowatych

W przeciwieństwie do zaawansowanych badań proteomicznych plazmy nasienia wyższych kręgowców w tym ssaków (Baker, 2011) oraz kilku gatunków ryb, takich jak karp (Dietrich i in. 2014), pstrąg tęczowy *(Oncorhynchus mykiss)* (Nynca i in. 2014), łosoś *(Salmo salar)* (Gombar i in. 2017) oraz sandacz *(Sander lucioperca)* (Dietrich i in. 2021), niewiele jest informacji na temat proteomu nasienia jesiotrów. Dotychczasowe badania dotyczyły głównie analizy plemników jesiotrów w tym analizy porównawczej proteomów plemników 5 gatunków jesiotrów (jesiotr syberyjski, jesiotr rosyjski *(Acipenser gueldenstaedtii),* jesiotr gwiaździsty (*Acipenser stellatus)*, sterlet i bieługa (*Huso huso*) i jednego wisłonosa *(Polyodon spathula)* z wykorzystaniem 2DE (Li i in. 2010a). W plemnikach wszystkich gatunków zidentyfikowano wiele izoform enolazy (ENO), w plemnikach wiosłonosa amerykańskiego wykazano obecność dehydrogenazy glicerolo-3-fosforanowej (GPDH), w plemnikach sterleta i bieługi kinazy fosfoglicerynianowej (PGK2), zaś w plemnikach jesiotra rosyjskiego i bieługi aldolazy fruktozo-bisfosforanowej A-2 (FBP A-2), fosforylazy glikogenowej typu IV (GP4) oraz GPDH. W kolejnych badaniach, Li i in. (2017a) przeprowadzili pogłębioną analizę proteomu plemników ryb jesiotrowatych wykorzystując podejście proteomiki różnicowej typu LC-MS/MS ze znakowaniem izobarycznym (iTRAQ). W plemnikach jesiotra syberyjskiego zidentyfikowano 1431 białek, natomiast u jesiotra amurskiego *(Acipenser schrenckii)* 1117 białek, w tym 291 i 359 białek specyficznych dla danych gatunków. Wykazano, że białka różnicujące zlokalizowane są głównie w mitochondriach i zaangażowane są w liczne szlaki metaboliczne, tj. glikolizę i cykl kwasu cytrynowego (TCA), odpowiadające za produkcję energii. Badania białek plazmy nasienia jesiotrów ograniczają się jedynie do analizy aktywności kilku enzymów, tj. LDH, ARS, ACP, BetaGlcNAc i enzymów antyoksydacyjnych (Sarosiek i in. 2004; Shaliutina i in. 2013; Alavi i in. 2014). Ponadto, Słowińska i in. (2015) wykazali obecność w plazmie nasienia jesiotra syberyjskiego żelatynaz (proteazy serynowe i metaloproteazy), ACR oraz inhibitorów proteaz serynowych. Dlatego też pogłębiona charakterystyka składu białkowego plazmy nasienia jesiotra syberyjskiego z wykorzystaniem metod proteomicznych pozwoli na zrozumienie roli plazmy nasienia oraz specyfiki procesów zachodzących w męskim układzie rozrodczym ryb jesiotrowatych.

1.6. Analiza proteomiczna płynu owaryjnego i ikry ryb jesiotrowatych

Techniki proteomiczne zostały z powodzeniem zastosowane do identyfikacji białek ikry u różnych gatunków ryb, tj. sandacz (1296 zidentyfikowanych białek) (Nynca i in. 2022) i danio pręgowany *(Danio rerio)* (2535 zidentyfikowanych białek) (Yilmaz i in. 2017), zaś proteom OF został zbadany jedynie u łososia (174 białka) (Johnson i in. 2014), sandacza (796 białek) (Nynca i in. 2022) i pstrąga tęczowego (54 białka) (Nynca i in. 2015a). Jednakże, informacje dotyczące proteomu ikry i OF jesiotrów są bardzo ograniczone. Już ponad 3 dekady temu za pomocą elektroforezy jednokierunkowej (1D/SDS-PAGE) wykazano obecność w otoczce jajowej jesiotra amerykańskiego *(Acipenser transmontanus)* pasma białkowego o masie 66 kDa, które uznano za naturalny induktor reakcji akrosomalnej (Cherr i Clark, 1984). Do chwili obecnej białko to nie zostało zidentyfikowane. Natomiast w przypadku jesiotra perskiego *(Acipenser persicus)*, zidentyfikowano 73 białka ikry wykorzystując 2DE w połączeniu ze spektrometrią mas MALDI-TOF/TOF. Analiza funkcjonalna tych białek wykazała ich udział w procesach translacji i transkrypcji, metabolizmie i wytwarzaniu energii, jak również w transporcie i podziale komórkowym (Keyvanshokooh i Vaziri, 2008). Niksirat i in. (2017) zidentyfikowali, przy użyciu elektroforezy 2D/SDS-PAGE, 36 białek zmienionych w ikrze sterleta w wyniku zapłodnienia. Wyróżniono białka, tj. białka proteasomu, ENO, białka szoku cieplnego i glikoprotein, których poziom w znacznym stopniu zmniejszył się w porównaniu do kontroli. Zmiany profilu białkowego podczas zapłodnienia u sterleta, wykazały, iż zewnętrzne zapłodnienie zachodzące u jesiotrów wiąże się z uwalnianiem białek do środowiska zewnętrznego otaczającego ikrę, którego celem jest przyciąganie i ochrona plemników (Niksirat i in. 2017). Dalsze badania nad kompletnym proteomem OF i ikry jesiotra syberyjskiego pozwoliłyby na poznanie roli białek w żeńskim układzie rozrodczym a także na lepsze zrozumienie specyfiki rozrodu ryb jesiotrowatych. Ponadto identyfikacja specyficznych białek dla OF i ikry może dostarczyć głębszej wiedzy na temat ich funkcji, lepszego zrozumienia interakcji między ikrą a OF oraz pochodzenia białek OF, a także wyselekcjonować potencjalne biomarkery jakości i starzenia się ikry.

1.7. Badania proteomiczne kriokonserwowanego nasienia jesiotra

Wprowadzenie technik proteomicznych pozwoliło także na szersze spojrzenie na zmiany zachodzące w nasieniu ryb w wyniku kriokonserwacji. Przeprowadzone dotychczas badania dotyczyły analizy proteomu plemników karpia (Li i in. 2010b) i okonia morskiego (*Dicentrarchus labrax*) (Zilli i in. 2005) oraz proteomu płynu zewnątrzkomórkowego (EM; *ang. extracellular medium)* karpia (Dietrich i in. 2015) i pstrąga tęczowego (Nynca i in. 2015b) w celu monitorowania białek uwolnionych z plemników w wyniku kriokonserwacji. EM zawiera plazmę nasienia, rozrzedzalnik oraz substancje uwalniane z plemników w wyniku kriokonserwacji (Dietrich i in. 2015). Ponieważ plazma nasienia ryb charakteryzuje się niskim stężeniem białka, stanowi ona niskie tło dla białek uwolnionych z plemników w wyniku kriokonserwacji (Ciereszko i in. 2017).

Nieliczne badania z wykorzystaniem technik proteomicznych dotyczą wpływu kriokonserwacji na profil białkowy plemników sterleta (Xin i in. 2018; Horokhovatskyi i in. 2018). Za pomocą elektroforezy żelowej 2D/SDS-PAGE w połączeniu ze spektrometrią mas MALDI- TOF/TOF, zidentyfikowano 6 białek plemnikowych oraz 2 białka w plazmie nasienia sterleta zmienione w wyniku kriokonserwacji (Xin i in. 2018). Natomiast Horokhovatskyi i in. (2018) dzięki elektroforezie różnicowej 2D-DIGE wykazali 16 białek różnicujących proteom nasienia świeżego i kriokonserwowanego sterleta. W wyniku kriokonserwacji, wykazano zmiany ilościowe białek zaangażowanych w procesy metaboliczne, produkcję energii oraz organizację struktury witki plemnika. Zmiany tych białek w kriokonserwowanym nasieniu mogą być związane z niedoborem ATP, zmniejszoną ruchliwością i symetrią ruchu plemników, w tym ich prędkości ruchu krzywoliniowego VCL, prostoliniowego VSL oraz liniowości LIN. W dostępnej literaturze brak jest informacji dotyczących wpływu kriokonserwacji na białka plemników jesiotra syberyjskiego. Pogłębione badania w tym kierunku pozwolą na poznanie mechanizmów kriouszkodzeń plemników ryb jesiotrowatych, co z kolei może prowadzić do opracowania bardziej skutecznych metod kriokonserwacji nasienia ryb jesiotrowatych. W przyszłości, dalsze badania w tym kierunku mogą umożliwić identyfikację specyficznych markerów białkowych, które mogłyby służyć jako wskaźniki jakości nasienia po kriokonserwacji.

2. Hipoteza i cele badawcze

W dostępnej literaturze niewiele jest danych dotyczących pogłębionej charakterystyki białek układu rozrodczego ryb jesiotrowatych w tym plemników, plazmy nasienia, ikry i OF. Poznanie składu białkowego gamet i otaczającego je środowiska (plazmy nasienia i OF) jest niezbędne w celu zrozumienia biochemicznych i fizjologicznych podstaw funkcjonowania układu rozrodczego u tych ryb i zrozumienia ich wzajemnych interakcji podczas zapłodnienia. Jest to istotne dla poszerzenia wiedzy na temat specyfiki rozrodu jesiotra syberyjskiego oraz mechanizmów kriouszkodzeń plemników w zależności od zastosowanego krioprotektanta.

W oparciu o badania własne i dostępne dane literaturowe, w ramach przedstawionej pracy doktorskiej, weryfikacji poddano następujące hipotezy:

1. Skład białkowy płynu owaryjnego jesiotra syberyjskiego różni się od składu białkowego ikry, odzwierciedlając ich specyficzne funkcje w procesach rozrodczych
2. Kriokonserwacja wpływa na proteom kriokonserwowanego nasienia jesiotra syberyjskiego.
3. Kriokonserwacja nasienia z użyciem DMSO oraz MeOH wpływa w zróżnicowany sposób na białka plemnikowe związane z procesem zapłodnienia oraz wczesnym rozwojem embrionalnym

Ze względu na opisowy charakter badań, nie postawiono hipotezy w przypadku analizy proteomu plazmy nasienia.

Aby zweryfikować wyżej wymienione hipotezy i zrealizować zamierzony cel badawczy, sformułowano następujące zadania badawcze:

1. Charakterystyka profilu białkowego oraz kompleksów białkowych plazmy nasienia jesiotra syberyjskiego (Publikacja: PI)
2. Porównanie proteomu ikry i OF jesiotra syberyjskiego (Publikacja: PII)
3. Analiza proteomiczna zmian w profilu białkowym nasienia jesiotra syberyjskiego po kriokonserwacji z użyciem MeOH i DMSO jako krioprotektantów (Publikacja: PIII)

3. Spis zastosowanych metod

Doświadczenie 1 (Publikacja PI, Kodzik i in. 2023)

- Pobranie materiału i ocena jakości nasienia (komputerowa analiza nasienia w systemie CASA (*ang. Computer Assisted Semen Analysis*), pomiar stężenia i osmolarności białka w plazmie nasienia, analizy cytometryczne (pomiar koncentracji i żywotności plemników)
- Elektroforeza 2D/SDS-PAGE, 2D BN/SDS-PAGE
- Skanowanie i barwienie obrazów 2DE
- Wycinanie, trawienie i identyfikacja spotów białkowych (spektrometria mas MALDI-TOF/TOF)
- Analiza LC-MS/MS
- Analiza funkcjonalna zidentyfikowanych białek: IPA (*ang. Ingenuity Pathway Analysis*), ShinyGo, String (*ang. Search Tool for the Retrieval of Interacting Genes*)

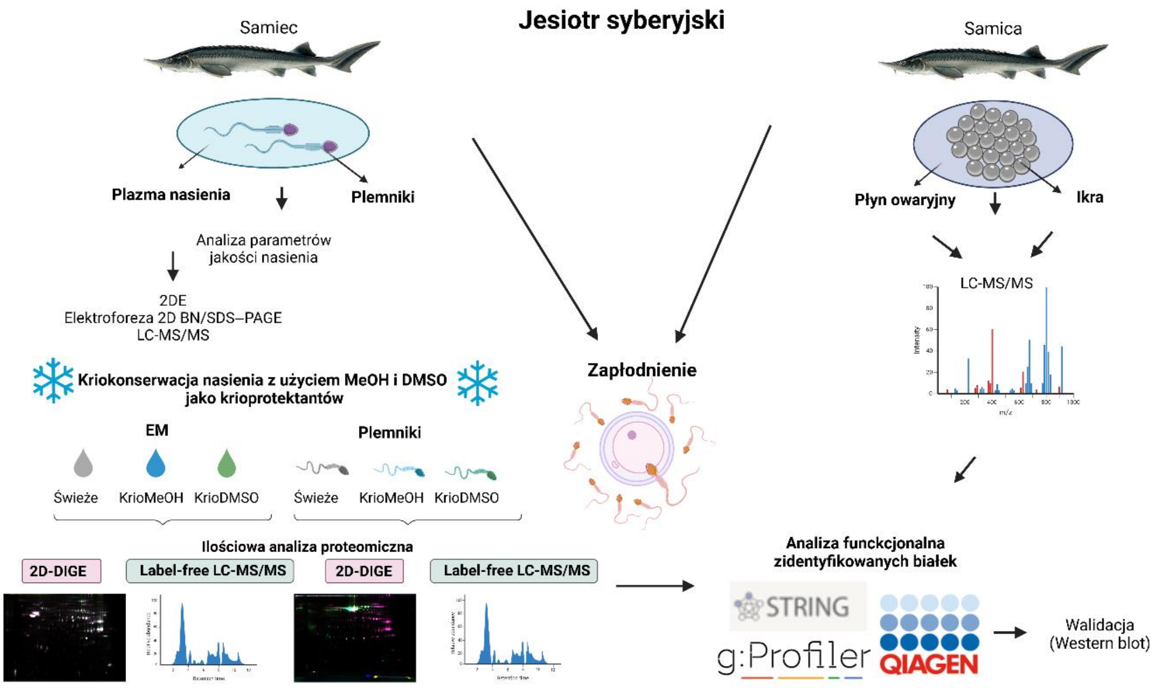
Doświadczenie 2 (Publikacja PII, Kodzik i in. 2024)

- Pobranie materiału i ocena jakości nasienia oraz płynu owaryjnego (komputerowa analiza nasienia w systemie CASA; pomiar pH, stężenia oraz osmolarności białka w płynie owaryjnym)
- Zapłodnienie
- Przygotowanie materiału oraz ekstrakcja białek
- Analiza LC-MS/MS
- Analiza funkcjonalna zidentyfikowanych białek: IPA, ShinyGo, String
- Walidacja wyników- analiza Western blot

Doświadczenie 3 (Publikacja PIII, Kodzik i in. 2024)

- Pobranie materiału i ocena jakości nasienia (CASA, cytometria przepływowa- koncentracja, żywotność i płynność błony komórkowej plemników, poziom reaktywnych form tlenu (ROS+), integralność akrosomu, osmolarność i stężenie białka plazmy nasienia)
- Przygotowanie materiału, kriokonserwacja nasienia
- Oznaczanie prób znacznikami fluorescencyjnymi
- Elektroforeza 2D-DIGE
- Barwienie i skanowanie żeli oraz analiza obrazów 2D

- Wycinanie, trawienie i identyfikacja spotów białkowych (spektrometria mas MALDI-TOF/TOF)
- Analiza LC-MS/MS
- Walidacja wyników- analiza Western blot
- Analiza statystyczna: GraphPad Prism
- Analiza funkcjonalna zidentyfikowanych białek: IPA, ShinyGo, String, g:Profiler



Rysunek 2. Graficzne przedstawienie doświadczeń przeprowadzonych w ramach pracy doktorskiej. 2DE- elektroforeza dwukierunkowa; 2D BN/SDS-PAGE- elektroforeza dwukierunkowa w warunkach denaturujących (analiza kompleksów białkowych); LC-MS/MS - wysokosprawna chromatografia cieczowa połączona ze spektrometrią mas; EM- płyn zewnątrzkomórkowy uzyskany w wyniku odwirowania nasienia świeżego oraz po kriokonserwacji z użyciem DMSO lub MeOH; KrioMeOH- nasienie kriokonserwowane z dodatkiem MeOH jako krioprotektanta; KrioDMSO- nasienie kriokonserwowane z dodatkiem DMSO jako krioprotektanta; 2D-DIGE- dwuwymiarowa fluorescencyjna elektroforeza różnicowa.

4. Wyniki

4.1. Charakterystyka profilu białkowego oraz kompleksów białkowych plazmy nasienia jesiotra syberyjskiego (publikacja PI)

W celu scharakteryzowania proteomu oraz poznania funkcji białek plazmy nasienia jesiotra syberyjskiego zastosowano dwie strategie identyfikacji białek. Pierwsza z nich była oparta na metodach żelowych (2DE oraz 2D BN/SDS–PAGE), natomiast druga z pominięciem żelu (LC-MS/MS) (PI, Fig. 1). Zastosowanie 2DE pozwoliło na detekcję 502 spotów białkowych, z których 339 zostało zidentyfikowanych (314 spotów białkowych wybarwionych za pomocą Coomasie Briliant Blue G250 oraz 25 spotów wybarwionych przy użyciu srebra) (PI, Fig. 2), natomiast analiza LC-MS/MS pozwoliła na identyfikację 657 białek. Łącznie obie metody pozwoliły na identyfikację 665 białek, co stanowi największy katalog białek plazmy nasienia ryb jesiotrowatych. Ponadto, wyróżniono dominujące białka w plazmie nasienia jesiotra syberyjskiego. Kryteria selekcji dominujących białek opierały się na stosunku intensywności widm białkowych do całkowitej ich ilości w próbie (analiza LC-MS/MS) oraz na intensywności zidentyfikowanych spotów białkowych danego białka w stosunku do łącznej intensywności wszystkich białek na żelu (%Intens > 1.0) (analiza 2DE). Dominujące białka w analizie LC-MS/MS to albumina (ALB), transferyna (TF), immunoglobulina (IGK), dipeptydaza Beta-Ala-His (CNDP1), dehydrogenaza aldehydu-3-fosfoglicerynowego (GAPDH), apolipoproteina A-I (APOA1), hemopeksyna (HPX), fosforylaza glikogenowa L (PYGL), białko z grupy przeciwdziałających zamarzaniu (afp4) i ovochymaza 2 (OVCH2). Dodatkowo, analiza 2DE wyłoniła jako dominujące białka mutazę fosfoglicerynianową (PGAM2), białko wiążące ryboflawinę (RfBP), lektynę (fel) i enolazę (ENO3). Na podstawie analizy 2DE wykazano, że ok. 60% zidentyfikowanych białek w plazmie nasienia jesiotra występowało w więcej niż w jednym spocie białkowym, co wskazuje na obecność proteoform. W szczególności ALB i TF, występowały odpowiednio w 86 i 48 proteoformach, różniących się wartością pI oraz masą cząsteczkową (PI, Tab. 1).

Nowatorskie zastosowanie elektroforezy 2D BN/SDS–PAGE, pozwoliło na identyfikację czterech kompleksów białkowych: kompleks nr 1 złożony był z TF i FEL, kompleks nr 2 z ALB i białka wiążącego retinol (RBP4), kompleks nr 3 z ALB i TF oraz kompleks nr 4 z APOA1, RfBP i mioglobiny (MB). Dodatkowo wykazano występowanie TF w formie dimeru, trimeru, tetrameru i pentameru oraz trzech różnych form immunoglobuliny (IGH o masie cząsteczkowej 55 kDa i 56 kDa oraz IGK- 26 kDa). Masa cząsteczkowa zidentyfikowanych IGH wskazuje również na występowanie immunoglobuliny w formach oligomerycznych (PI, Fig. 3, Tab. 2).

Analiza funkcjonalna przeprowadzona przy użyciu programów bioinformatycznych, tj. IPA oraz ShinyGo wykazała, że białka plazmy nasienia odgrywają kluczową rolę w odpowiedzi ostrej fazy, koagulacji, glikolizy i glukoneogenezy oraz w regulacji metabolizmu lipidów. Dodatkowo zidentyfikowane białka związane były z ruchem komórkowym, apoptozą, syntezą i degradacją białek oraz sygnalizacją i interakcją międzykomórkowej (PI, Fig. 5, 6, 7). Klasyfikacja na podstawie kryterium „procesy biologiczne" wykazała dominujący udział białek plazmy nasienia w odpowiedzi immunologicznej, odpowiedzi na stres oraz w procesach katabolicznych (PI, Fig. 8, Tab. 3). Ponadto, wykazano udział białek plazmy nasienia w procesach rozrodczych, w tym w spermatogenezie, zapłodnieniu, reakcji akrosomalnej, wiązaniu do osłonki jajowej oraz ruchliwości plemników (PI, Fig. 9, Tab. 4).

Przeprowadzono analizę porównawczą proteomu plazmy nasienia jesiotra syberyjskiego z człowiekiem (*Homo sapiens*) oraz trzema gatunkami ryb, tj. czawyczą (*Oncorhynchus tshawytscha*), sandaczem oraz pstrągiem tęczowym, która wykazała białka zarówno wspólne jak i specyficzne gatunkowo (PI, Fig. 4). Analiza funkcjonalna 98 białek unikalnych dla plazmy nasienia jesiotra syberyjskiego wykazała ich zaangażowanie w wiązanie pochodnych węglowodanów oraz aktywność katalityczną (głównie hydrolazy). Dodatkowo białka te były także zaangażowane w procesy immunologiczne i metabolizm. Zidentyfikowano również 82 białka wspólne dla jesiotra i człowieka, w tym ACR i enzymy akrosomalne, co potwierdza obecność akrosomu w plemnikach jesiotra syberyjskiego. Występowanie wspólnych białek z rybami doskonałokostnymi oraz ssakami sugeruje ich funkcjonalne podobieństwo pomiędzy wyższymi i niższymi kręgowcami, podczas gdy różnice w proteomie są najprawdopodobniej związane ze specyfiką rozrodu jesiotra. Zbiór zidentyfikowanych białek występujących w plazmie nasienia jesiotra syberyjskiego został zdeponowany w bazie ProteomeXchange o numerze identyfikacyjnym PXD035351. Uzyskane wyniki weszły w skład publikacji PI.

4.2. Porównanie proteomu ikry i płynu owaryjnego jesiotra syberyjskiego (publikacja PII)

W celu poznania proteomu ikry i płynu owaryjnego jesiotra syberyjskiego zastosowano metodę opartą na spektrometrii mas LC-MS/MS. Analiza ta umożliwiła identyfikację 565 białek w ikrze oraz 617 białek w OF. W ikrze wyróżniono główne białka takie jak witelogenina (VTG2), kinaza difosforanowa nukleozydów (NME2), hydrolaza ubikwityny (UCHL2), kofilina (CFL2) oraz cystatyna-B (CSTB), natomiast w OF dominowały ALB, APOA1, TF, hemoglobina (HBE1) oraz HPX (PII, Tab. 1).

Analiza porównawcza proteomu ikry i płynu owaryjnego wykazała, że 287 białek było specyficznych dla ikry, a 339 białek było unikalnych dla OF jesiotra syberyjskiego. Analiza funkcjonalna tych białek wskazała na różnorodne role biologiczne. Białka unikalne dla OF były związane z egzocytozą i odpowiedzią immunologiczną, w tym z aktywacją leukocytów i neutrofili, kaskadą dopełniacza i procesem krzepnięcia, a także regulacją cytoszkieletu aktynowego oraz metabolizmem cholesterolu. Natomiast białka unikalne dla ikry były zaangażowane w procesy metaboliczne, w tym w fosforylację oksydacyjną i metabolizm kwasów tłuszczowych, oraz w ubikwitynację i translacją białek (PII, Fig. 2, 3, 4, 6, Tab. 3). Ponadto, wykazano udział białek ikry i OF w procesach rozrodczych, w tym w zapłodnieniu, dojrzewaniu oocytów oraz zapobieganiu polispermii.

W celu walidacji uzyskanych wyników proteomicznych ikry i OF przeprowadzono analizę Western blot dla wybranych 4 białek, tj. ALB, VTG2, fibrynogenu (FIBB) i fibronektyny 1 (FN1). Analiza Western blot potwierdziła wyniki analiz LC-MS/MS, wykazując brak FGB i FN1 w ikrze oraz ich obecność w OF. Wykryto także silny sygnał ALB w OF lecz ze względu na niskie stężenie ALB w ikrze (140 razy niższe niż w OF), białko to nie zostało wykryte w analizie Western blot (tzn. poniżej granicy wykrywalności). Ponadto analiza Western blot wykazała sześciokrotnie wyższy poziom VTG2 w ikrze w porównaniu do OF (PII, Fig. 5).

Wśród zidentyfikowanych białek, na podstawie literatury wyłoniono białka, które mogą pełnić rolę potencjalnych biomarkerów umożliwiających ocenę jakości ikry, należą do nich: hydrolaza ubikwityny (UCHL1, UCHL3), białka rybosomalne (RPL36, RPL22), białka szoku cieplnego (HSP5, HSP9, HSP90AB1) oraz białka akrosomalne (SPACA4). Katalog białek występujących w ikrze i OF jesiotra syberyjskiego został zdeponowany w bazie ProteomeXchange o numerze identyfikacyjnym PXD044168. Uzyskane wyniki weszły w skład publikacji PII.

4.3. Analiza proteomiczna zmian w profilu białkowym nasienia jesiotra syberyjskiego po kriokonserwacji z użyciem MeOH i DMSO jako krioprotektantów (publikacja PIII)

W celu poznania zmian w składzie białkowym plemników jesiotra syberyjskiego zachodzących podczas procesu kriokonserwacji nasienia, przeprowadzono ilościową analizę porównawczą proteomu plemników i EM nasienia świeżego i kriokonserwowanego z użyciem

MeOH lub DMSO jako krioprotektantów. Wykorzystano dwie metody 2D-DIGE oraz LC-MS/MS bez znakowania prób (label-free LC-MS/MS) (PIII, Fig. 1). Połączenie tych metod umożliwiło identyfikację 224 białek różnicujących plemniki świeże i kriokonserwowane z użyciem MeOH (PIII, Fig. 7A) oraz 118 białek różnicujących plemniki świeże i kriokonserwowane z użyciem DMSO (PIII, Fig. 7B). W nasieniu kriokonserwowanym z użyciem MeOH, 113 białek plemnikowych miało podwyższony poziom, a 103 białka miały obniżony poziom w porównaniu do nasienia świeżego. Natomiast w nasieniu kriokonserwowanym z użyciem DMSO, 49 białek miało podwyższony poziom, a 62 białka miały obniżony poziom w porównaniu do nasienia świeżego. Dodatkowo, analiza 2D-DIGE wykazała 8 białek (MeOH) i 7 białek (DMSO), które wykazywały zarówno podwyższony jak i obniżony poziom, co może świadczyć o występowaniu modyfikacji posttranslacyjnych w czasie kriokonserwacji (PIII, Fig. 7C). Analiza proteomiczna EM nasienia świeżego oraz po kriokonserwacji pozwoliła na identyfikację 342 i 363 białek uwolnionych z plemników do EM po mrożeniu z MeOH i DMSO (PIII, Fig. 7F).

Uzyskane wyniki wskazują, iż w wyniku mrożenia nasienia z MeOH najbardziej zmienione białka w plemnikach to wimentyna (VIM), białko związane z rzęskami i witką (CFAP96), izomeraza peptydyloprolilowa (PPIL3), histony (H2AZ), białko regulowane przez fosforylację tyrozyny wiążące wapń (CABYR), fosforylaza glikogenu (PYGM), tubulina (TUBB4B), fosfoglukomutaza (PGM1). Natomiast w plemnikach kriokonserwowanych z DMSO największą zmienność wykazywały białka takie jak nukleoporyna (NUP85), H2AZ1, białko wiążące Acyl-CoA (DBI), CABYR, PYGM, enzym usuwający rozgałęzienia glikogenu (AGL). Na podstawie największego wzrostu poziomu białek w EM w wyniku kriokonserwacji z użyciem MeOH wyróżniono białko szoku cieplnego (HSPD1), syntazę semialdehydowo alfa-aminoadypinową (AASS), dehydrogenazę izocytrynianową [NADP] (IDH2), składnik kompleksu dehydrogenazy 2-oksoglutaranu E1 (OGDH), podjednostkę zeta białka kompleksu T1 (CCT6A), białko związane z aktyną T3 (ACTRT3), hydratazę fumaranową (FH), kinazę kreatynową typu U (CKMT1A). W nasieniu kriokonserwowanym z DMSO największy wzrost poziomu białek w EM wykazały izomeraza disiarczkowa białka A3 (PDIA3), syntaza argininobursztynianowa (ASS1), cykliczna fosfodiesteraza AMP (PDE7A), profilina (PFN3), kinaza pirogronianowa (PKM) (PIII, Fig. 6). Białka te mogą być potencjalnymi wskaźnikami uszkodzeń kriogenicznych plemników i/lub przydatności nasienia do kriokonserwacji.

Przeprowadzono analizę porównawczą białek uwolnionych z plemników (identyfikowanych jako białka o obniżonym poziomie w plemnikach i podwyższonym poziomie w EM) w wyniku kriokonserwacji z DMSO i MeOH. Analiza wykazała, że 340 białek zmieniało się zarówno w wyniku kriokonserwacji z DMSO, jak i MeOH, natomiast 36 białek zmieniało się specyficznie w wyniku kriokonserwacji nasienia z MeOH, a 39 białek w wyniku kriokonserwacji z DMSO. Funkcjonalna analiza przy użyciu narzędzi bioinformatycznych wykazała, że specyficzne białka uwolnione z plemników po kriokonserwacji z MeOH są głównie zaangażowane w metabolizm kwasów organicznych, w tym kwasów tłuszczowych, organizację cytoszkieletu, aktywność oksydoreduktaz oraz wiązanie jonów magnezowych. Te białka były zlokalizowane głównie w mitochondriach i mikrotubulach. Z kolei białka uwolnione z plemników kriokonserwowanych z użyciem DMSO były istotnie zaangażowane w procesy związane z transportem mRNA, zapłodnieniem, aktywnością kinaz oraz ARSA. Białka te były zlokalizowane w porach jądrowych, pęcherzykach wydzielniczych, lizosomach oraz kompleksie białkowym BBSome (*ang. Bardet-Biedl Syndrome Protein Complex*) (PIII, Fig. 10).

W celu potwierdzenia wyników proteomicznych przeprowadzono walidację za pomocą analizy Western blot dla wybranych białek: hydrogenazy mleczanowej A (LDHA), kinazy kreatynowej (CKB), ENO3, aldolazy fruktozo-bisfosforanowej A (ALDOA), H2A.Z, hydratazy fumaranowej (FH) oraz ACR. Badania wykazały spadek poziomu LDHA i H2A.Z w plemnikach po kriokonserwacji zarówno z użyciem MeOH, jak i DMSO. Z kolei obniżenie poziomu CKB zaobserwowano wyłącznie w grupie z MeOH. Dodatkowo, stwierdzono wzrost poziomów wszystkich badanych białek w EM po kriokonserwacji, niezależnie od użytego krioprotektanta (PIII, Fig. 12). W przypadku akrozyny, przeciwciała identyfikowały charakterystyczne pasma białkowe o masach 38 kDa i 24 kDa w plemnikach świeżych oraz kriokonserwowanych, bez zmiany ich intensywności po kriokonserwacji. Co ciekawe, w EM nasienia świeżego, przeciwciała wykrywały pojedyncze pasmo o masie 73 kDa, prawdopodobnie odpowiadające proakrozynie. Po kriokonserwacji, obok pasma 73 kDa, pojawiły się dodatkowe pasma o masach cząsteczkowych od 24 do 53 kDa (PIII, Fig. 13). Obserwowany spadek pasma o masie 73 kDa, a także wzrost intensywności pasm o niższej masie cząsteczkowej po mrożeniu z użyciem MeOH i DMSO, sugerują zaburzenia w systemie akrosyna-proakrozyna wynikające z kriokonserwacji.

W ramach kompleksowych badań nad wpływem kriokonserwacji na plemniki jesiotra syberyjskiego przeprowadzono analizy kluczowych parametrów jakości nasienia, tj. ruchliwość, żywotność, stres oksydacyjny, płynność błon komórkowych oraz integralność akrosomu. Nasze wyniki wskazują, że kriokonserwacja obniżała procent plemników ruchliwych, ich prędkość ruchu krzywoliniowego VCL oraz odsetek plemników o ruchu progresywnym. Nie stwierdzono istotnych różnic między zastosowaniem MeOH a DMSO w wymienionych parametrach (PIII, Fig. 2). Wykazano również spadek żywotności plemników po kriokonserwacji, przy czym żywotność była wyższa w przypadku prób kriokonserwowanych z DMSO w porównaniu do MeOH (PIII, Fig. 3B). Proces kriokonserwacji indukował również stres oksydacyjny niezależnie od rodzaju użytego krioprotektanta (PIII, Fig. 3C). Ponadto, obserwowano zmiany w płynności błon komórkowych (PIII, Fig. 3A) oraz uszkodzenia integralności akrosomu plemników, choć w tym parametrze nie zaobserwowano znaczących różnic między MeOH a DMSO (PIII, Fig. 4C).

Kolejnym krokiem było przeprowadzenie zapłodnienia z użyciem nasienia kriokonserwowanego z MeOH i DMSO. Ponieważ doświadczenie przeprowadzono w kolejnym roku, nasienie świeże użyto wyłącznie do oceny jakości ikry. Użycie nasienia kriokonserwowanego z DMSO spowodowało dramatyczny spadek odsetka wylęgniętych larw w porównaniu z nasieniem kriokonserwowanym z MeOH (PIII, Fig. 5). Nasze wyniki dostarczają istotnych informacji na temat specyficznych wpływów krioprotektantów na jakość oraz proteom kriokonserwowanych plemników, co ma kluczowe znaczenie dla poznania mechanizmu kriouszkodzeń oraz optymalizacji procedur kriokonserwacji w praktyce hodowlanej i ochronie gatunków. Uzyskane wyniki weszły w skład publikacji PIII.

5. Dyskusja

5.1. Charakterystyka proteomu plazmy nasienia jesiotra syberyjskiego

5.1.1. Główne funkcje białek plazmy nasienia jesiotra syberyjskiego

Jednym z zadań badawczych (zadanie badawcze 1) było scharakteryzowanie proteomu plazmy nasienia jesiotra syberyjskiego. Przedstawione wyniki zawarte w publikacji nr 1 reprezentują największy opisany proteom plazmy nasienia (665 białek) dla ryb jesiotrowatych. Białka dominujące w plazmie nasienia jesiotra syberyjskiego, tj. APOA1, TF, HPX, ALB i IGK są związane głównie z odpowiedzią immunologiczną oraz ochroną przed stresem oksydacyjnym (Dietrich i in. 2014, 2021; Nynca i in. 2014). Białka te, z wyjątkiem IGK, zostały wcześniej zidentyfikowane jako główne białka plazmy nasienia innych gatunków ryb, tj. sandacz, karp czy pstrąg tęczowy (Dietrich i in. 2014, 2021; Nynca i in., 2014). Podobnie jak w przypadku sandacza, AFP4 zostało sklasyfikowane jako główne białko plazmy nasienia jesiotra, sugerując istotną rolę AFP4 w ochronie przed uszkodzeniami wywołanymi niskimi temperaturami podczas zimowania u tych gatunków (Dietrich i in. 2021). Do głównych białek zakwalifikowano także GAPDH i PYGL, zaangażowane w metabolizm glukozy (Rahi i in. 2020), co wskazuje na istotną rolę glikolizy jako źródła energii dla plemników zarówno w stanie spoczynku jak i podczas ruchu. CNDP1 i OVCH2 również zostały zidentyfikowane jako główne białka. CNDP1 może wpływać na ruchliwość plemników (Tan i Han, 1995; Adami i in. 2020) oraz pełnić funkcje antyoksydacyjną (Aydın i in. 2018), natomiast OVCH2 jest związane z właściwościami antybakteryjnymi (Gao i Zhang, 2009), dojrzewaniem plemników i zapłodnieniem (Kiyozumi i in. 2020). Jednym z interesujących odkryć było zidentyfikowanie wśród dominujących białek w plazmie nasienia jesiotra białek wiążących ryboflawinę (RfBP) oraz RBP4, które nie zostały zidentyfikowane u innych gatunków ryb. RfBP uczestniczy w spermatogenezie, biogenezie akrosomu oraz interakcji plemnik-komórka jajowa (Bhat i in. 1995), natomiast RBP4 odgrywa kluczową rolę w transporcie składników przez barierę krew-jądro, co jest istotne dla rozwijających się plemników (Adiga i in. 1997). Białka te są kluczowe dla mechanizmów transportu oraz homeostazy witamin B2 i A, co jest niezbędne dla spermatogenezy oraz ochrony antyoksydacyjnej (Davis i Ong, 1992). Podsumowując, główne białka plazmy nasienia jesiotra syberyjskiego pełnią kluczowe rolę w ochronie przed stresem oksydacyjnym, odpowiedzi immunologicznej, metabolizmie, transporcie, spermatogenezie i dojrzewaniu plemników. Obecność tych białek w wysokich stężeniach w plazmie nasienia jesiotra syberyjskiego sugeruje ich istotny udział w ochronie plemników i tkanek męskiego układu rozrodczego jesiotrów. Natomiast różnice w składzie głównych białek plazmy nasienia ryb mogą odzwierciedlać specyfikę rozrodu jesiotrów.

W plazmie nasienia jesiotra zidentyfikowaliśmy również 70 białek związanych z procesami rozrodczymi, takimi jak spermatogeneza, zapłodnienie, wiązanie plemnika z osłonką przejrzystą, ruchliwość plemników, reakcja akrosomalna i rozwój gonad męskich, w tym składniki kaskady ubikwitynacji, systemu degradacji białek zależnego od ubikwityny, który jest zaangażowany na różnych etapach spermatogenezy (Mochida i in. 2002). Co ciekawe, wykazaliśmy obecność białek związanych z egzosomami, co może wskazywać na ich rolę w procesie spermatogenezy, dojrzewaniu i ruchliwości plemników, reakcji akrosomalnej, kapacytacji oraz interakcji plemnik-komórka jajowa u jesiotrów (Sullivan i in. 2005). Przyszłe badania powinny skoncentrować się na izolacji egzosomów z plazmy nasienia jesiotra oraz ich proteomicznej i funkcjonalnej charakterystyce. Obecnie, wiedza na temat fizjologii plemników i mechanizmów zapłodnienia u jesiotrów jest bardzo ograniczona. Dotyczy to m.in. mechanizmów aktywacji ruchliwości plemników oraz reakcji akrosomalnej i jej roli w zapłodnieniu. Nasze wyniki wskazują na obecność w plazmie nasienia białek potencjalnie zaangażowanych w te procesy, co jest istotne dla planowania przyszłych badań mających na celu poznania specyfiki rozrodu samców jesiotra.

5.1.2. Kompleksy białkowe w plazmie nasienia jesiotra syberyjskiego

Otrzymane wyniki umożliwiły po raz pierwszy szczegółowe scharakteryzowanie i opisanie kompleksów białkowych występujących w plazmie nasienia jesiotra. W naszych badaniach zwizualizowaliśmy cztery kompleksy, z których dwa zawierały ALB połączoną z RBP4 (kompleks nr 3) oraz APOA1 (kompleks nr 4). Według dostępnej wiedzy, ALB należy do białek transportowych, wiążąc różne ligandy i przenosząc je (Li i in. 2017b), oraz zaangażowana jest w wiązanie peptydów poprzez mostki disiarczkowe, szczególnie w pozycji cysteiny SH-34 (Laurell i Thulin, 1975). Ponadto, po raz pierwszy został zidentyfikowany kompleks składający się z TF połączonej z białkiem specyficznym dla ryb FEL (kompleks nr 1) oraz kompleks zawierający MB, APOA1 i RfBP (kompleks nr 2). Przyszłe badania powinny być ukierunkowane na szczegółową charakterystykę kompleksów białkowych, co pozwoli na zrozumienie interakcji białko-białko oraz pomoże wyjaśnić funkcje i regulacje białek plazmy nasienia w procesach rozrodczych jesiotra.

5.2. Charakterystyka proteomu płynu owaryjnego i ikry jesiotra syberyjskiego

5.2.1. Rola białek specyficznych dla płynu owaryjnego samic jesiotra syberyjskiego

W ramach niniejszej pracy doktorskiej przeprowadzono także szczegółową analizę składu białkowego OF i ikry, ich roli w procesach rozrodczych oraz porównania proteomu OF oraz ikry jesiotra syberyjskiego (zadanie badawcze nr 2). Nasze badania, opisane w publikacji nr 2, stanowią dotychczas największą bazę zidentyfikowanych białek w ikrze i OF jesiotrów. Wyselekcjonowano także białka specyficzne dla ikry i OF, co pozwoliło na dokładniejsze zrozumienie ich unikalnych funkcji. Nasze wyniki wykazały dominujący udział białek specyficznych dla OF jesiotrów w procesach odpornościowych, w tym w odpowiedzi typu humoralnego i komórkowego. Zidentyfikowano także trzy szlaki aktywacji układu dopełniacza: klasyczny, lektynowy oraz alternatywny, a także białka, które kontrolują jego aktywność. Zidentyfikowano również białka ostrej fazy, które biorą udział w naprawie tkanek i ochronie przed stresem oksydacyjnym. Białka związane z kaskadą krzepnięcia i stanem zapalnym mogą odgrywać także rolę w owulacji, traktowanej jako kontrolowany stan zapalny u ssaków i ryb (Espey, 1994; Lubzens i in. 2010). Nasze wyniki sugerują także, że białka OF jesiotra uczestniczą w aktywacji makrofagów oraz degradacji neutrofili, które u jesiotrów klasyfikowane są jako heterofile (Palić i in. 2011), co wskazuje, że są one istotnymi elementami wrodzonej odporności OF typu komórkowego. Ponadto, obecność wśród specyficznych białek OF, białek należących do macierzy zewnątrzkomórkowej (ECM; ang. *extracellular matrix*) oraz białek zaangażowanych w transport i metabolizm lipidów podkreśla ich istotną rolę w wielu procesach zachodzących w jajniku, w tym w utrzymaniu prawidłowej struktury, interakcji komórka-macierz oraz regulacji procesów związanych z rozwojem pęcherzyków, dojrzewaniem oocytów oraz uwalnianiu dojrzałych oocytów (Ambekar i in. 2013; Akhavan i in. 2020). Podsumowując, nasze badania podkreślają istotną rolę białek specyficznych dla OF jesiotra syberyjskiego w regulacji procesów immunologicznych, w utrzymaniu struktury i funkcji jajnika oraz owulacji. Złożony system immunologiczny obecny w OF zapewnia ochronę gamet przed patogenami oraz stresem, wspiera naprawę tkanek i może odgrywać kluczową rolę w owulacji oraz utrzymaniu homeostazy po stanach zapalnych.

5.2.2. Rola białek specyficznych dla ikry samic jesiotra syberyjskiego

Nasze wyniki wykazały, że białka specyficzne dla ikry związane są przede wszystkim z procesami translacji i transkrypcji, w szczególności czynniki inicjacji translacji (EIFs; ang. *eukaryotic initiation factors*) i białka rybosomalne, które są kluczowe dla syntezy matczynego

RNA i białek, kontrolując wczesne etapy rozwoju zarodka (Nynca i in. 2022; Yilmaz i in. 2017). Kluczowe szlaki sygnalizacyjne związane z białkami ikry obejmują sygnalizację EIF2 oraz regulację sygnalizacji EIF4 i p70S6K, które są istotne dla inicjacji translacji poprzez rozwijanie struktur drugorzędowych mRNA i wiązanie rybosomów. Nasze badania wykazały także udział białek ikry w szlakach fosforylacji oksydacyjnej (OxPhos) i β-oksydacji kwasów tłuszczowych kluczowych dla produkcji energii niezbędnej dla rozwoju zarodka (Fillmore i in. 2011). Metabolizm oksydacyjny, w tym OxPhos, jest ważnych wskaźnikiem jakości ikry, co było wcześniej obserwowane w badaniach ikry pstrąga tęczowego (Ma i in. 2019; Wendling i in. 2004). Podsumowując, nasze badania podkreślają istotną rolę białek specyficznych dla ikry jesiotra syberyjskiego w początkowym etapie rozwoju zarodka jak również w dojrzewaniu oocytów, metabolizmie i zachowaniu homeostazy u ryb jesiotrowatych.

5.3. Podobieństwo składu białkowego płynu owaryjnego i plazmy nasienia jesiotra syberyjskiego

Szczególną uwagę warto zwrócić na podobieństwo składu białkowego OF i plazmy nasienia jesiotra syberyjskiego. Obecność dominujących białek, tj. ALB, TF, HPX, APOA1 i FEL w obu płynach wskazuje na ich udział w odpowiedzi immunologicznej, procesach metabolicznych, detoksykacji komórkowej i ochronie przed stresem (Kodzik i in. 2023). Natomiast białka specyficzne dla OF odgrywają kluczową rolę w procesach immunologicznych, adhezji komórkowej i metabolizmie, co dodatkowo podkreśla funkcję ochronną i odżywczą OF w dojrzewaniu oocytów. Z drugiej strony, białka specyficzne dla plazmy nasienia, w tym białka związane z metabolizmem, proteolizą, adhezją komórkową i organizacją macierzy zewnątrzkomórkowej, pełnią istotna rolę w procesie spermatogenezy i dojrzewania plemników (Kodzik i in. 2023). Warto podkreślić, że analiza funkcjonalna wykazała, iż zarówno w plazmie nasienia, jak i w OF, obecne są białka kluczowe dla zapłodnienia, wskazując na możliwe interakcje między plemnikiem a komórką jajową. Obecność wspólnych białek sugeruje, że środowisko zewnętrzne, w którym znajdują się komórki jajowe i plemniki, odgrywa kluczową rolę w ich ochronie, szczególnie w kontekście utrzymania wysokiej żywotności i prawidłowego funkcjonowania gamet (Johnson i in. 2014; Nynca i in. 2015; 2022; Ciereszko i in. 2017). Wyniki naszych badań dostarczają cennych informacji na temat złożonych mechanizmów rozrodczych jesiotra syberyjskiego, wskazując na kluczowe role, jakie pełnią białka w OF i plazmie nasienia w regulacji procesu zapłodnienia.

5.4. Analiza proteomiczna zmian w profilu białkowym nasienia jesiotra syberyjskiego po kriokonserwacji z użyciem MeOH i DMSO jako krioprotektantów

W niniejszej pracy doktorskiej poruszono również kwestię kompleksowej analizy zmian proteomu nasienia jesiotra syberyjskiego w wyniku kriokonserwacji z użyciem MeOH i DMSO jako krioprotektantów (zadanie badawcze nr 3). Wyniki przedstawione w publikacji nr 3 stanowią najobszerniejszy dotychczas zbiór danych dotyczących białek plemników jesiotra syberyjskiego, które ulegają zmianom podczas kriokonserwacji. Dodatkowo, nasze wyniki wykazały, że standardowe parametry jakości nasienia w tym ruchliwość, żywotność czy integralność akrosomu, nie są wystarczające do określenia zdolności zapładniającej plemników po kriokonserwacji, co podkreśla istotną rolę czynników molekularnych, tj. skład białkowy, w tym procesie. Nasze wyniki po raz pierwszy wykazały zróżnicowany wpływ krioprotektantów MeOH i DMSO na proteom plemników oraz umożliwiły identyfikację białek, które zostały zmieniane specyficznie tylko w wyniku kriokonserwacji z użyciem DMSO lub MeOH, co może odgrywać kluczową rolę w zrozumieniu przyczyn znacznie obniżonej zdolności zapładniającej plemników w wyniku kriokonserwacji.

5.4.1. Białka zmieniające się specyficznie w plemnikach kriokonserwowanych z użyciem DMSO

Kriokonserwacja z użyciem DMSO w dużym stopniu wpłynęła na zmiany w poziomie ACR oraz białku wiążącym akrozynę (ACRBP), które reguluje aktywność akrozyny i odgrywają kluczową rolę w reakcji akrosomalnej (Ciereszko i in. 1996; Ciereszko i in. 2000). Wykazano również, że system akrozyna/proakrozyna, który został już wcześniej zidentyfikowany w plemnikach jesiotra syberyjskiego (Słowińska i in. 2015), ulega zaburzeniu w wyniku kriokonserwacji. Nasze wyniki wskazują również na zmiany w białkach kluczowych dla rozpoznania i wiązania plemnika do komórki jajowej, tj. białko wiążące osłonkę przejrzystą 2 (ZPBP2), ARSA, serynowa proteaza 55 (PRSS55) oraz sialidaza-1 (NEU1), która jest odpowiedzialna za desjalilację glikoprotein na powierzchni plemnika, umożliwiając prawidłową reakcję akrosomalną i wniknięcie plemnika do komórki jajowej (Sarosiek i in. 2004; Shang i in. 2018; Vallet-Buisan i in. 2023). Warto podkreślić, że PRSS55 zidentyfikowano we wcześniejszych badaniach jako białko markerowe plemników kluczowe dla zapłodnienia oocytów i wczesnego rozwoju zarodka u ssaków (Vallet-Buisan i in. 2023). Dodatkowo nasze badania wskazują, że kriokonserwacja z użyciem DMSO powodowała specyficzne zmiany nukleoporyn, tj. NUP54, NUP155 i NUP85, które wchodzą w skład kompleksów porów jądrowych (NPC; ang. *nuclear pore complexes*) i są kluczowe dla ich integralności i funkcji. Nukleoporyny odgrywają ważną rolę w spermatogenezie, w tym w upakowaniu chromatyny, biogenezie witki oraz wydłużaniu jądra (Major i in. 2011; Guglielmi i in. 2020; Arafah i in. 2021). Zakłócenia w ich funkcjonowaniu mogą wpływać na stabilność genomu oraz proces zapłodnienia. Ponadto, kriokonserwacja z DMSO powodowała zmiany w białkach budujących kompleks BBSome, w tym TTC8, BBS1 i BBS, które uczestniczą w transporcie wewnątrzrzęskowym, ruchu białek błonowych oraz aktywności mitochondrialnej. Zaburzenia tych białek prowadzą do defektów witki i obniżonej ruchliwości plemników (Jin i in. 2009; Guo i in. 2023). W badaniach wykryto także spadek poziomu dyneiny (DNAI2) i tioredoksyny (NME9), kluczowych dla ruchu witki i organizacji aksonemy, co sugeruje naruszenie integralności witki podczas kriokonserwacji (Koscinski i in. 2020; Walton i in. 2021). Wykazano także, że kriokonserwacja z użyciem DMSO wpływa na białka związane z centrosomem tj. IFT25 i KIFC3, które są niezbędne dla transportu wewnątrzkomórkowego i organizacji wrzeciona mitotycznego (Hu i in. 2015; Amargant i in. 2021). Zakłócenia w funkcji centrosomu mogą wpływać na organizację centrosomów zygoty po zapłodnieniu, co może prowadzić do zaburzeń w dalszych podziałach komórkowych zygoty i wczesnym rozwoju zarodka (Firat-Karalar i in. 2014; Aljiboury i in. 2023). Podsumowując, kriokonserwacja z DMSO prowadzi do specyficznych zmian w białkach zaangażowanych w procesy zapłodnienia, w tym reakcję akrosomalną, wiązanie plemnika do osłonki przejrzystej, organizację porów jądrowych oraz strukturę witki i centrosomu, co z kolei prowadzi do zaburzenia procesu zapłodnienia, stabilizacji genomu, naruszenia integralności witki, metabolizmu i wczesnego rozwoju zarodkowego.

5.4.2. Białka zmieniające się specyficznie w plemnikach kriokonserwowanych z użyciem MeOH

Kriokonserwacja nasienia z użyciem MeOH wpłynęła specyficznie na białka związane z funkcjonalnością mitochondriów, w tym te zaangażowane w produkcję energii, jak również w strukturę witki. Zmiany w kluczowych enzymach mitochondrialnych, tj. ALDH5A1, ECHDC1, GCDH, NUDT19, MCCC2 oraz HADH, które odgrywają rolę w katabolizmie kwasów karboksylowych i tłuszczowych, mogą prowadzić do zakłóceń w syntezie ATP oraz zwiększenia poziomów reaktywnych form tlenu (ROS) (Amaral i in. 2013). To z kolei może powodować zaburzenia w produkcji energii i nasilać stres oksydacyjny (Ribas-Maynou i in. 2020; Mańkowska i in. 2022). W naszych badaniach zaobserwowano również istotne zmiany w białkach zaangażowanych w metabolizm lipidów oraz odpowiedź na stres oksydacyjny, co może zwiększać podatność plemników na uszkodzenia podczas kriokonserwacji (Samanta i in.

2011). Zmiany w białkach związanych z aksonemą plemników, tj. CFAP20, CFAP96, MAPRE1 oraz TPM3, sugerują zaburzenia w integralności strukturalnej witki i mogą bezpośrednio wpływać na obniżenie ruchliwości plemników (Inaba i in. 2015). Podsumowując, kriokonserwacja z użyciem MeOH wpływa na funkcjonalność plemników, zwłaszcza poprzez obniżenie ich ruchliwości i potencjalne obniżenie zdolności zapładniającej w porównaniu do nasienia świeżego. Niemniej jednak, zmiany te wydają się być subletalne, ponieważ nie powodują one tak znaczącego spadku zdolności zapładniającej, jak w przypadku użycia DMSO.

6. Podsumowanie

W niniejszej pracy po raz pierwszy przeprowadzono szczegółową charakterystykę proteomiczną białek układu rozrodczego ryb jesiotrowatych. Niniejsze badania dostarczyły największą bazę danych proteomicznych dotyczących białek plazmy nasienia, ikry i płynu owaryjnego jesiotra i wykazały obecność kompleksów białkowych. Nasze badania wniosły istotny wkład w dziedzinę rozrodu jesiotrów, dostarczając po raz pierwszy kompleksowej charakterystyki proteomicznej plemników jesiotra syberyjskiego po kriokonserwacji z użyciem dwóch różnych krioprotektantów - DMSO i MeOH. Badania potwierdziły, że MeOH jest najskuteczniejszym krioprotektantem w mrożeniu nasienia jesiotra. Biorąc pod uwagę kluczową rolę kriokonserwacji w zachowaniu różnorodności genetycznej wśród zagrożonych gatunków, nasze wyniki są szczególnie ważne i stanowią podstawę do dalszych badań mających na celu doskonalenie tej techniki. Uzyskana wiedza jest całkowicie nowa i może być kluczowa dla lepszego zrozumienia, jak krioprotektanty wpływają na funkcjonalność plemników i sukces zapłodnienia u jesiotrów. Dalsze badania nad specyficznymi białkami, które zostały zidentyfikowane w badaniach mogą wskazywać na potencjalne białkowe markery jakości nasienia i ikry oraz przydatności nasienia do zamrażania.

7. Wnioski

Wyniki uzyskane w ramach niniejszej rozprawy doktorskiej zostały szczegółowo zawarte w oryginalnych pracach naukowych wchodzących w skład niniejszej rozprawy doktorskiej. Poniżej zaprezentowane zostały wnioski, które uzyskano w trakcie realizacji poszczególnych zadań badawczych.

Zadanie badawcze nr 1:

1. Główną funkcją białek plazmy nasienia jesiotra syberyjskiego jest ochrona plemników i tkanek męskiego układu rozrodczego przed atakiem drobnoustrojów oraz stresem.
2. Główne białka plazmy nasienia odgrywają również kluczową rolę w metabolizmie lipidów i produkcji energii niezbędnej dla zachowania żywotności, ruchliwości plemników oraz integralności błon.
3. Białka plazmy nasienia biorą udział w procesach rozrodczych, w tym w spermatogenezie, zapłodnieniu, reakcji akrosomalnej oraz ruchliwości plemników.
4. Obecność kompleksów białkowych w plazmie nasienia jesiotra syberyjskiego wskazuje na istotną rolę interakcji białko-białko w procesach związanych z funkcjonowaniem nasienia.

Zadanie badawcze nr 2:

1. Białka ikry jesiotra syberyjskiego są kluczowe dla procesów transkrypcji, translacji i metabolizmu energetycznego w celu utrzymania homeostazy i prawidłowego rozwoju zarodka.
2. Białka OF jesiotra syberyjskiego uczestniczą głównie w procesach odpornościowych, oraz metabolizmie cholesterolu oraz regulacji struktury cytoszkieletu aktynowego, co jest kluczowe dla ochrony oocytów i wspierania ich rozwoju.
3. Podobieństwo w składzie białkowym OF i plazmy nasienia jesiotra syberyjskiego wskazuje, że oba płyny pełnią podobne funkcje z zakresie odpowiedzi immunologicznej, procesów metabolicznych, detoksykacji komórkowej oraz ochrony przed stresem oksydacyjnym, co podkreśla ich kluczową rolę w ochronie i rozwoju gamet.

Zadanie badawcze nr 3:

1. Kriokonserwacja nasienia nie tylko negatywnie wpływa na ruchliwość, żywotność i integralność akrosomu plemników jesiotra, ale także prowadzi do istotnych zmian w proteomie.
2. Zastosowanie DMSO i MeOH jako krioprotektantów skutkuje odmiennymi zmianami w proteomie plemników, które wpływają na ich funkcjonalność i zdolność zapładniającą.
3. Standardowe parametry jakości nasienia takie jak ruchliwość, żywotność czy integralność akrosomu nie są wystarczające do określenia zdolności zapładniającej plemników po kriokonserwacji.
4. Zmiany w białkach kluczowych dla procesów zapłodnienia, organizacji porów jądrowych, struktury wici i centrosomu w wyniku kriokonserwacji z DMSO wpływają na obniżoną zdolność zapładniającą, destabilizację genomu oraz zaburzenia na etapie wczesnego rozwoju zarodkowego.

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9. Publikacje wraz z oświadczeniami autorów

Olsztyn, 15.09.2024

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Niniejszym oświadczam, że wymienione publikacje powstały w wyniku mojego poniżej określonego wkładu:

1. **Kodzik N**, Ciereszko A, Szczepkowski M, Karol H, Judycka S, Malinowska A, Świderska B, Dietrich MA. Comprehensive proteomic characterization and functional annotation of Siberian sturgeon seminal plasma proteins. *Aquaculture*. 2023;568:739326. https://doi.org/10.1016/j.aquaculture.2023.739326

Udział w pobieraniu materiału biologicznego, przygotowanie materiału do analiz, wykonanie elektroforezy 2D SDS/PAGE oraz 2D BN/SDS–PAGE, identyfikacja białek przy użyciu spektrometrii mas MALDI-TOF/TOF, wykonanie analiz bioinformatycznych, współudział w interpretacji wyników, wizualizacja uzyskanych wyników, przygotowanie manuskryptu oraz udział w czynnościach związanych z procesem publikacji

2. **Kodzik N,** Ciereszko A, Szczepkowska B, Malinowska A, Dietrich M.A. Comparative proteomic analysis of the ovarian fluid and eggs of Siberian sturgeon. *BMC Genomics, 2024;* 25, 451. https://doi.org/10.1186/s12864-024-10309-y

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Udział w pobieraniu materiału biologicznego, przygotowanie materiału do analiz, współudział w ekstrakcji białek z materiału biologicznego, cytometryczna analiza żywotności, koncentracji plemników oraz integralności akrosomu, wykonanie elektroforezy 2D-DIGE, identyfikacja białek przy użyciu spektrometrii mas MALDI-TOF/TOF, wykonanie analiz bioinformatycznych, współudział w analizie statystycznej, współudział w interpretacji wyników, wizualizacja uzyskanych wyników, przygotowanie manuskryptu oraz udział w czynnościach związanych z procesem publikacji

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Utrzymanie hodowli ryb, współudział w pobieraniu materiału biologicznego

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Pomiar osmolalności i koncentracji białka, pomoc techniczna w wykonaniu elektroforezy 2D SDS-PAGE oraz 2D BN/SDS–PAGE.

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Wykonanie analizy LC-MS/MS oraz przygotowanie części metodycznej manuskryptu dotyczącej wykonanej analizy.

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Wykonanie analizy LC-MS/MS oraz przygotowanie części metodycznej manuskryptu dotyczącej wykonanej analizy.

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Utrzymanie hodowli ryb, współudział w pobieraniu materiału biologicznego, przeprowadzenie zapłodnienia

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Opracowanie koncepcji badań, nadzór merytoryczny nad badaniami, współudział w pobieraniu materiału biologicznego i kriokonserwacji nasienia, wybór stosowanych metod, cytometryczna analiza płynności błon, współudział w analizie statystycznej, administracja projektu, współudział w interpretacji wyników i sformułowaniu wniosków, przygotowaniu manuskryptu, odpowiedzi na recenzje oraz czynnościach związanych z procesem publikacji

Mariola Dietrich

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Comprehensive proteomic characterization and functional annotation of Siberian sturgeon seminal plasma proteins

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ABSTRACT

Siberian sturgeon (*Acipenser baerii*) is considered the most valuable species in aquaculture. The uniqueness of sturgeon reproduction implicates the need for studying semen composition at the protein level. Seminal plasma supports and protects the viability, motility and fertilizing capacity of spermatozoa by creating the optimal environment for storage and might be a rich source of biomarkers of reproductive physiology and/or disorders in male fishes. To our knowledge, there are no reports on the in-depth characterization of sturgeon seminal plasma proteins. The present study is the first to describe the in-depth proteomic characterization of Siberian sturgeon seminal plasma using two complementary proteomic approaches: gel-based (2DE and 2D blue native (BN)/SDS–PAGE) and gel-free (LC–MS/MS). LC–MS/MS allowed the identification of 657 proteins and 339 protein spots were identified after 2DE (almost 60% were present in various proteoforms). Combining the two proteomic strategies, we identified 665 proteins with high confidence (false positive rate below 1%), producing the largest catalogue of proteins from sturgeon seminal plasma to date. For the first time, we demonstrated interactions between seminal plasma proteins; four multiprotein complexes (MC1-MC4) were identified after 2D BN/SDS–PAGE, including (MC1) serotransferrin-2 (TF) and fish-egg lectin (FEL); (MC2) serum albumin 2 (ALB) and retinol-binding protein 4 (RBP4); (MC3) ALB and TF; and (MC4) apolipoprotein A-I (APOA1), riboflavin-binding protein (RfBP) and myoglobin (MB). BN/SDS–PAGE also revealed that immunoglobulin and TF formed homo-multimeric (dimer, trimer, tetramer and pentamer) complexes in seminal plasma. ALB, TF, beta-Ala-His dipeptidase (CNDP1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IGH, APOA1, hemopexin (HPX), type-4 ice-structuring protein (AFP4), FEL, RfBP, ovochymase 2 (OVCH2) and glycogen phosphorylase, liver form-like (PYGL) were selected as major seminal plasma proteins. The functional analysis of the identified proteins indicated their involvement mainly in immune system processes and response to stimulus, metabolism, vesicle-mediated transport, proteolysis, and cadherin binding involved in cell–cell adhesion. Moreover, 69 proteins were associated with reproductive processes, including spermatogenesis, fertilization, acrosomal reaction and sperm motility. Comparative proteomic analysis of sturgeon seminal plasma showed common proteins between fish and humans and 172 proteins specific for sturgeons, which may reflect the specificity of sturgeon reproduction. To conclude, this integrative proteomic view offers a deeper insight into the physiological function of seminal plasma and processes occurring in the sturgeon reproductive tract. The availability of a catalogue of sturgeon seminal plasma proteins opens the way to future research into development of new biomarkers and possible application in the improvement of methods of controlled reproduction. The mass spectrometry proteomics data have been deposited into ProteomeXchange with the identifier PXD035351.

1. Introduction

Sturgeon ancestors existed >200 million years ago, and the extant species are called living fossils (Birstein et al., 1997). Sturgeons belong to the order *Acipenseridae*, family *Chondrostei,* and occupy a key evolutionary position between teleost and cartilaginous fishes. The

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population of sturgeons has dramatically decreased because of overfishing and water pollution (Dettlaff et al., 1993). As a result, most sturgeons are considered endangered species (27 species of sturgeon are on the Red list). Siberian sturgeon (*Acipenser baerii*) is considered the most valuable species in aquaculture, as they are a source of caviar and high-quality meat with the current production in aquaculture of about 442 tons in 2020 (FAO, 2022). Compared to other sturgeon species, Siberian sturgeon shows a rapid growth rate, resistance to pathogens, and a relatively short reproductive cycle (7–8 years). Regarding its commercial importance and special evolutionary status, it is worth studying its reproduction to improve restocking programs, which are of great interest for this species (Babaei et al., 2017).

The biology and physiology of sturgeon semen differ substantially from those of teleost fish by a more complex structure of spermatozoa, longer sperm motility duration, lower sperm concentration, sperm maturation process, atypical testicular morphology and characteristics of seminal plasma. Sturgeon spermatozoa, which have an acrosome, may represent a transitional stage before the simplification of sperm structure in teleost fishes, which leads to the loss of the acrosome (Pšenička and Ciereszko, 2018). Moreover, in sturgeon, sperm maturation occurs outside the testis via dilution of sperm by urine during passage of semen through the kidneys to the Wolffian ducts, while in teleosts, sperm maturation occurs due to factors in the environment of the spermatic duct, such as pH and bicarbonate ions (Dzyuba et al., 2014a). Correspondingly, seminal plasma and spermatozoa proteins in teleostean and chondrostean fishes have evolved adaptations in response to the reproductive environment (Li et al., 2009). The uniqueness of sturgeon semen implicates the need for studying semen composition at the protein level. In addition, seminal plasma might be a rich source of biomarkers of reproductive physiology and/or disorders in male fishes. To identify potential protein biomarkers in seminal plasma, construction of a comprehensive protein catalogue in this fluid is a necessary first step.

Sturgeon seminal plasma has a specific chemical and biochemical composition and plays an important role in supporting and protecting viability and creating an optimal environment to maintain spermatozoa in the quiescent state. It is characterized by low concentration of substantial mineral compounds (Na^+, K^+, Ca^{2+}, Mg^{2+}), low protein concentrations (not higher than 1 mg ml^{-1}) and low osmolality (50–80 mOsmol kg^{-1}). Interestingly, the composition of seminal plasma is very similar to that of urine (Dzyuba et al., 2014b). To date, knowledge about sturgeon seminal plasma proteins is limited to the characterization of a few enzymes, such as lactic dehydrogenase (LDH), arylsulfatase (ARS), acid phosphatase (ACP), β-*N*-acetylglucosamisase (BetaGlcNAc), and antioxidative enzymes (Sarosiek et al., 2004; Shaliutina et al., 2013; Alavi et al., 2014). In addition, gelatinases (serine proteases and metalloproteases), acrosin and serine protease inhibitors were studied using zymography and western blot in sturgeon seminal plasma (Słowińska et al., 2015). However, in most cases, such proteins have not yet been specifically identified.

To study proteins on a large scale, proteomic methods are necessary. Proteomic techniques have been widely used to identify seminal plasma proteins of mammalian species, for example, in humans (Pilch and Mann, 2006) and dolphins (Fuentes-Albero et al., 2021), and recently in several fish species, such as carp (Dietrich et al., 2014), rainbow trout (Nynca et al., 2014), Chinook salmon (Gombar et al., 2017) and pike-perch (Dietrich et al., 2021). It should be emphasized that the composition of seminal plasma proteins varies among teleost fish species, likely reflecting species-specific characteristics of reproduction in fish. To our knowledge, there are no reports on the in-depth characterization of sturgeon seminal plasma proteins, and this knowledge is limited to the spermatozoa proteome (Li et al., 2010, 2011; Li et al., 2017a, 2017b; Horokhovatskyi et al., 2018). Therefore, the proteomic characterization of Siberian sturgeon seminal plasma is important for better understanding the male reproductive processes in sturgeon and creating possibilities for improving artificial reproductive techniques, including short- and long-term semen storage and fertilization.

The aim of this study was to identify, describe, and classify for the first time the seminal plasma proteins of Siberian sturgeon and analyze their possible functions. Two complementary proteomic approaches: gel-based (2DE and 2D blue native (BN)/SDS–PAGE) and gel-free (LC–MS/MS) were applied to maximize the identification of proteins present in sturgeon seminal plasma. The presented protein catalogue provides new information on the composition of seminal plasma and its potential utility in diagnosis of reproductive disorders.

2. Materials and methods

2.1. Experimental design and workflow

For in-depth characterization of protein composition of sturgeon seminal plasma we applied three different proteomic strategies,

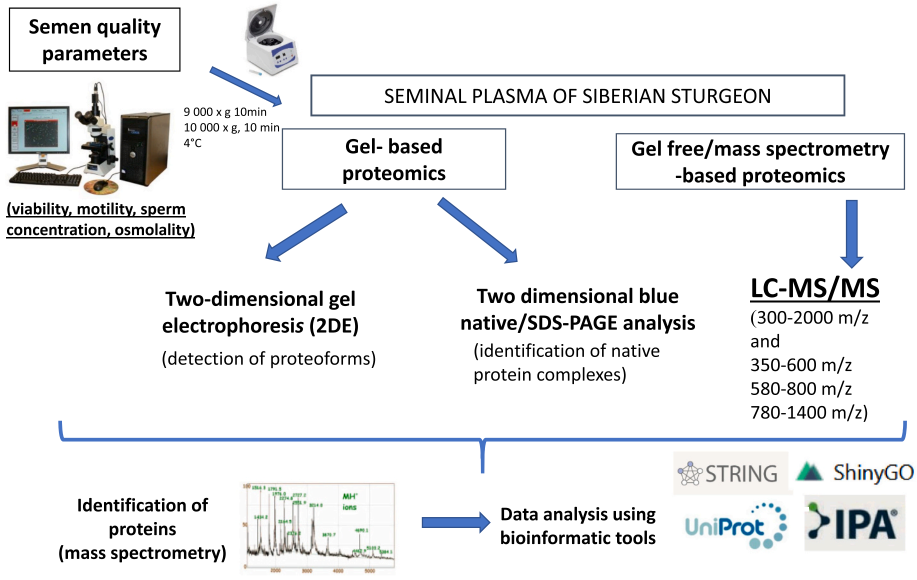

Fig. 1. Experimental design and workflow for the proteomic analysis of Siberian sturgeon seminal plasma using two complementary proteomic approaches: gel-based (2DE and 2D blue native (BN)/SDS–PAGE) and gel-free (LC–MS/MS) performed in wide ion mass range of 300–2000 m/z, and three narrow mass ranges (i.e., 350–600, 580–800, 780–1400 m/z). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including 1) two-dimensional gel electrophoresis (2D SDS–PAGE) combined with identification of proteins using matrix-assisted laser desorption/ionization time of flight/time of flight mass spectrometry (MALDI TOF/TOF), 2) liquid chromatography-based analysis coupled with MS/MS (LC-MS/MS) in wide ion mass range of 300–2000 m/z, and three narrow mass ranges (i.e., 350–600, 580–800, 780–1400 m/z) and 3) 2D Blue Native/SDS–PAGE (2D BN/SDS–PAGE) combined with MALDI-TOF/TOF to visualize and identify native protein complexes in seminal plasma. The experimental design and workflow are illustrated in Fig. 1.

2.2. Semen collection

Semen was collected from six mature Siberian sturgeons, weighing 9 ± 2 kg and aged 7–9 years, maintained at the Department of Sturgeon Fish Breeding Inland Fisheries Institute in Pieczarki, Poland. Lighting for 24 h/0 h provided photostimulation, and the water temperature in the tanks was 16 °C. Twenty-four hours before the collection of sturgeon semen, the males were stimulated hormonally with an injection of 0.03 mg kg^{-1} luteinizing hormone releasing hormone (LH-RH, Genscript, USA; Judycka et al., 2015). The milt was collected in the spawning season (May 2021) using a syringe with an attached rigid tube inserted into the urogenital opening, taking care not to pollute the semen with blood. After semen collection, samples were transported at 4 °C to Olsztyn, Poland.

2.3. Measurement of semen quality parameters

Sperm motility parameters were examined by computer-assisted sperm analysis with the CEROS II system (Hamilton-Thorne, USA) using 12-well multitest slide (well diameter 4mm, depth 30 μm; ICN Biomedicals Inc., Aurora, OH, USA). Semen was activated by dilution (1:20) with activation solution (10 mM Tris, 20 mM NaCl and 2 mM $CaCl_2$, 0.5% bovine serum albumin, pH 8.5; Jähnichen et al., 1999) at 8 °C. The value for each sample represents a mean from two separate measurements of motility parameters of 200 spermatozoa. The sperm motility parameters were as follows: straight-line velocity (VSL, μm s^{-1}), curvilinear velocity (VCL, μm s^{-1}), average path velocity (VAP, μm s^{-1}), linearity ($LIN = 100 \times VSL/VCL$, %), amplitude of lateral head displacement (ALH, μm) and percentage of motile sperm (MOT, %). The total volume of semen (ml) was measured directly after collection using a syringe (1 ml accuracy). The sperm concentration and viability were measured by flow cytometry using a Guava EasyCyte Flow Cytometer (Guava Technologies Inc., Luminex, Austin, TX, USA). Before analysis, the semen was diluted (1:100) with immobilizing solution (Park and Chapman, 2005) and measured according to the Guava ViaCount protocol (Luminex, USA). Sperm were rediluted with ViaCount Reagent to obtain a final concentration of 3×10^5 cells ml^{-1}

2.4. Measurement of seminal plasma osmolality and protein concentration

Seminal plasma was obtained by centrifugation of semen at 9000 ×g (4 °C, 10 min), and then the supernatant was centrifuged again at 10000 ×g (4 °C, 10 min). Osmolality was measured using a Minitübe Abfüll-u Labortechnik Löser apparatus (Tiefenbach, Germany). The protein concentration was measured by the Bradford method using a Coomassie Plus Kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as a standard.

2.5. Liquid chromatography-mass spectrometry

LC–MS analysis and sample preparation were performed at the Mass Spectrometry Laboratory at the Institute of Biochemistry and Biophysics PAS.

2.5.1. Sample preparation and measurement

One hundred micrograms of protein mixture from each seminal plasma sample was dried using a SpeedVac and resuspended in 100 μl of 100 mM ammonium bicarbonate buffer. Cysteine residues were reduced with 10 mM tris(2-carboxyethyl)phosphine through 1 h of incubation at 60 °C and blocked with 20 mM *s*-methylmethanethiosulfonate. Samples were digested overnight with trypsin (Promega, Madison, WI, USA) and resulting peptides were desalted with 10 mg Oasis HLB columns (Waters), then dried and resuspended in 0.1% FA. Peptide concentration was measured with Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific).

Samples were analyzed with an LC–MS system composed of Evosep One (Evosep Biosystems) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto disposable Evotips C18 trap columns (Evosep Biosystems) according to the manufacturer's protocol with minor modifications. For each sample, a total of four raw files were acquired in data-dependent mode using the following MS1 scan ranges: 350–600, 580–800, 780–1400 and 300–2000 m/z. Chromatography was performed at a flow rate of 250 nl min^{-1} using an 88 min (15 samples per day) gradient on an EV1106 analytical column (Dr Maisch C18 AQ, 1.9 μm beads, 150 μm ID, 15 cm long, Evosep Biosystems). MS1 resolution was set at 60000 with a normalized automatic gain control (AGC) target of 300% and auto maximum injection time. For MS2, the resolution was set at 15000 with a standard normalized AGC target, auto maximum injection time and the top 40 precursors within an isolation window of 1.6 m/z considered for MS/MS analysis. Dynamic exclusion was set at 20 s with an allowed mass tolerance of ±10 ppm and a precursor intensity threshold of 5e-3. Precursors were fragmented in HCD mode with a normalized collision energy of 30%.

2.5.2. Data analysis

Raw files were preprocessed with Mascot Distiller (version 2.7, Matrix Science, London, UK) software. The resulting peak lists were searched with Mascot Server against the *Acipenser* proteins derived from the NCBI database (created on 2021.07.26, 93,060 sequences; 56,935,290 residues). The following parameters were used: enzyme – Trypsin, fixed modification – Methylthio(C), variable modification – Oxidation(M), missed cleavages – 1, instrument – HCD. Fragment and parent mass recalibration was performed with in-house MScan software (http://proteom.ibb.waw.pl/mscan/), with average parent tolerances of 5 ppm and 0.01 Da for fragments. FDR was controlled using a target-decoy strategy and kept below 1%. Sector runs for each sample were merged and analyzed together with the full m/z range runs. Subset proteins and single-peptide proteins were excluded from the analysis. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifiers PXD035351 and https://doi.org/10.6019/PXD035351.

2.6. 2D SDS–PAGE

Before electrophoresis seminal plasma samples ($n = 6$) was concentrated using spin concentrators (cut off 3 kDa), and purified with a Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. After clean up seminal plasma proteins (500 μg) were resuspended in rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 2% IPG buffer 3-10NL, 50 mM dithiothreitol and bromophenol blue) to a final volume of 450 μl. 2D electrophoresis was performed as previously described by Horokhovatskyi et al. (2018). To visualize spots, gels were stained using Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA) and silver. The protein spot volume was calculated by Image Master 2D Platinum software by normalizing the volume of each spot from each gel against the total spot volume.

2.7. MALDI-TOF/TOF identification

Briefly, all protein spots were manually cut off from the gels and prepared for digestion and protein identification using MALDI-TOF/TOF. Spots were digested and identified using the protocol described earlier (Horokhovatskyi et al., 2018). MS and MS/MS spectra were searched using MascotServer (Matrix Sciences) in the *Acipenser* database, created on 04.12.2019 with the following MASCOT settings: cleavage enzyme, trypsin; max missed cleavages, 2; fragment ion mass tolerance, 0.7 Da; parent ion mass tolerance, 100 ppm; alkylation of cysteine by carbamidomethylation as a fixed modification; and oxidation of methionine as a variable modification. For the peptide mass fingerprinting (PMF) and MS/MS ion search, statistically significant ($p \leq 0.05$) matches by MASCOT were regarded as correct hits.

2.8. 2D BN/SDS–PAGE

2D BN/SDS–PAGE electrophoresis was performed as described earlier (Thangthaeng et al., 2011) with some modifications. All semen samples ($n = 3$) were stored at 4 °C before centrifugation at 1500 ×*g* (10 min, 4 °C). Each sample containing 40 μg seminal plasma protein was loaded on a gradient gel (4% stocking gel, 6–13% resolving gel and BN electrophoresis was performed at 4 °C using Bio-Rad Mini-PROTEAN III (Richmond, CA) as described earlier (Thangthaeng et al., 2011). After BN, gel strips were equilibrated for 10 min at RT in 62.5 mM Tris–HCl (pH 6.8), 2% SDS and 10% glycerol, then 5 min at 95 °C in the same buffer supplemented with 5% 2-mercaptoethanol. Finally, gel strips were placed onto SDS–PAGE gels (12.5%), and electrophoresis was performed at 200 V and 20 mA for 30 min and then 200 V and 40 mA for 1.5 h. The gels were stained with Serva blue G-250 (SERVA Electrophoresis GmbH, USA).

2.9. Bioinformatic analysis

Ingenuity Pathway Analysis (IPA) (Qiagen, CA, US) of the identified proteins was used to interpret the identified proteins in the context of molecular and cellular functions and canonical pathways. The GI numbers of the identified proteins were matched to the UniProtKB database (www.UniProt.org) to obtain Gene Ontology (GO) annotation using the categories “molecular function”, “biological processes” and “cellular component”. GO analysis was carried out using ShinyGo (v0.75; http://bioinformatics.sdstate.edu/go/). Protein–protein interaction network analysis of proteins associated with reproductive processes was performed using the Search Tool for the Retrieval of Interacting Genes (STRING) database (v. 11.5; http://string-db.org/) with a medium confidence score cutoff of 0.4. The search for interactions was restricted to *Homo sapiens* protein pairs. The reliability of the interactions between proteins was assessed by a combined score (edge score).

3. Results

3.1. Characteristics of sturgeon semen

Semen parameters for all six males are provided in Supplementary Table S1. Briefly, the mean semen volume was 35 ± 4.6 ml, the sperm concentration was $1.83 \pm 1.25 \times 10^9$ ml^{-1}, the sperm viability was 98.8 ± 0.3% and the sperm motility 89 ± 3% with the following sperm kinetic parameters VCL 157.8 ± 18.7 μm s^{-1}, VAP 141.6 ± 23.3 μm s^{-1}, VSL 130.1 ± 24.2 μm s^{-1}, LIN 78.4 ± 7.6%, and ALH 4.8 ± 0.6 μm. The seminal plasma characteristics were as follows: osmolality 83 ± 18 mOsm kg^{-1} and protein concentration 301.1 ± 113.5 μg ml^{-1}.

3.2. LC–MS/MS analysis of sturgeon seminal plasma proteins

From a total of 77,758 MS/MS spectra, 657 annotated proteins were identified from seminal plasma of Siberian sturgeon (FDR 1%, Supplementary Table S2). A total of 528 proteins were identified using a wide precursor range of 300–2000 m/z, and 129 proteins were additionally identified with narrow precursor ranges (i.e., 350–600, 580–800, 780–1400 m/z). A Venn diagram representing the overlap of proteins identified by LC–MS/MS using different m/z range strategies is presented in Supplementary Fig. S1. Based on the ratio of protein spectra to total spectra, ten of the most dominant seminal plasma proteins were selected (Supplementary Table S3), including serum albumin 2-like (ALB), transferrin (TF), immunoglobulin kappa light chain (IGK), beta-Ala-His dipeptidase-like isoform X1 (CNDP1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), apolipoprotein A-I (APOA1), hemopexin (HPX), glycogen phosphorylase, liver form-like (PYGL), type-4 ice-structuring protein LS-12 (AFP4), and ovochymase-2 (OVCH2).

3.3. Two-dimensional electrophoresis protein profile of sturgeon seminal plasma

A total of 502 protein spots were detected, of which 339 spots (314 spots were detected after Coomassie Brilliant Blue G250 staining and 25 spots were detected after silver staining) were successfully identified by MALDI-TOF/TOF (Fig. 2, Supplementary Table S4). In comparison to LC-MS approach, 2DE allowed the identification of an additional eight proteins (cathelicidin (CAMP), nucleoside diphosphate kinase A2 (NME7), nuclear transport factor 2 (NUTF2), 6-phosphogluconolactonase (PGLS), trefoil factor 2 (TFF2), transthyretin (TTR), ubiquitin-60S ribosomal protein L40 (UBA52), and parvalbumin 7 (PVALB). 48 proteins were identified in more than one protein spot, likely representing protein proteoforms with different isoelectric points and/or molecular weights; for example, ALB was identified in 87 spots, accounting for the highest percentage of total spot volume detected in gels, TF in 48 spots, CNDP1 in 11 spots, APOA1 in 10 spots and immunoglobulin mu subclass 2 heavy chain variable region, partial (IGH) in 9 spots. Spot quantification by the relative volume (%Vol > 1.0) showed that ALB, TF, IGH, APOA1, CNDP1, phosphoglycerate mutase 2 (PGAM2), riboflavin-binding protein (RfBP), fish-egg lectin (FEL), AFP4 and enolase B (ENO3) were the most ten dominant proteins in sturgeon seminal plasma (Table 1).

3.4. Two-dimensional blue native/SDS–PAGE analysis of sturgeon seminal plasma protein complexes

After 2D BN/SDS–PAGE, we visualized 13 protein bands, of which 9 bands represented homoprotein complexes (HC1-HC9) and 4 bands represented multiprotein complexes (MC1-MC4) (Fig. 3). Multiprotein complexes were composed of (MC1) TF and FEL; (MC2) ALB and retinol-binding protein 4 (RBP4); (MC3) ALB and TF and (MC4) APOA1, RfBP and myoglobin (MB) (Table 2). Additionally, TF with a molecular mass of 80 kDa in the second dimension (spots 4, 5, 7, 8, 18–22) corresponded to the expected molecular weight of a monomeric molecule. In the first dimension, TF had mobilities of 406, 380, 339, 325, 282, 246, 218 and 177 kDa. These bands corresponded to a TF pentamer, tetramer, trimer, and dimer, respectively. After the second dimension, 3 different forms of immunoglobulin were identified (spots 1–3): IGH (55 kDa, 56 kDa) and IGK (26 kDa). The molecular weight indicated that the immunoglobulins occurred in oligomeric forms. The results of all identified proteins of homoprotein complexes are reported in Supplementary Table S5.

3.5. Overlap of Siberian sturgeon seminal plasma proteome with Chinook salmon, pikeperch and human

The Venn diagram indicated 184 proteins present in seminal plasma of sturgeon and other fish species (pikeperch and Chinook salmon), of which 127 proteins were common to both fish and humans (Fig. 4). In addition, we identified 172 proteins characteristic of sturgeon seminal

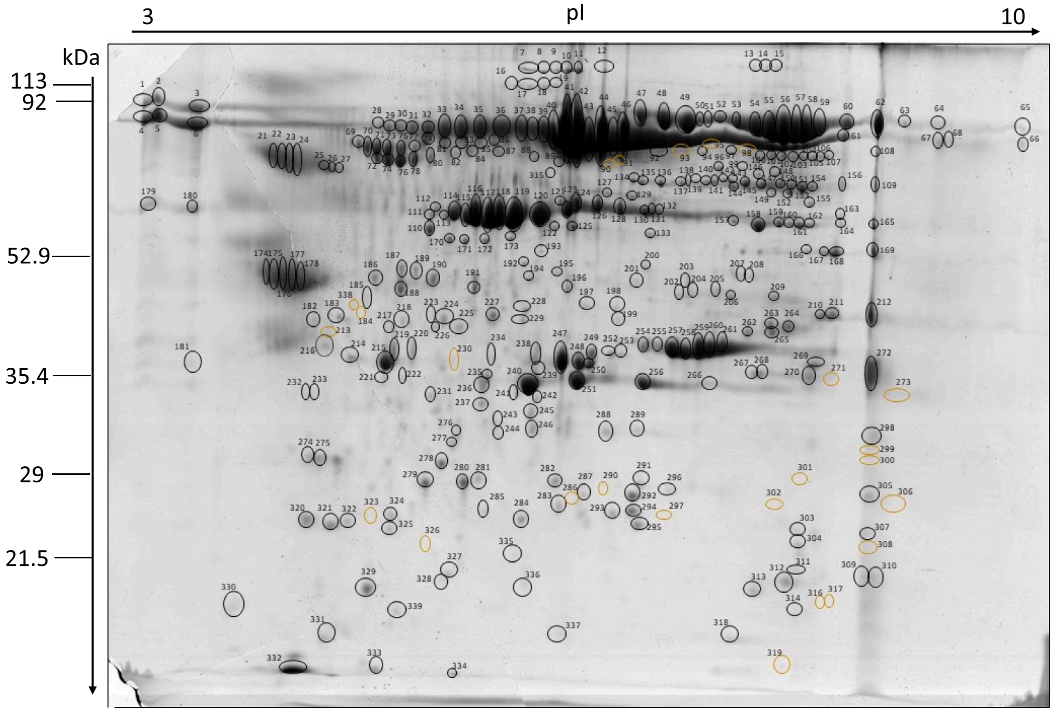

Fig. 2. Two-dimensional SDS–PAGE gel of Siberian sturgeon seminal plasma. The numbers correspond to spot numbers presented in Supplementary Table S2. A total of 315 proteins were detected after Coomassie Brilliant Blue G250 staining (marked in black), and 24 proteins were detected after silver staining (marked in yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

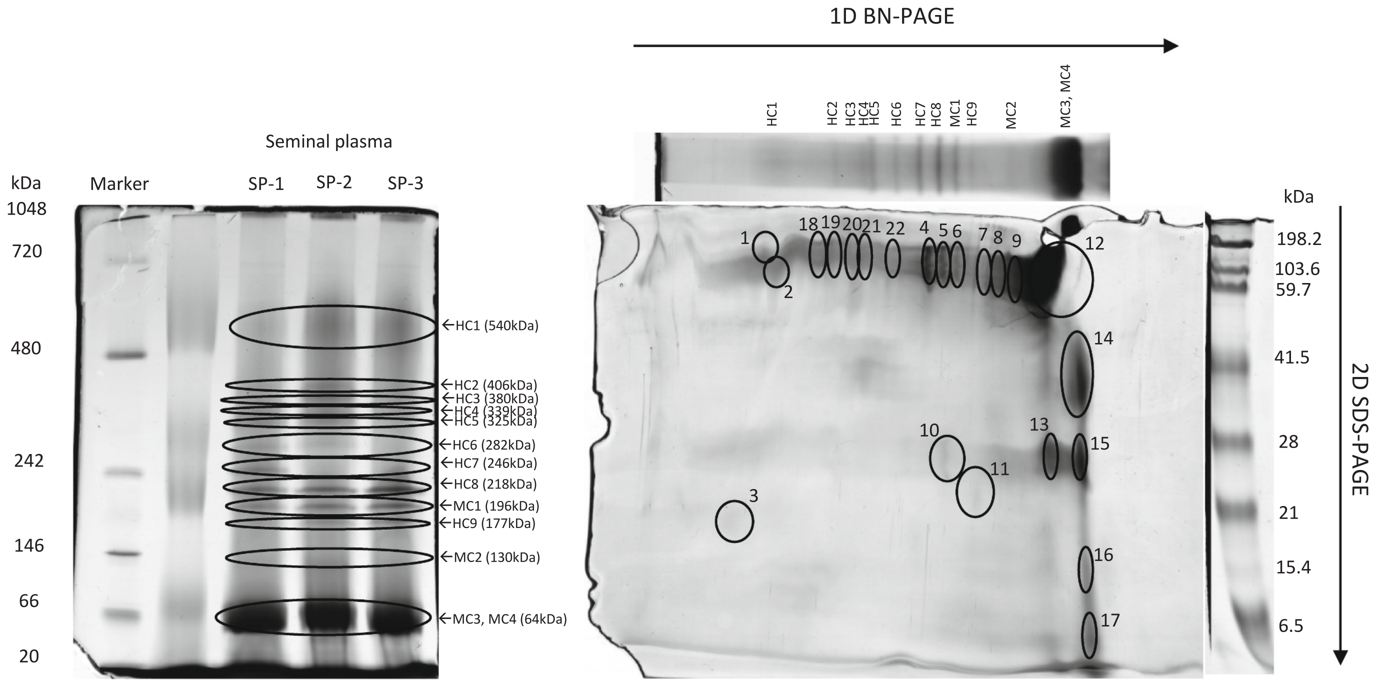

Fig. 3. Two-dimensional Blue Native (BN)/SDS–PAGE of sturgeon seminal plasma. Seminal plasma from three males (SP1–3) was separated by first dimensional gradient (3–12%) BN-PAGE (a) and further analyzed by second dimensional SDS–PAGE under denaturing conditions (b). Multiprotein complexes are numbered from 1 to 4 (MC1-MC4), and homoprotein complexes are numbered from 1 to 9 (HC1-HC9). Gels were stained with Serva Blue G. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Top ten most abundant proteins in sturgeon seminal plasma identified by MALDI TOF/TOF.

No.	Identified protein ID	Gene	Human Gene Homolog	Accession number	Spot number (Fig. 2)	Total volume spot %
1.	Serum albumin 2-like *(Acipenser ruthenus)*	*alb*	ALB	XP_033869101	4–12, 40–46, 61, 66–68, 111–121, 123, 124, 126–132, 134–141, 143, 145, 150, 156, 163, 170–173, 179, 180, 187, 189, 225, 226, 229, 231, 238, 245–250, 252–255, 257–261, 271, 274–275, 278, 280	43.13
2.	Transferrin a precursor, partial (*Acipenser gueldenstaedtii*)	*tf*	TF	QOI31301	1–3, 49–60, 62–65, 94, 97, 103–109, 142, 144, 147, 149, 151–154, 197–199, 208, 218, 224, 227–228, 284, 333	19.74
3.	Immunoglobulin mu subclass 2 heavy chain variable region partial *(Acipenser baerii)*	*igh*	IGH	AGO06423	28, 30–37	5.70
4.	Apolipoprotein A-I-like *(Acipenser ruthenus)*	*apoa1*	APOA1	XP_034773370	181, 232, 233, 235, 236, 240, 251, 256, 266, 273	4.58
5.	Cytosolic non-specific dipeptidase *(Acipenser ruthenus)*	*cndp1*	CNDP1	XP_033848222	69–78, 80	3.14
6.	Phosphoglycerate mutase 2 *(Acipenser ruthenus)*	*pgam2*	PGAM2	RXM92634	269, 272	1.70
7.	Fish-egg lectin-like isoform X2 *(Acipenser ruthenus)*	*fel*	Fish specific	XP_034766664	214, 215	1.42
8.	Riboflavin-binding protein-like *(Acipenser ruthenus)*	LOC117962300	RfBP	XP_034769630	174, 176–178, 281, 282	1.39
9	Type-4 ice-structuring protein LS-12-like *(Acipenser ruthenus)*	*afp4*	Fish specific	XP_034765998	332	1.28
10	Enolase B (*Acipenser baerii*)	*eno3*	ENO3	ABF60009	157–162, 164–165	1.21

Table 2
Proteomic identification of individual components of multiprotein complexes MC1-MC4 presented on Fig. 3.

No. complex	No. spot	Protein name	Accession no.	Calculated MW (Da)/pI
MC1 (196 kDa)	6	serotransferrin-2	XP_03389495	78,198/6.68
	10	fish-egg lectin-like isoform X2	XP_034766664	27,847/5.36
MC2 (130 kDa)	9	serum albumin 2-like	XP_033869101	71,229/5.94
	11	retinol-binding protein 4 isoform X2	XP_034782008	23,209/5.91
MC3 (64 kDa)	12	serum albumin 2-like	XP_033869101	71,229/5.94
	13	apolipoprotein A-I-like	XP_033852070	30,325/6.95
MC4 (62kDa)	14	riboflavin-binding protein-like	XP_034769630	28,961/5.32
	15	apolipoprotein A-I-like	XP_033852070	30,325/6.95
	16	myoglobin	XP_033910440	16,558/7.88
	17	myoglobin	XP_033910440	16,558/7.88

plasma; 74 of them represented different isoforms of proteins identified in other fish species, whereas 98 proteins have not yet been identified. Specific proteins of sturgeon seminal plasma were mainly involved in carbohydrate derivative binding (cyclic nucleotide-gated cation channel beta-1 (CNGB1), ficolin-1-B (FCN1), adiponectin (ADIPOQ), leucine-rich repeat serine/threonine-protein kinase 2 (LRRK2), GTP-binding protein SAR1a (SAR1A), rho-associated protein kinase 2 (ROCK2), anti-Mullerian hormone, partial (AMHR2), diacylglycerol kinase delta (DGKD), hydrolase activity pantetheinase (VNN1), DIS3-like exonuclease 2 (DIS3L2), intestinal-type alkaline phosphatase (ALPI), pancreatic triacylglycerol lipase (PNLIP), LRRK2, (MST1) and immune system process (FCN1, vascular cell adhesion protein 1 (VCAM1), ADIPOQ, tyrosinase (TYR), VNN1, proteoglycan 4 (PRG4), roquin-1 (RC3H1), MST1, deleted in malignant brain tumors 1 protein (DMBT1), LRRK2, MB, resistin (RETN) and interleukin-27 subunit beta (EBI3)). We also found 82 proteins of sturgeon seminal plasma common to humans, including hydrolytic enzymes, such as acrosin binding protein (ACRBP) and acrosin (ACR).

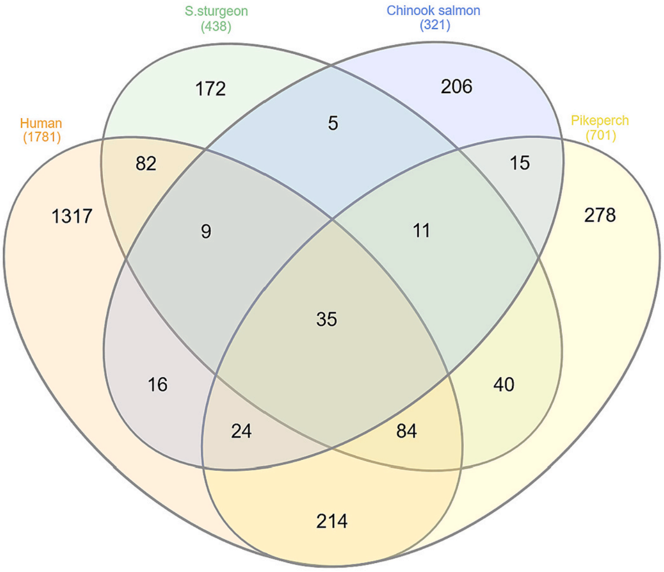

Fig. 4. Venn diagram representing the overlap of the identified seminal plasma proteins for the Siberian sturgeon (665 proteins), Chinook salmon (345 proteins), pikeperch (850 proteins) and human (1781 proteins). The numbers correspond to the unique genes due to the presence of multiple homologs of the same protein.

3.6. *Functional annotation of sturgeon seminal plasma proteins*

To obtain an overview of the biological associations of the sturgeon seminal plasma proteins we searched against NCBI database and mapped 649 out of the total 665 Siberian sturgeon seminal plasma proteins (657 proteins identified using LC–MS/MS and additional 8 proteins identified using 2D-SDS–PAGE) to 425 unique human homologs due to the presence of multiple homologs of the same human protein. Four

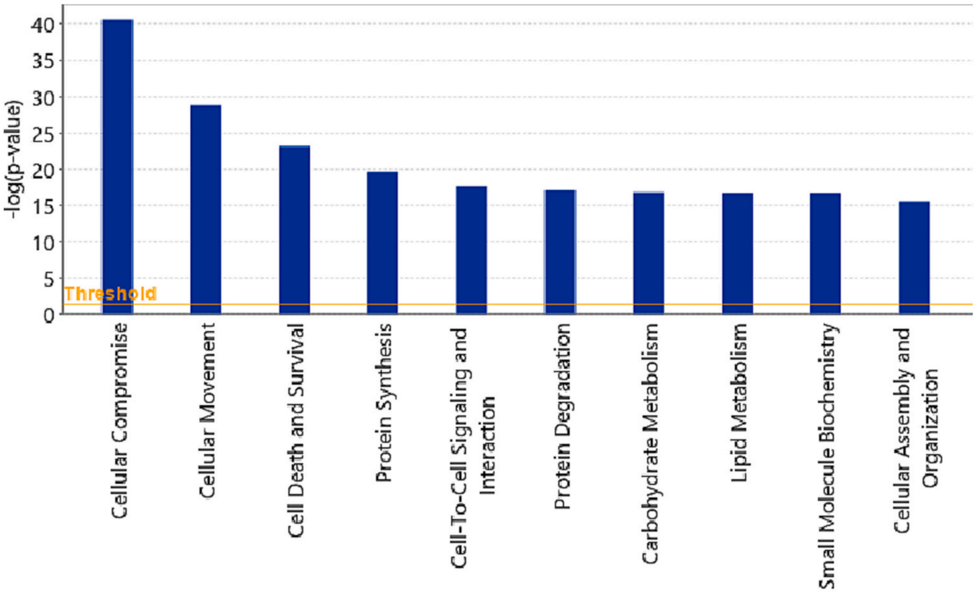

Fig. 5. The top ten molecular and cellular functions based on IPA analysis.

proteins were identified as uncharacterized/hypothetical with no similarity to mammals, and 6 proteins were specific to fish, including FEL (three isoforms), putative methyltransferase, gonadal soma-derived growth factor, aerolysin-like protein (two isoforms), glycine-rich cell wall structural protein 1.8 (three isoforms) and AFP4 (two isoforms). 425 proteins corresponding to human homologs were submitted to ShinyGo v0.741 (http://bioinformatics.sdstate.edu/go/) and Ingenuity Pathway Analysis (IPA; *Homo sapiens* was selected as the target organism).

On the basis of IPA, we categorized the identified seminal plasma proteins based on their canonical pathways and molecular and cellular functions. The top five molecular and cellular functions and canonical pathways are shown in Fig. 5 and Table 3. Fig. 6 depicts identified proteins mapped to the most significant enriched canonical pathways. The most connected proteins were associated with the following networks: Network 1- Energy production and nucleic acid metabolism (score 34), consisting of 22 seminal plasma proteins and Network 2- cellular compromise, free radical scavenging, Inflammatory response (score 29), consisting of 20 seminal plasma proteins (Fig. 7). Complete sets of all the IPA results are presented in Supplementary Table S6. GO analysis was carried out using ShinyGo and assigned to three groups of protein annotations: cellular component, biological process, molecular function and KEGG pathways. The top 10 significant terms for these groups are presented in Fig. 8. The top processes involved in the immune system were platelet degranulation, immune response, neutrophil-mediated immunity, myeloid leukocyte-mediated immunity, leukocyte-mediated immunity, immune effector process, neutrophil degranulation, neutrophil activation involved in immune response, and granulocyte and leukocyte degranulation (Supplementary Table S7).

3.7. Seminal plasma proteins associated with reproduction

ShinyGo analysis showed that 70 identified seminal plasma proteins were involved in the reproductive process. An additional search of seminal plasma proteins using the UniProt GO annotation database revealed their involvement in reproductive processes such as developmental processes involved in reproduction, reproductive structure development, binding of sperm to the zona pellucida, spermatogenesis, single fertilization and acrosome reaction (Table 4). Using STRING analysis, we found that 68 proteins associated with reproduction interacted with each other (142 edges, 63 nodes, clustering coefficient, 0.382; PPI enrichment *p* value, <1.0e-16, medium confidence 0.400). The majority of connected proteins were involved in the binding of sperm to the zona pellucida (red nodes, 2.56e-09), single fertilization (blue nodes, 4.45e-08), cell development (yellow nodes, 6.48e-06) and spermatogenesis (green nodes, 4.16e-05; Fig. 9).

4. Discussion

This work represents the first in-depth proteomic characterization of sturgeon seminal plasma protein and, to the best of our knowledge, the largest seminal plasma proteome (665 proteins) reported for sturgeons (Actinopterygii). A major drawback to the identification of Siberian sturgeon seminal plasma proteins is a lack of a complete genome of this species. The number of protein sequences in NCBInr *Acipenser baerii* database is still extremely limited (566 sequences) compared to *Homo sapiens* database (1,744,524 sequences), and therefore *Acipenseridae* database (94,359 sequences) was created. Our studies significantly extended previous findings, which were restricted to the identification of a limited number of proteins in sturgeon seminal plasma (Ciereszko et al., 1996; Piros et al., 2002; Shaliutina et al., 2013; Słowińska et al., 2015; Xin et al., 2019). To our knowledge, the proteomic dataset obtained in the present work for sturgeon seminal plasma is the second largest (after pikeperch) available seminal plasma proteome reported for fish species. The overlap of sturgeon seminal plasma proteins with teleost fish and mammals reflects the functional similarity between higher and lower vertebrates, while differences in protein composition may be related to the specific mode of fish reproduction.

4.1. Major proteins in the seminal plasma of sturgeon and their function

The abundant proteins of sturgeon seminal plasma identified in our study are mainly related to immune and stress responses, such as APOA1, TF, HPX, ALB and IGK. These proteins, except IGK, were previously identified as major proteins in other fish species, such as pikeperch, carp and rainbow trout (Dietrich et al., 2014, 2021; Nynca et al., 2014). Similar to pikeperch, AFP4 was classified as a major seminal protein of sturgeon, suggesting an important role of AFP4 in protection against cold-induced damage at extremely low temperatures during the wintering period in these species (Dietrich et al., 2021). The substantial overlap in the composition of major proteins between sturgeon and teleost fish suggests that the general protective mechanisms against

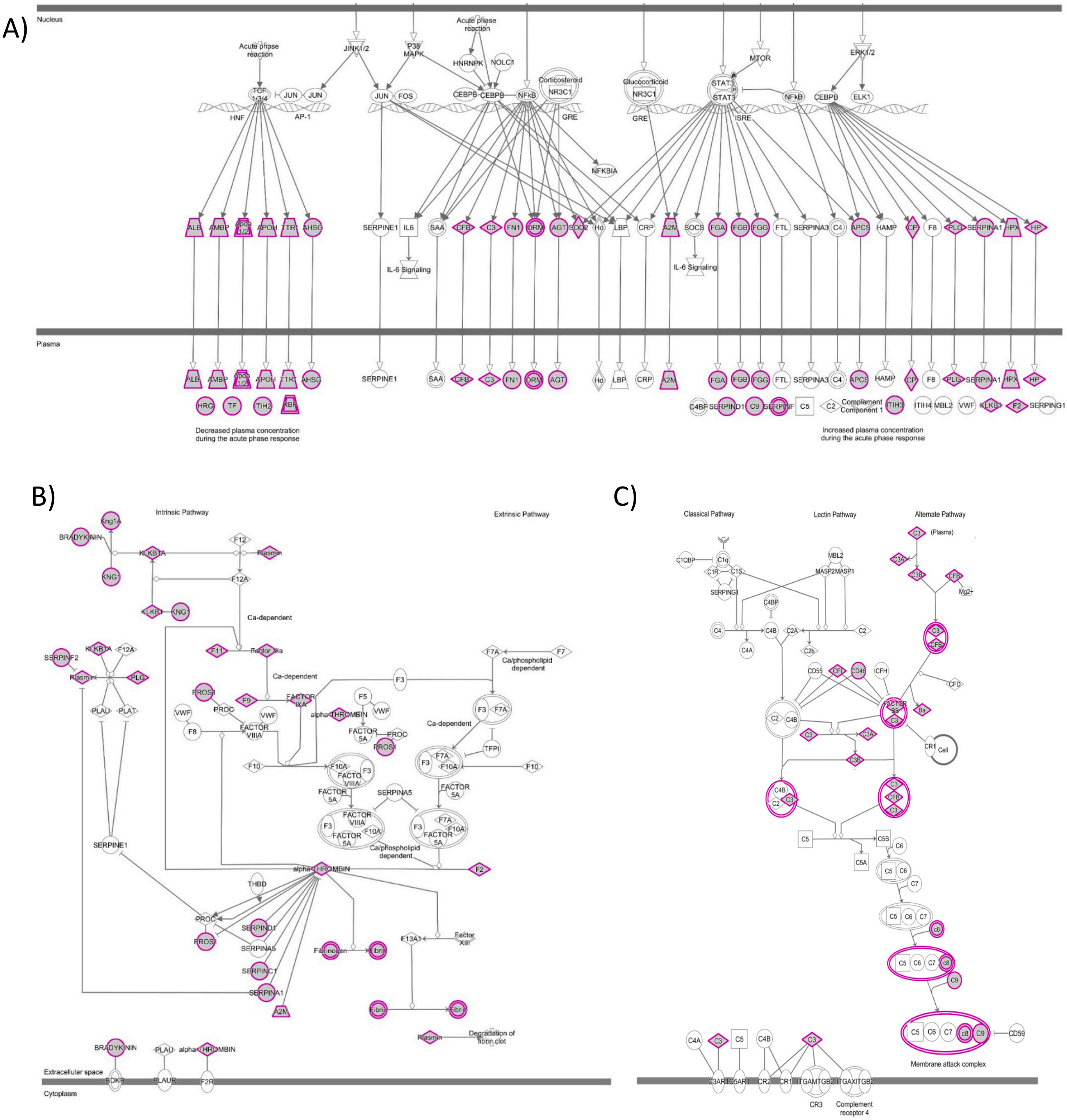

Fig. 6. The top canonical pathways significantly enriched in sturgeon seminal plasma indicated by IPA analysis. Acute phase response signaling (A), coagulation (B) and complement (C) system overlap with identified proteins. White-proteins were not identified in our proteomics study, but are incorporated as part of the network.

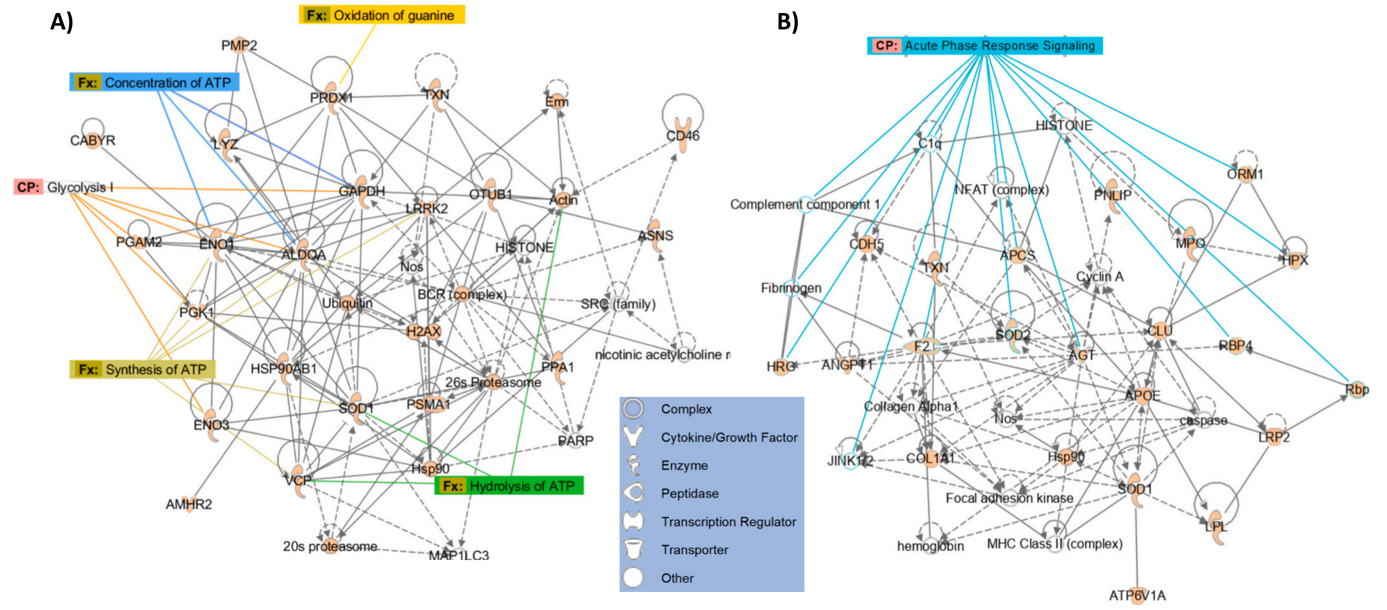

Fig. 7. The top scoring IPA networks A) energy production and nucleic acid metabolism, B) cellular compromise, free radical scavenging, inflammatory response. Nodes represent proteins and lines show the relationship (solid and dotted lines represent direct and indirect association, respectively). Nodes shapes indicate protein's primary function. Non-colour nodes are added by the IPA through relationships with other molecules. The legend explaining node shape is given in the upper right panel.

pathogens, oxidative and cold stress are well conserved in the seminal plasma of fish.

Among abundant proteins, we identified GAPDH, PYGL, CNDP1 and OVCH2, which were previously found, but to a lesser extent, in the seminal plasma of other fish species. The abundance of GAPDH and PYGL involved in glucose metabolism confirms the importance of glycolysis for energy supply for sperm in the quiescent state and during long-lasting motility (Rahi et al., 2020). CNDP1 is the extracellular secreted carnosinase degrading histidine-containing dipeptides such as carnosine (β-alanyl-l-histidine), anserine (β-alanyl-3-methyl-l-histidine) and homocarnosine (γ-aminobutyryl-l-histidine) (Peters et al., 2018). Carnosine seems to have beneficial effects on the male reproductive system, including sperm movement (Tan and Han, 1995), prevention of oxidative stress (Aydın et al., 2018), and improvement of sperm mitochondrial activity (Adami et al., 2020). Our results indicated the presence of the carnosine-carnosinase system and suggest its importance in the male reproductive tract of sturgeon; however, further studies are needed to unravel the effect of carnosine metabolism on sturgeon spermatozoa. OVCH2 is a secreted chymotrypsin-like serine protease associated with antibacterial properties (Gao and Zhang, 2009), sperm maturation and fertilization (Kiyozumi et al., 2020). It is possible that OVCH2 in sturgeon seminal plasma can be involved in sperm maturation and the immune response against pathogens. The presence of high concentrations of ovochymase in sturgeon seminal plasma suggests its importance for protection of the male reproductive system in chondrostei. The differences in the composition of major proteins may reflect the specificity of sturgeon reproduction.

Interestingly, riboflavin binding protein present in abundance in sturgeon seminal plasma has not been detected in seminal plasma of other fish species. We also found other vitamin-binding protein, RBP4, albeit to a much lower extent. RfBP plays important roles in sperm development, acrosome biogenesis and sperm–egg interactions (Bhat et al., 1995). RBP4 is crucial for developing sperm (Davis and Ong, 1992) and is involved in the transportation of micronutrients across the blood-testis barrier (Adiga et al., 1997). This protein was significantly involved in LXR/RXR activation, predicted by IPA as a top-ranked pathway in sturgeon seminal plasma. In summary, our results suggest the involvement of RfBP and RBP4 in the transport mechanism and homeostasis of vitamins (B2 and A) in sturgeon seminal plasma, which are crucial factors in spermatogenesis, germ cell development, and antioxidative protection.

4.2. *Proteins involved in immune responses*

In sturgeon, we found domination of immune-related proteins in seminal plasma and acute phase response signaling was selected as significant canonical pathway. The acute phase response is the first rapid mechanism of protection against microbes and pathogens, repair of tissue damage and inactivation of proteases in fish (Bayne and Gerwick, 2001). As mentioned above, this function was exerted by major proteins as well as several other minor proteins associated with the complement system and the coagulation process. Moreover, we identified 14 specific proteins in sturgeon seminal plasma involved in immune processes that have not yet been detected in seminal plasma of other fish species such as MST1, leucocyte activation (vascular cell adhesion protein 1 (VCAM1) and roquin-1 (RC3H)), humoral immune response (DMBT1, NCAM1, EBI3, FCN1), innate immune response (TYR, RETN), anti-inflammatory activities (ADIPOQ, LRRK2, PRG4, VNN1). These proteins may reflect the specificity of the immune response in sturgeon. In conclusion, our results strongly suggest that sturgeon seminal plasma proteins are important for the defense of semen against pathogens and additionally allow for a broader understanding of the roles of proteins involved in immune response mechanisms.

4.3. *Exosome-related proteins*

One of the most enriched functions of sturgeon seminal plasma proteins was vesicle-mediated transport and exocytosis, with a large number of proteins (250 proteins) linked to exocytosis (Caponnetto et al., 2017). In addition to extracellular proteins, a substantial number of proteins identified in seminal plasma are cytoplasmic, associated with seminal exosomes present in mammalian and fish seminal plasma,

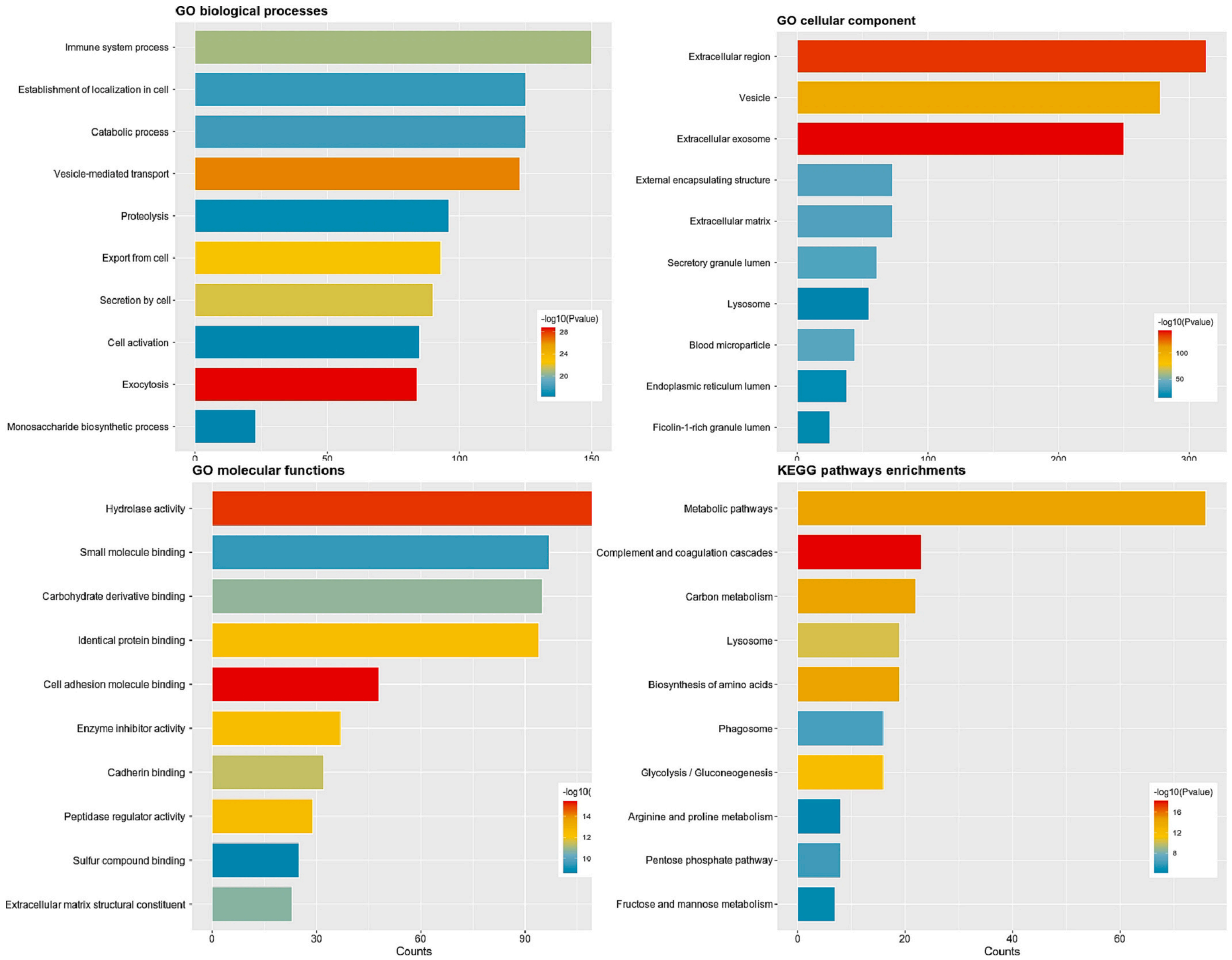

Fig. 8. Gene Ontology (GO) analysis of biological process, molecular function, cellular component and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification of sturgeon seminal plasma proteins.

Table 3
IPA analysis overview of sturgeon seminal plasma proteins.

Top canonical pathways	P value	No of molecules	Molecules
Acute Phase Response Signaling	6.31E-22	31	A2M, AGT, AHSG, ALB, AMBP, APCS, APOA1, APOH, C3, C9, CFB, CP, F1, F2, FGA, FGB, FGG, FN1, HP, HPX, HRG, ITIH2, ITIH3, KLKB1, PLG, RBP4, SERPINA1, SERPIND1, SERPINF1, SERPINF2, SOD2, TF
Complement and Coagulation System	5.01E-18	15	A2M, F11, C3, C4, C8A, C8G, C9, F2, F9, CLU, F11, FGA, FGB, FGG, KLKB1, KNG1, PLG, PROS1, SERPINA1, SERPINC1, SERPIND1, SERPINF2
LXR/RXR Activation	1.54E-17	23	AGT, AHSG, ALB, AMBP, APOA1, APOC1, APOC2, APOH, C3, C9, CLU, FGA, GC, HPX, KNG1, LPL, LYZ, MMP9, RBP4, SERPINA1, SERPINF1, SERPINF2, TF
FXR/RXR Activation	1.99E-17	23	AGT, AHSG, ALB, AMBP, APOA1, APOC1, APOC2, APOH, C3, C9, CLU, FBP1, FETUB, FGA, GC, HPX, KNG1, LPL, RBP4, SERPINA1, SERPINF1, SERPINF2, TF
Glycolysis	8.64E-14	13	ALDOA, ALDOB, ENO1, ENO3, FBP1, GAPDH, GPI, PFKP, PGAM1, PGAM2, PGK1, PKM, TPI1

Table 4

List of all identified proteins involved in male reproductive processes based on ShinyGO and UniProt database analysis.

GO category	Genes	No of molecules
Reproductive process:	ACR, ACRBP, ACTL7A, ACTL9, AGT, ALDOA, AMBP, AMHR2, ANTXR1, ARSA, BBS2, C3, CABYR, CADM1, CCT2, CCT3, CCT8, CD46, CLU, FETUB, H2AX, HEL-S-100n, HEL-S-44, HEL-S-87p, HEL-S-62p, HSP90AB1, HTRA1, IDH1, IGFBP7, KRT8, LRP2, LRRK2, MMP9, MST1, MYH9, NAMPT, OVCH2, PARK7, PBEF1, PGAM2, PRKAR1A, PRKAR2A, PSAP, PPP2R1A, RAN, RBP4, RETN, ROPN1L, SPACA9, SELENOP, SERPINF1, SLC9C1, SOD1, SOD2, SPINK2, SPINT2, STK4, ZAN, ZPBP2, UCHL1, UCHL3, UTAB1, UBA1, SKP1, KLKB1, KNG1, TUBA1A, ENO1, GSTM3, RfBP	70
Spermatogenesis	SLC9C1, ROPN1L, ZPBP2, CABYR, H2AX, CADM1, BBS1, LRRK2, SPINK2, PGAM2, SOD1, SOD2, HEL-S-44, CABYR, MST1, SELENOP, KRT8, MMP9, NAMPT, RBP4, RETN, BBS2, RAN, ACTL7A, ACTL9, UCHL1, UCHL3, UTAB1, UBA1, SKP1, RfBP, STK4, PPP2R1A, SERPINF1, ZAN	34
Single fertilization	ACRBP, CCT3, CCT2, CCT8, ZAN, ZPBP2, ALDOA, ACR, FETUB, PARK7, CD46, MYH9, PRKAR2A, ARSA, ZPBP2, HEL-S-100n, SELENOP, HEL-S-87p, OVCH2, ACTL7A, ACTL9, GSTM3	21
Binding of sperm to zona pellucida	ACRBP, CCT3, CCT2, CCT8, ZAN, ZPBP2, ALDOA, ACR, FETUB, PRKAR2A, PRKAR1A, ARSA, HEL-S-100n, SERPINF1, HEL-S-62p, IDH1	15
Spermatid development	ROPN1L, ZPBP2, CABYR, BBS2, SPINK2, RAN, ACTL7A, ACTL9	8
Flagellated sperm motility	SLC9C1, ROPN1L, BBS2, MST1, AGT, ANTXR1, HSP90AB1, IGFBP7, BBS2, KLKB1, KNG1, PGAM2, TUBA1A, CABYR, AMBP, CLU, HTRA1, PBEF1	18
Sperm axoneme assembly	BBS2	1
Acrosome reaction	ACR, ACRBP, SPACA9, CD46, ROPN1L, CABYR, ENO1	4
Male gonad development	LRP2	1
Male sex differentiation	AMHR2	1

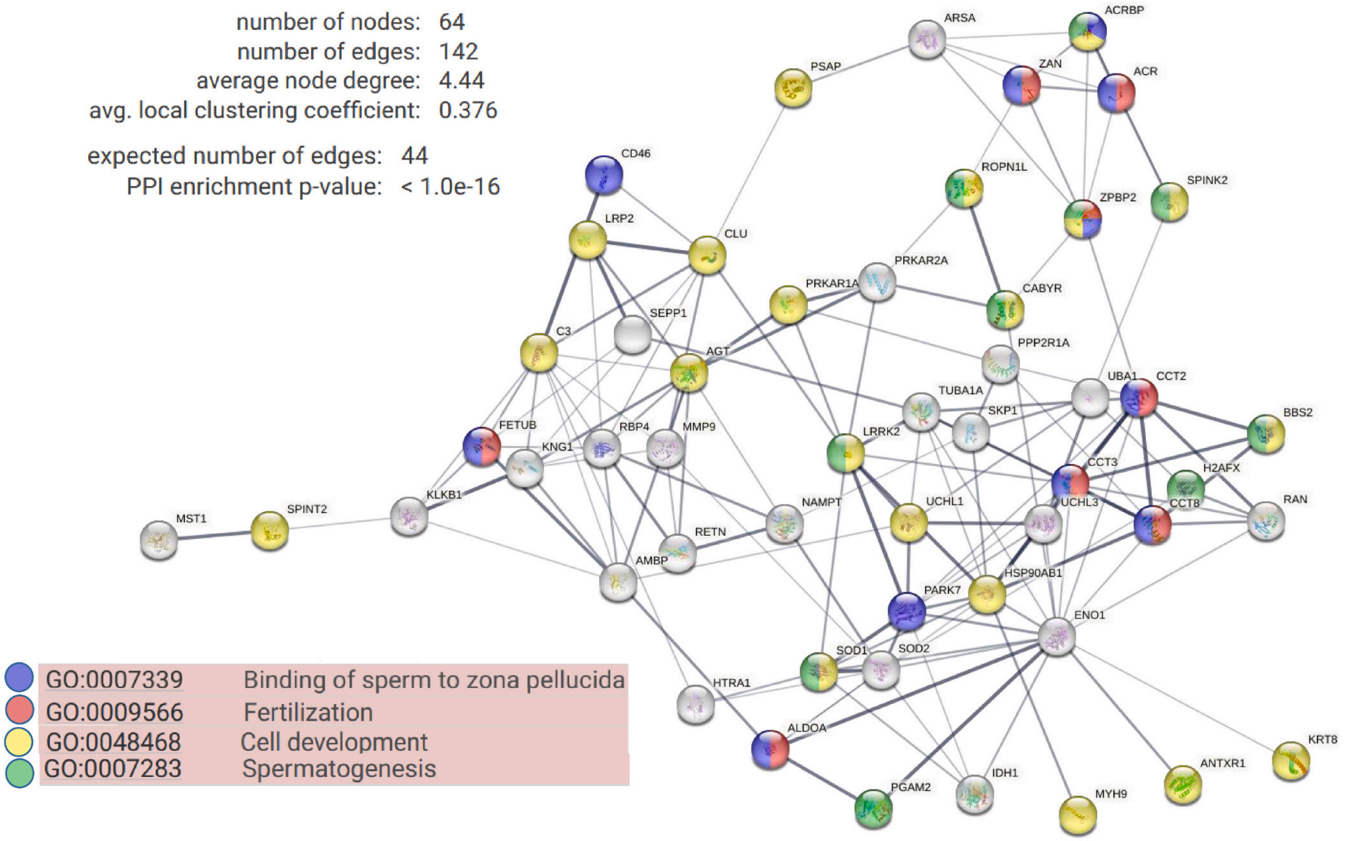

Fig. 9. Protein–protein interaction networks among proteins involved in the reproductive process: binding of sperm to the zona pellucida (red nodes), single fertilization (blue nodes), cell development (yellow nodes) and spermatogenesis (green nodes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including salmon (Gombar et al., 2017), sole (Zhang et al., 2020) and pikeperch (Dietrich et al., 2021). Exosomes in seminal plasma play important roles in mammalian sperm maturation and motility, acrosome reaction, sperm-oocyte interaction, sperm protection from oxidative stress and immunomodulation (Sullivan et al., 2005). The presence of exosome-associated proteins in sturgeon seminal plasma suggests that exosomes could play active roles in the male reproductive process, such as spermatogenesis and sperm maturation. Future studies should focus on the isolation of exosomes from sturgeon seminal plasma and their proteomic and functional characterization.

4.4. Energy metabolism

In our study, GO and IPA analyses revealed that energy-producing processes such as glucose metabolic pathways (glycolysis and gluconeogenesis, glycogen metabolism, pentose phosphate pathway), fructose and mannose metabolic pathways and amino acid (arginine, proline) metabolism are significant for sturgeon seminal plasma. Glucose and fructose present in fish seminal plasma, including sturgeon, are utilized by spermatozoa as the main energy substrates to support sperm motility and survival (Lahnsteiner et al., 1993; Soengas et al., 1993; Aramli et al., 2013). Our results emphasize the contribution of different metabolic pathways to energy production and the importance of glucose, fructose and amino acid metabolism for providing energy in sturgeon spermatozoa.

According to IPA, liver X receptor/retinoid X receptor (LXR/RXR) activation and farnesoid X receptor/retinoid X receptor (FXR/RXR) activation pathways associated with lipid metabolism were the most significant canonical pathways of sturgeon seminal plasma. These pathways were also identified among the major canonical pathways of seminal plasma proteins of teleost fish (Ciereszko et al., 2017). In addition to playing a role in lipid metabolism, the LXR/FXR pathway also participates in inflammation and cholesterol catabolism (Calkin and Tontonoz, 2012), and the FXR/RXR pathway is additionally involved in glucose metabolism, bile acid regulation and the inflammatory response (Ding et al., 2015). The contributions of major seminal plasma proteins, such as APOA1, TF, ALB, and HPX, to the FXR/RXR and LXR/FXR pathways suggest an important role of the retinoid receptor activation pathway in sturgeon reproduction and the involvement of lipid metabolism in providing energy to spermatozoa and maintaining sperm membrane integrity during storage in the spermatic duct.

4.5. Proteins involved in reproduction

In our study, 70 seminal plasma proteins were described as being involved in reproductive functions such as spermatogenesis, fertilization, binding of sperm to the zona pellucida, motility, acrosome reaction, and male gonad development. Importantly, we found 30 proteins linked with spermatogenesis, including components of the ubiquitination cascade, such as ubiquitin carboxyl-terminal hydrolase isozyme L1 and L3 (UCHL1, UCHL3), ubiquitin-like modifier-activating enzyme 1 (UBA1), and S-phase kinase-associated protein 1 (SKP1). The ubiquitin-mediated protein degradation system was found in the fish reproductive tract and is involved in different stages of spermatogenesis, destruction of sperm mitochondria and control of sperm quality (Mochida et al., 2002). Moreover, SKP1 is well known as a crucial protein in the production of mature spermatozoa (Guan et al., 2020). There are also many other important proteins in seminal plasma that are involved in sperm motility, such as angiotensinogen (AGT), anthrax toxin receptor 1 (ANTXR1), heat shock protein 90 beta (HSP90AB1), insulin-like growth factor-binding protein 7 (IGFBP7), KLKB1, kininogen-1 (KNG1), PGAM2 and TUBA1A. Interestingly, KNG1 is a specific substrate for KLKB1 (Fink et al., 1989) and is associated with the stimulation of postejaculation sperm motility (Somlev et al., 1996). Apart from the effects of kinins on sperm motility, the kallikrein-kinin system is involved in the regulation of spermatogenic functions of the testis (Schill and Miska, 1992). We also showed the presence of a set of proteins involved in acrosome reaction including ropporin-1-like (ROPN1L), calcium-binding tyrosine phosphorylation-regulated protein (CABYR), enolase A (ENO1), ACR, ACRBP, sperm acrosome-associated protein 9 (SPACA9), and membrane cofactor protein (CD46). Moreover, during the acrosome reaction, proteins such as zona pellucida-binding protein 2 (ZPBP2), zonadhesin (ZAN), protein kinase cAMP-dependent type I regulatory subunit alpha (PRKAR1A) and RfBP also participate in the binding of sperm to the zona pellucida. To date, knowledge concerning the details of sperm physiology and the mechanism of fertilization in sturgeons is extremely limited, including the mechanism of sperm motility activation, the acrosome reaction and its role in fertilization. In our opinion, our results indicated the presence of proteins potentially involved in these processes and are important for the design of future studies aiming to unravel a detailed description of specific reproductive processes in male sturgeon.

4.6. Protein complexes

Most proteins can be found in functional or regulatory complexes with other proteins (Sali et al., 2003). However, to our knowledge, the presence of protein complexes in fish seminal plasma has not yet been studied. Proteins often occur in oligomeric form composed of multiple subunits, which may be identical (homooligomers) or different (heterooligomers) (Danielli et al., 2020). In our study, the molecular mass of the complex indicated that immunoglobulin occurred in homomultimeric form as a trimer and was identified as consisting of light and heavy chains, while TF was present in homooligomeric forms such as dimers, trimers, tetramers and pentamers formed through covalent (S—H) bonds in seminal plasma. We also visualized four multiprotein complexes (MC1-MC4). Two of them contained ALB attached to RBP4 (MC3) and APOA1 (MC4). Albumins play a role as transporter proteins by binding to various ligands and carrying them around (Li et al., 2017a) and may attach peptides through S—S bridges, particularly at position SH-34 cysteine (Laurell and Thulin, 1975). For the first time, we identified a complex composed of TF connected with fish-specific protein FEL (MC1) and complex 2 (MC2) containing MB, APOA1 and RfBP. The future detailed characterization of multiprotein complexes should be an important step toward an integrative view of the protein–protein interaction network to help understand seminal plasma protein function and regulation in sturgeon reproductive processes.

4.7. Potencial biomarkers of semen quality

In our opinion, several identified seminal plasma proteins may potentially serve as candidate biomarkers for sperm quality and fertilization in sturgeons, because these proteins were indicated as specific biomarkers in mammalian semen. The acrosome related proteins (ACR, ACRBP, SPACA9, CD46, ZAN, APBP2) may serve as marker of sperm acrosome status, proteins associated with immune (HP, C3, LGALS3BP, IGs, EPX) and stress response (PRDX, HPX, S100, SOD) as markers of sperm oxidative stress or inflammation/infection, ECM1 is a proposed biomarker for spermatogenesis while CLU and ubiquitin as markers of semen quality (Samanta et al., 2018; Kumar and Singh, 2020). In addition the presence of sperm proteins in seminal plasma may be potentially useful for evaluation of sperm dysfunction and damaging. Moreover, we detected in sturgeon seminal plasma a fibronectin (FN1) which was proposed as marker of freezability of mammalian semen (Vilagran et al., 2015). Further studies are needed to test their usefulness as potential biomarkers of male fertility in sturgeon for improving artificial reproductive biotechnologies, such as cryopreservation of semen.

In summary, this study provides the largest proteomic data of sturgeon seminal plasma proteins and demonstrates the presence of their native complexes. Our results significantly extend current knowledge regarding the composition of sturgeons' seminal plasma proteins and their relationship to seminal plasma proteins of teleost fish and higher vertebrates. This complex view allowed us to gain advanced insight into the physiological function of seminal plasma and improve our knowledge of the processes that occur in the sturgeon reproductive tract.

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CRediT authorship contribution statement

Natalia Kodzik: Formal analysis, Data curation, Visualization, Methodology, Writing – original draft. **Andrzej Ciereszko:** Conceptualization, Writing – original draft, Writing – review & editing. **Mirosław Szczepkowski:** Resources. **Halina Karol:** Investigation. **Sylwia Judycka:** Investigation. **Agata Malinowska:** Formal analysis, Data curation. **Bianka Świderska:** Formal analysis, Data curation. **Mariola A. Dietrich:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Comparative proteomic analysis of the ovarian fluid and eggs of Siberian sturgeon

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Abstract

Background Sturgeon species are living fossils that exhibit unique reproductive characteristics, and elucidation of the molecular processes governing the formation and quality of sturgeon eggs is crucial. However, comprehensive data on the protein composition of sturgeon ovarian fluid (OF) and eggs and their functional significance are lacking. To address this knowledge gap, the aim of the present study was to conduct a comprehensive comparative proteomic analysis of Siberian sturgeon OF and eggs using liquid chromatography–mass spectrometry (LC–MS/MS).

Results A total of 617 proteins were identified in OF, and 565 proteins were identified in eggs. A total of 772 proteins showed differential abundance. Among the differentially abundant proteins, 365 were more abundant in OFs, while 407 were more abundant in eggs. We identified 339 proteins unique to OFs and 287 proteins specific to eggs, and further investigated the top 10 most abundant proteins in each. The functional annotation of the OF proteins highlighted their predominant association with immune system processes, including the complement and coagulation cascade, neutrophil and leukocyte-mediated immunity, cholesterol metabolism, and regulation of the actin cytoskeleton. Analysis of egg proteins revealed enrichment in metabolic pathways, such as oxidative phosphorylation and fatty acid metabolism, and protein ubiquitination and translation. OF-specific proteins included extracellular matrix and secretory vesicles, and eggs were enriched in proteins localized to mitochondria and ribosome components.

Conclusions This study presents the first comprehensive characterization of the protein composition of sturgeon OF and eggs and elucidates their distinct functional roles. These findings advance our understanding of sturgeon reproduction, OF-egg signaling and the origin of OF proteins. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium with the dataset identifier PXD044168 to ensure accessibility for further research.

Keywords *Acipenser baerii*, Ovarian fluid, Eggs, Proteome, Mass spectrometry

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Background

Sturgeon ancestors existed more than 200 million years ago, and the extant species are living fossils [1] with unique reproductive systems, including gametes, compared to modern teleost fish. For example, sturgeon eggs are encased by a large and thick (50 μm) egg envelope composed of three or four distinct layers compared to the typical one or two envelope layers found in teleost fish. The different structures and numerous micropyles, from 3 to 15, of sturgeon eggs are unusual among fish [2] because the eggs of teleost fish possess only a single funnel-shaped micropyle. Sturgeons are important from an evolutionary perspective and highly valued for their black caviars and high-quality meat. Unfortunately, overfishing for meat and caviar production has led to a severe decline in the sturgeon population, and 27 sturgeon species are currently listed as endangered on the Red List. Therefore, gaining a deeper understanding of the molecular processes underlying sturgeon egg formation and quality is of great scientific and practical importance.

Ovarian fluid (OF) is the maternally derived fluid that surrounds fish eggs, and it plays an important role in creating an optimal environment for egg maturation and fertilization success. OF modulates sperm velocity as a mechanism of cryptic female choice [3]. The biochemical composition of sturgeon OF, including ions, proteins, amino acids and sugars, supports and protects fish gametes against the harmful effects of low freshwater osmolality [4]. The pH of OF and the contents of potassium, sodium and calcium ions are similar to other fish species, but osmolarity varies between fish species. Sturgeon OF has an average osmolality of approximately 200 mOsm kg^{-1}, and salmonids exhibit an average osmolality of greater than 250 mOsm kg^{-1} [5]. The concentration of proteins in sturgeon OF is greater (2.41 ± 0.30 mg mL^{-1} for Siberian sturgeon) [6] than that cyprinids (1.58 mg mL^{-1} for bleak) [7]. There is a gap in our knowledge for the identification of particular proteins of sturgeon OF, which is a prerequisite for a better understanding of their specific roles.

Despite the importance of sturgeon oocytes for aquaculture, little information is available on their protein composition. Proteomic techniques are powerful tools for studying proteins on a large scale and have been successfully used to identify egg proteins in various fish species, such as pikeperch (1296 proteins) [8] and zebrafish (2535 proteins) [9]. Similarly, OF proteins have been identified in chinook salmon (174 proteins) [10], pikeperch (796 proteins) [8] and rainbow trout (54 proteins) [11]. However, only a limited number of proteins have been identified in the OF and eggs of sturgeon species, such as sterlets and Persian sturgeon, and protein counts ranged from 30 to 80 [12, 13]. A comprehensive proteomic study of sturgeon eggs and their environment is lacking because most studies focused solely on the OF or eggs. A comprehensive approach would elucidate the relationship between the OF and eggs. Only one recent proteomic study on pikeperch (a teleost fish) investigated OF and eggs and addressed this issue [8]. In-depth proteomic studies of eggs successfully identified potential biomarkers for egg quality in pikeperch [8] and zebrafish [9]. Comparative proteomic analysis of sturgeon OF and eggs allows determination of the important roles of proteins in the female reproductive system, development and ovarian physiology of fish.

The ecological and economic importance of sturgeon support a clear need to examine the proteomic composition of the OF and eggs of sturgeon species. Therefore, the present study provides a comprehensive overview of the OF and egg proteomes of Siberian sturgeon and compared their proteomes using an efficient LC–MS/MS approach to elucidate their composition and functional significance. Western blot analysis was used to validate proteins specific to the OF and eggs. This knowledge provides new information on the factors influencing egg quality and reproductive success and may have implications for biomarker discovery and aquaculture practices.

Results

Characteristics of sturgeon OF and egg quality

The protein concentration, osmolality and pH of the OF were 2.49 ± 0.28 mg mL^{-1}, 235 ± 11 mOsm kg^{-1} and 7.74 ± 0.01, respectively. The eggs collected for analysis were characterized by high quality with a fertilization rate at the second cleavage (4 h postfertilization) of $97.5 \pm 0.3\%$ and a hatching rate of $78.4 \pm 6.5\%$. The parameters for all five females are provided in Supplementary Table S1.

LC–MS/MS identification of OF and egg proteins

In the OF, a total of 732 proteins, containing 3519 peptides, were identified from 12,721 MS/MS matched spectra, and 692 proteins, comprised of 3150 peptides, were identified in eggs from 12,406 MS/MS matched spectra with high confidence ($FDR < 1\%$). Among these identified proteins, 617 and 565 proteins with a minimum of two peptides were detected in at least three of five biological replicates of the OF and eggs, respectively (Supplementary Table S2). The mass spectrometry proteomics data were deposited into ProteomeXchange with the dataset identifier PXD044168. The list of the 10 most abundant proteins (exponentially modified protein abundance index ($emPAI \geq 9$) in the OF and eggs is presented in Table 1.

Functional annotation of the OF and egg proteomes

To elucidate the biological functions of the sturgeon OF and egg proteins, we performed a search in the NCBI

Table 1 The 10 most abundant proteins in the ovarian fluid (OF) and eggs of Siberian sturgeon (*exponentially modified protein abundance index* (emPAI) ≥ 9)

Protein	Gene name	Calculated MW (kDa)	Accession number	Organism	emPAI
OF					
serum albumin 2-like	*ALB*	69.1	XP_033869101.2	*Acipenser ruthenus*	420.7
apolipoprotein A-I-like	*APOA1*	30.3	RXM92209.1	*Acipenser ruthenus*	99.0
serotransferrin isoform 3	*TF*	77.1	QHQ72345.1	*Acipenser ruthenus*	65.6
hemoglobin subunit beta-2-like, partial	*HBE1*	16.2	XP_033887121.2	*Acipenser ruthenus*	30.6
hemopexin precursor, partial	*HPX*	50.7	QOI31294.1	*Acipenser gueldenstaedtii*	17.9
nucleoside diphosphate kinase A-like	*NME2*	17.2	XP_033895527.2	*Acipenser ruthenus*	14.9
fish-egg lectin-like isoform X1	*FEL*	26.6	XP_033912133.2	*Acipenser ruthenus*	10.6
alpha-1-antitrypsin homolog	*SERPINA1*	47.5	XP_033892557.2	Acipenser ruthenus	9.1
histone H3-like	*H3C13*	11.4	XP_033853079.1	Acipenser ruthenus	9.0
actin, cytoplasmic 2	*ACTG1*	41.7	XP_033886943.1	*Acipenser ruthenus*	9.0
EGGS					
vitellogenin-like (similar to vitellogenin *AB2a [Acipenser schrenckii])*	*vtg2*	193.6	XP_033858533.2	*Acipenser ruthenus*	73.4
nucleoside diphosphate kinase A2 isoform X2	*NME2*	17.2	XP_033895521.1	*Acipenser ruthenus*	38.8
ubiquitin carboxyl-terminal hydrolase isozyme L1	*UCHL1*	24.9	RXM35207.1	*Acipenser ruthenus*	18.3
vitellogenin-like isoform X2 (similar to vitellogenin *AB1 [A. schrenckii])*	*vtg1*	194.3	XP_034780761.1	*Acipenser ruthenus*	14.1
cofilin-2-like	*CFL2*	18.7	RXM93845.1	*Acipenser ruthenus*	14.2
cystatin-B-like	*CSTB*	11.3	XP_033864077.1	*Acipenser ruthenus*	12.9
zona pellucida sperm-binding protein 3-like	*ZP3*	28.9	XP_034773453.1	*Acipenser ruthenus*	10.0
peroxiredoxin-1	*PRDX1*	23.7	XP_033885460.2	*Acipenser ruthenus*	9.0
creatine kinase B-type-like	*CKB*	42.6	XP_033897145.1	*Acipenser ruthenus*	9.0
triosephosphate isomerase B	*TPI1*	26.9	XP_033899162.1	*Acipenser ruthenus*	9.0

database and mapped 602 and 553 proteins from the OF and eggs, respectively, to 465 and 471 unique human gene homologs, respectively due to the presence of multiple homologs of the same human protein. Among the identified proteins, gene homologs were not found for 30 and 16 proteins of the OF and eggs, respectively (mostly vitellogenins (VTGs), fish-egg lectin-like, riboflavin-binding protein, protein rapunzel-like, type-4 ice-structuring protein, cell-surface glycoprotein 1, microtubule-associated protein futsch, high choriolytic enzyme 1, gonadal soma-derived growth factor, glycine-rich cell wall structural protein 1.8, stonustoxin and 15 uncharacterized proteins).

GO analysis of biological process terms revealed that most of the OF proteins were associated with exocytosis and secretion (162 proteins) and immune system processes (182 proteins), especially neutrophil-, leucocyte- and granulocyte-mediated immunity, and various cellular metabolic and catabolic process-related terms (182 proteins) and protein localization, targeting and translation (48 proteins) were enriched among the egg proteins (Fig. 1). Enrichment analysis using Ingenuity Pathway Analysis (IPA) identified 40 and 58 canonical pathways that were significantly enriched in the OF and eggs, respectively. The top 10 canonical pathways are presented in Table 2. KEGG pathway analysis highlighted similar pathways. The full functional annotations for the OF and eggs are available in Supplementary Tables S3 and S4, respectively.

Comparison of OF and egg proteins

Using the emPAI-based estimates of the quantitative relationships within each type of sample, a total of 772 differentially abundant proteins (DAPs) between the OF and eggs were identified, including 365 proteins that were more abundant in the OF and 407 proteins that were more abundant in eggs (Supplementary Table S5). Of these DAPs, 339 proteins were exclusively detected in OF samples, and 287 were unique to eggs (i.e., not detected in the OF samples) (Fig. 2A; Supplementary Tables S6). Among the DAPs shared by the OF and eggs, the top proteins (FC>20) in OF were albumin (ALB) (140-fold), serotransferrin (TF) (72-fold), hemopexin (HPX) (51-fold), alpha-1-antitrypsin homolog (SERPINA1) (30-fold), and cytosolic nonspecific dipeptidase (CNDP2) (29-fold), where as zona pellucida sperm-binding proteins (ZPs) (40-fold), peroxiredoxin 4 (PRDX4) (31-fold), vitellogenin-like (VTG2) (23-fold), ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) (23-fold), and endoplasmin (HSP90B1) (20-fold) were highly abundant in the eggs (Supplementary Table S7).

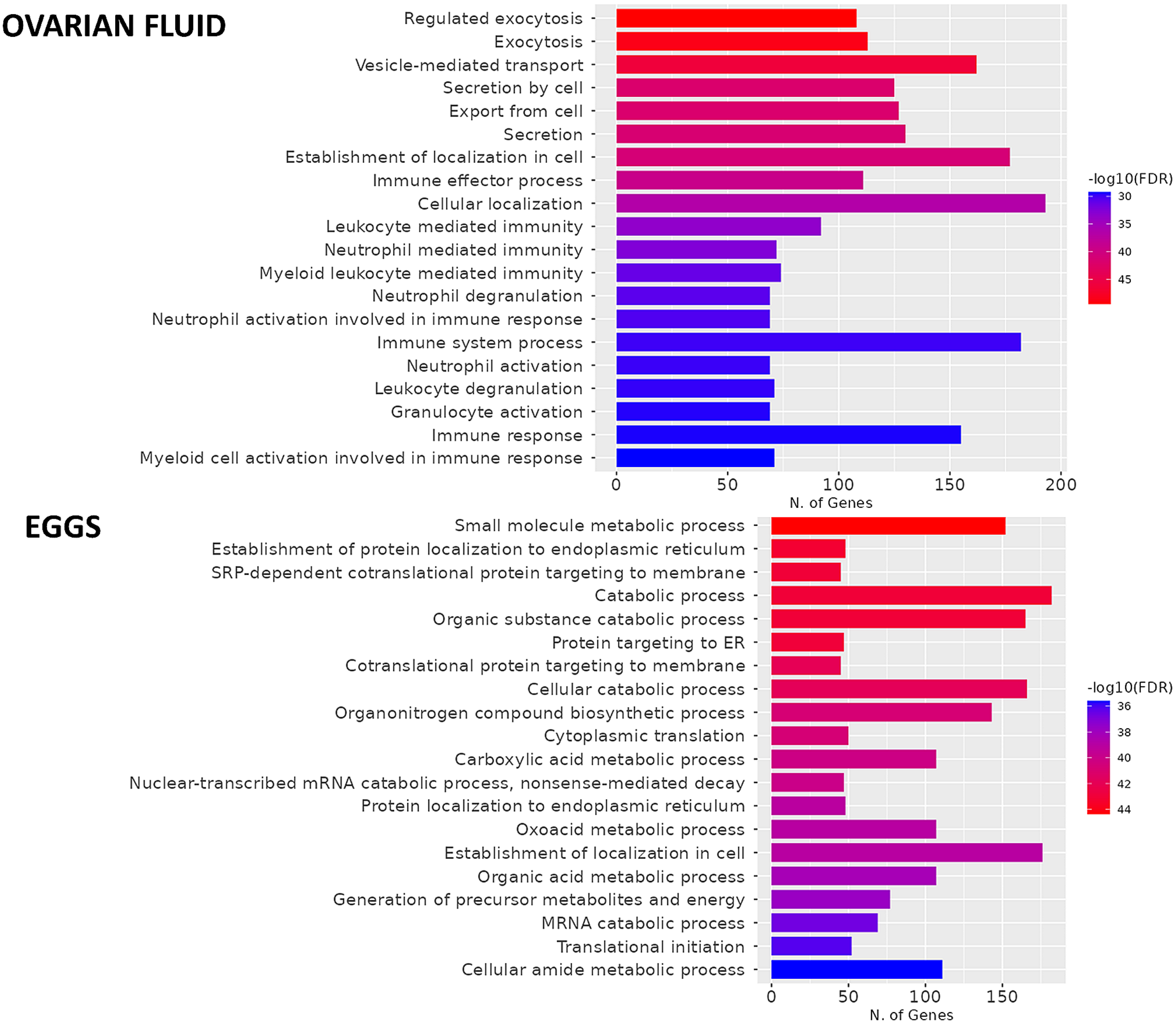
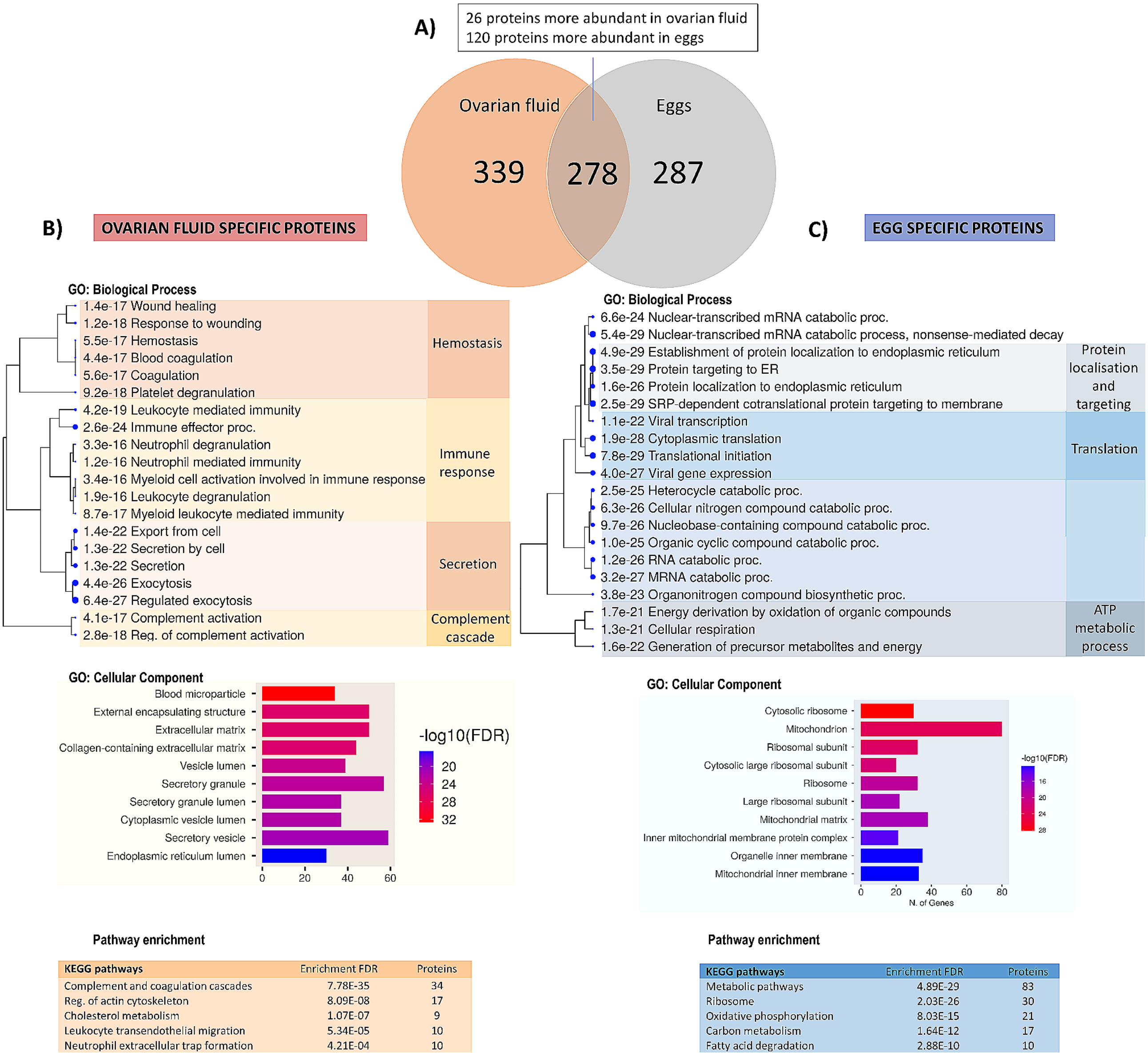


Fig. 1 GO biological process enrichment analysis of total identified proteins in the ovarian fluid (OF) and eggs of Siberian sturgeon. Most OF proteins were associated with exocytosis and secretion and immune system processes, but the top-ranked biological processes were cellular metabolic and catabolic processes in eggs

Table 2 Top 10 canonical pathways significantly enriched in ovarian fluid (OF) and egg proteins

OF			Eggs		
Pathway	***p* value***	**Proteins**	**Pathway**	***p* value***	**Proteins**
LXR/RXR Activation	1.44E-24	30	EIF2 Signaling	7.32E-44	55
Acute Phase Response Signaling	2.32E-23	34	Oxidative Phosphorylation	1.19E-19	25
FXR/RXR Activation	4.17E-23	29	Protein Ubiquitination Pathway	1.89E-19	29
Complement System	1.49E-21	18	Regulation of eIF4 and p70S6K Signaling	1.89E-14	25
Coagulation System	4.03E-17	15	Inhibition of ARE-Mediated mRNA Degradation Pathway	1.18E-12	22
Actin Cytoskeleton Signaling	1.33E-16	31	Glycolysis I	1.51E-12	11
Clathrin-mediated Endocytosis Signaling	7.43E-15	27	Fatty Acid β-oxidation I	1.64E-12	12
Intrinsic Prothrombin Activation Pathway	1.71E-11	12	Sirtuin Signaling Pathway	6.59E-12	28
Integrin Signaling	3.24E-11	23	TCA cycle II	2.62E-11	9
Epithelial Adherens Junction Signaling	3.24E-11	20	Gluconeogenesis	2.82E-11	10

* The *p*-value associated with a function or a pathway is a measure of the likelihood that the association between a set of focus genes in the experiment and a given process or pathway is due to random chance. The smaller the *p*-value the less likely that the association is random and the more significant the association. In general, a *p*-value (calculated using the right-tailed Fisher exact test) < 0.05 indicates a statistically significant, nonrandom association



Pathway enrichment

KEGG pathways	Enrichment FDR	Proteins
Complement and coagulation cascades	7.78E-35	34
Reg. of actin cytoskeleton	8.09E-08	17
Cholesterol metabolism	1.07E-07	9
Leukocyte transendothelial migration	5.34E-05	10
Neutrophil extracellular trap formation	4.21E-04	10

Pathway enrichment

KEGG pathways	Enrichment FDR	Proteins
Metabolic pathways	4.89E-29	83
Ribosome	2.03E-26	30
Oxidative phosphorylation	8.03E-15	21
Carbon metabolism	1.64E-12	17
Fatty acid degradation	2.88E-10	10

Fig. 2 Functional analysis of proteins unique to ovarian fluid (OF) and eggs. Venn diagram representing the overlap of all identified proteins in the OF and eggs of Siberian sturgeon (**A**). GO biological processes and cellular components; KEGG pathway analysis of the OF (**B**) and egg (**C**) proteins. Pathways with many shared genes are clustered together. Larger dots indicate more significant *P* values

GO and KEGG enrichment analyses of proteins specific to the OF and eggs

GO term enrichment analysis of the proteins unique to the OF and eggs revealed significant enrichment of 500 and 338 biological processes, respectively (Supplementary Table S8 and Table S9). We present the top 20 significantly enriched GO biological processes, which shows that most proteins unique to the OF were associated with exocytosis (61 proteins) and immune and stress response (107 proteins), especially leukocyte- and neutrophil-mediated immunity, complement activation and coagulation (Fig. 2B, C). In contrast, metabolic processes (100 proteins; including catabolic processes, such as RNA catabolic process and nitrogen compound catabolic process and energy derivation), protein localization and targeting (31 proteins), and translation (33 proteins) were enriched in eggs. KEGG pathway analysis revealed that proteins involved in complement and coagulation cascades (34 proteins), regulation of the actin cytoskeleton (17 proteins), cholesterol metabolism (9 proteins) and immune-related pathways were specifically enriched in the OF, and metabolic pathways (83 proteins), including oxidative phosphorylation (21 proteins), carbon metabolism (17 proteins), fatty acid degradation (10 proteins) and ribosomes (30 proteins), were most enriched in eggs (Fig. 2B, C). Among the proteins involved in the complement cascade, we identified proteins related to the classical, alternative and lectin pathways (Supplementary Figure S1). IPA highlighted similar pathways and emphasized the representation of proteins involved in eukaryotic initiation factor (EIF) signaling and protein ubiquitination pathways for egg-specific proteins. IPA revealed functional enrichment of OF proteins involved in organismal survival, inflammatory response, immune cell trafficking, hematological system development and organismal development, and egg-specific proteins were mostly involved in tissue morphology, embryonic development, organismal survival, connective tissue development and nervous system development (Table 3).

Among the OF-specific proteins related to immune and stress responses, there was a high degree of connectivity between each other (Fig. 3). Among the egg-specific proteins linked to metabolism, six distinct interaction networks were found, among which 28 ribosomal proteins and proteins involved in oxidative phosphorylation (19 proteins) exhibited the highest degree of connectivity (Fig. 4).

OF and egg proteins associated with reproduction

ShinyGo and GO analyses revealed that 102 OF proteins and 117 egg proteins were involved in reproductive processes. An additional search of OF and egg proteins using the Panther database (http://www.pantherdb.org/) revealed their involvement in the ovulation cycle, oocyte maturation, prevention of polyspermy, ovarian aging, fertilization, sperm-egg interaction and embryonic development. All identified proteins and their female reproductive functions are available in Supplementary Table S10.

Validation of mass spectrometry data using Western blotting

To validate the LC‒MS/MS results, we selected four proteins for further analysis using 1D Western blotting: ALB, fibrinogen beta chain (FGB), fibronectin 1 (FN1) and vitellogenin-like (XP_033858533.2), which shows 97.89% identity to vitellogenin AB2a from *Acipenser schrenckii*, corresponding to the fish VTG2 [14] (Supplementary Figure S2). As presented in Fig. 5, the changes in the abundance of the selected proteins were consistent with the changes in the LC‒MS/MS analysis. Western blot analysis confirmed the absence of FGB and FN1 proteins in the eggs and demonstrated their exclusive presence in the OF (Fig. 5A). A strong signal of ALB was also detected in OF. However, due to its low concentration in eggs (140 times lower than in the OF), it was not detected using the current conditions (i.e., below the limit of detection). Western blot analysis revealed a six-fold greater abundance of VTG2 in eggs compared to the OF (Fig. 5B). The full-length blots are presented in Supplementary Figure S2. The specificity of the antibodies used was confirmed using mass spectrometry analysis (Supplementary Table S11).

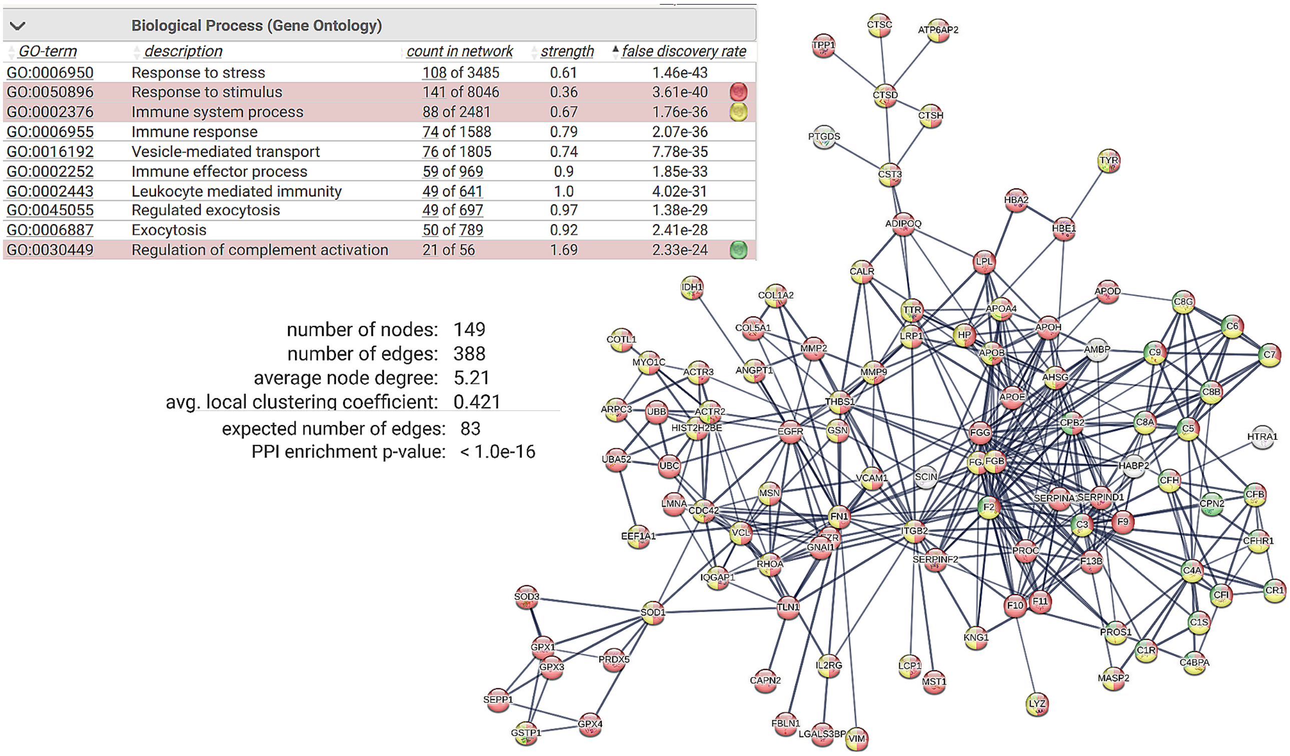
Discussion

The present study presents the first comprehensive characterization of the OF and mature egg proteomes of sturgeon and their comparison. Our findings contribute to the largest proteomic catalog of the OF and eggs in sturgeons (*Actinopterygii*), with a total of 602 and 553 proteins identified in the OF and eggs, respectively. Pathways related to mRNA translation (EIF signaling), protein degradation (ubiquitin‒proteasome pathway) and metabolic pathways (oxidative phosphorylation, glycolysis, fatty acid β-oxidation, and the sirtuin signaling pathway) were

Table 3 Functional analysis of proteins specific to ovarian fluid (OF) and eggs using Ingenuity pathway analysis (IPA)

OF specific proteins			**Egg specific proteins**		
Top canonical pathway	***p* value***	**Proteins**	**Top canonical pathway**	***p* value***	**Proteins**
Complement System	3.73E-26	18	EIF2 Signaling	4.17E-31	36
Acute Phase Response Signaling	3.34E-25	29	Oxidative Phosphorylation	7.28E-19	20
LXR/RXR Activation	5.02E-22	23	Protein Ubiquitination Pathway	8.14E-12	14
FXR/RXR Activation	8.98E-22	23	Fatty accid β-oxidation	1.14E-12	10
Coagulation System	4.37E-19	14	Regulation of eIF4 and p70S6K Signaling	1.14E-12	16
Top molecular and cellular functions	**p value***	**Proteins**	**Top molecular and cellular functions**	**p value***	**Proteins**
Organismal Survival	2.5E-28-1.3E-16	135	Tissue Morphology	3.4E-10-9.7E-03	25
Inflammatory Response	6.7E-28-4.5E-09	148	Embryonic Development	1.2E-06-9.2E-04	9
Immune Cell Trafficking	1.4E-26-3.3E-09	87	Organismal Survival	4.8E-05-4.8E-05	75
Hematological System Development and Function	2.1E-25-3.3E-09	119	Connective Tissue Development and Function	2.7E-04-6.2E-03	9
Organismal Development	1.2E-23-3.9E-09	129	Nervous System Development and Function	4.6E-04-9.7E-03	20

* the *p* value associated with a function or a pathway is a measure of the likelihood that the association between a set of focus genes in the experiment and a given process or pathway is due to random chance. The smaller the *p* value, the less likely that the association is random and the more significant the association. In general, a *p* value (calculated using the right-tailed Fisher exact test) < 0.05 indicates a statistically significant, nonrandom association



Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate	
GO:0006950	Response to stress	108 of 3485	0.61	1.46e-43	
GO:0050896	Response to stimulus	141 of 8046	0.36	3.61e-40	●
GO:0002376	Immune system process	88 of 2481	0.67	1.76e-36	●
GO:0006955	Immune response	74 of 1588	0.79	2.07e-36	
GO:0016192	Vesicle-mediated transport	76 of 1805	0.74	7.78e-35	
GO:0002252	Immune effector process	59 of 969	0.9	1.85e-33	
GO:0002443	Leukocyte mediated immunity	49 of 641	1.0	4.02e-31	
GO:0045055	Regulated exocytosis	49 of 697	0.97	1.38e-29	
GO:0006887	Exocytosis	50 of 789	0.92	2.41e-28	
GO:0030449	Regulation of complement activation	21 of 56	1.69	2.33e-24	●

number of nodes: 149
number of edges: 388
average node degree: 5.21
avg. local clustering coefficient: 0.421
expected number of edges: 83
PPI enrichment p-value: < 1.0e-16

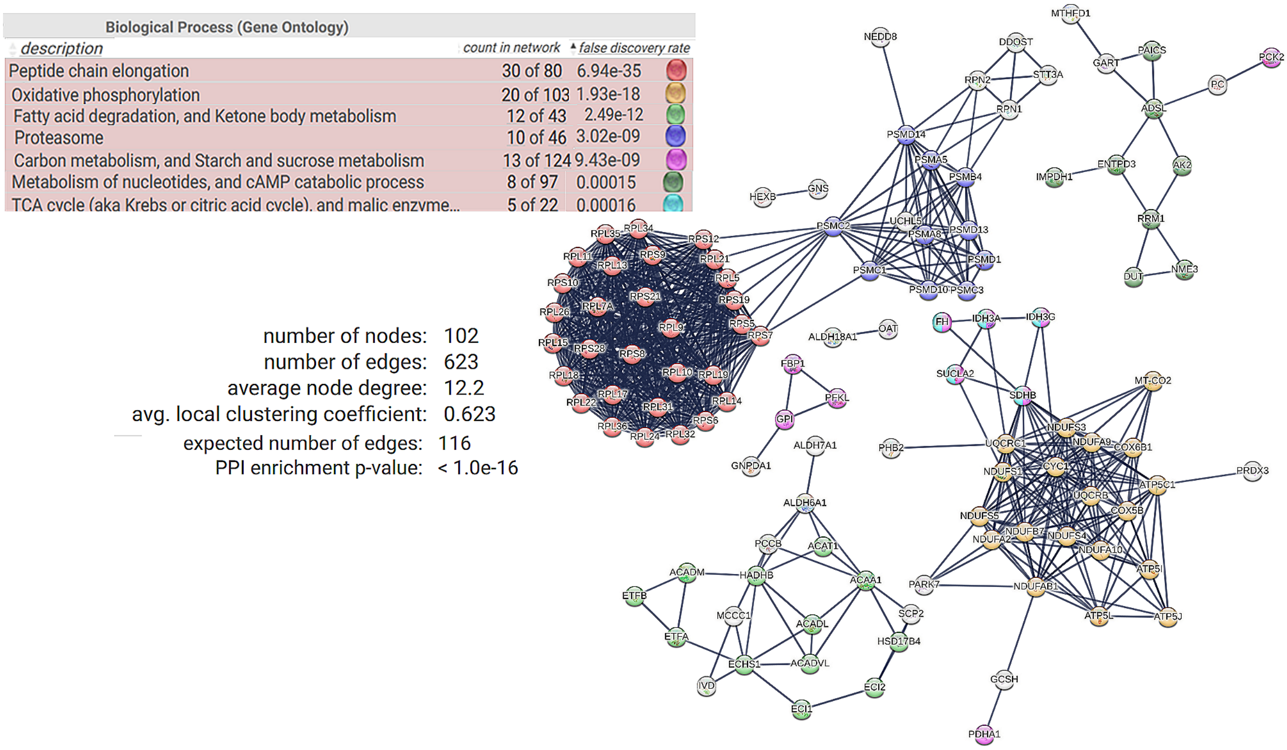
Fig. 3 Results of the Search Tool for the Retrieval of Interacting Genes/Proteins (v 11.0) analysis showing the protein association network of proteins specific to ovarian fluid assigned to immune system processes with a high confidence score. The nodes correspond to the proteins, and the edges represent the interactions (thick lines indicate a high score > 0.9; thin lines indicate a medium score > 0.7). Model statistics are presented on the left. An explanation of the edge colors is provided above the figure

the most significant canonical pathways in eggs. OF was enriched in various pathways, such as LHR/RXR activation, acute phase response signaling, complement and coagulation system, actin cytoskeleton signaling, clathrin-mediated endocytosis signaling, and integrin and epithelial adherens junction signaling. We also identified proteins that were unique to the OF and eggs, which elucidated their distinct molecular profiles.

Most abundant proteins in the OF and eggs and their functions

The most abundant proteins in the sturgeon OF were predominantly involved in the humoral innate immune response, including acute phase proteins (APPs), antimicrobial peptides (AMPs) and pattern recognition receptors (PRRs). Acute phase response proteins (ALB, TF, HPX, SERPINA1 and apolipoprotein A-I (APOA1)) are multifunctional proteins that participate in various processes, such as iron ion homeostasis (TF, ALB, HPX), oxidative protection (TF, HPX, ALB, APOA1), lipid metabolism (APOA1) and protease activity regulation (SERPINA1). Histone H3 (H3) and hemoglobin subunit beta (HBB) are precursors of AMPs, which play a fundamental role in innate immunity by killing pathogens and modulating the immune response in fish [15]. Fish egg lectin (FEL) was previously identified in the eggs of several teleost fish species, and it acts as a PRR that specifically binds to bacteria and as an opsonin to protect developing embryos/larvae from pathogenic invasion [16]. Among the abundant proteins in sturgeon OF, actin cytoplasmic 2 (ACTG1) and nucleoside diphosphate kinase A-like (NME1), which play important roles in oogenesis and embryo development in fish, were identified [17, 18]. The functions of ACTG1 and NME1 may also be linked to the immune response because ACTG1 is involved in the motility, phagocytosis and antigen presentation of immune cells [19], and NME1 is involved in T-cell activation [20]. Most of the dominant proteins in sturgeon OF (ALB, TF, HPX, HBB, SERPINA1, APOA1) were also found among the highly abundant proteins in the OF of teleost fish [8, 10, 11], which suggests that the general protective mechanisms of oocytes against pathogens and oxidative stress are evolutionarily conserved in the OF and similar to teleost fish. However, unlike teleost fish, the levels of histones H1 (H1), ACTG1, NME1 and FEL were more abundant in sturgeon OF, which indicates the importance of these proteins in the development and protection of sturgeon oocytes and embryos.

The dominant egg proteins were associated with various functions, including development, immune response, antioxidative protection, metabolism and fertilization. Similar to teleost fish, numerous VTG family members



Biological Process (Gene Ontology)

description	count in network	false discovery rate	
Peptide chain elongation	30 of 80	6.94e-35	🔴
Oxidative phosphorylation	20 of 103	1.93e-18	🟡
Fatty acid degradation, and Ketone body metabolism	12 of 43	2.49e-12	🟢
Proteasome	10 of 46	3.02e-09	🔵
Carbon metabolism, and Starch and sucrose metabolism	13 of 124	9.43e-09	🟣
Metabolism of nucleotides, and cAMP catabolic process	8 of 97	0.00015	🟢
TCA cycle (aka Krebs or citric acid cycle), and malic enzyme...	5 of 22	0.00016	🔵

number of nodes: 102
number of edges: 623
average node degree: 12.2
avg. local clustering coefficient: 0.623
expected number of edges: 116
PPI enrichment p-value: < 1.0e-16

Fig. 4 Results of the Search Tool for the Retrieval of Interacting Genes/Proteins (v 11.0) analysis showing the protein association network of proteins specific to eggs assigned to metabolic processes with a high confidence score. The nodes correspond to the proteins, and the edges represent the interactions (thick lines indicate a high score > 0.9; thin lines indicate a medium score > 0.7). Model statistics are presented on the left. An explanation of the edge colors is provided above the figure

were among the most abundant proteins in sturgeon eggs [3, 8, 10–13]. VTGs play multiple roles in providing an energy reserve for developing embryos and protecting them against microbial attacks and oxidative stress [21]. Cystatin-B (CSTB) is a reversible inhibitor of cysteine proteases, and it is involved in cellular protection against proteolysis and immune defense mechanisms in fish eggs, which indicates internal and external protection of eggs against pathogens. The abundance of PRDX1, which is an enzyme that reduces hydrogen peroxide, highlights the importance of the peroxiredoxin system in protecting sturgeon eggs against oxidative stress. ZP3 is an important component of the white sturgeon egg envelope, and it plays a role in fertilization [12, 22]. ZP3 functions in teleosts are also related to oogenesis and embryonic development [23–25]. Cofilin-2 (CFL2) is an actin-depolymerizing factor that is essential for actin cytoskeleton organization in fish oocytes and eggs. CFL2 may function with ZPs in envelope hardening and egg adhesion after fertilization in sturgeon [26]. NME was also identified as a major protein in the OF in this study, and UCHL1 is a deubiquitinating enzyme in the ubiquitin−proteasome system that is involved in oocyte and embryo development in fish. Ubiquitin-related genes are used as biomarkers of good-quality sea bass eggs [17, 27]. Among the abundant proteins, we also identified the metabolic enzymes creatine kinase B (CKB) and triosephosphate isomerase (TPI), which are involved in energy production via the creatine-phosphocreatine system and glycolysis, respectively. Notably, our study revealed differences in the composition of major proteins in eggs between teleosts [8] and sturgeons (current study). Only VTGs and ZPs were common, which may reflect the distinct structure of sturgeon oocytes, including the presence of multiple micropyles, a complex envelope structure, and a specific embryo development pattern (holoblastic cleavage) that is more similar to Xenopus than teleost fish.

Role of proteins unique to the OF in the immune response

Bioinformatic analysis revealed that most proteins unique to the OF were associated with immune system processes, including humoral and cellular responses. Our findings indicated the presence of three activation pathways of the complement system in sturgeon OF: classical (C1r-C1s, C1q, C2, and C4B); lectin (mannan-binding lectin serine protease 2 (MASP2) and C4B); and alternative (complement factor H-related protein 1 (CFHR1) and complement factor B (CFB)). We also identified component C3, which is crucial for these pathways, and components of the common terminal pathway (C5, C6, C7, C9, C8A, C8B, anC8G) and regulatory proteins (complement receptor type 1 and 2 (CR1, CR2), complement

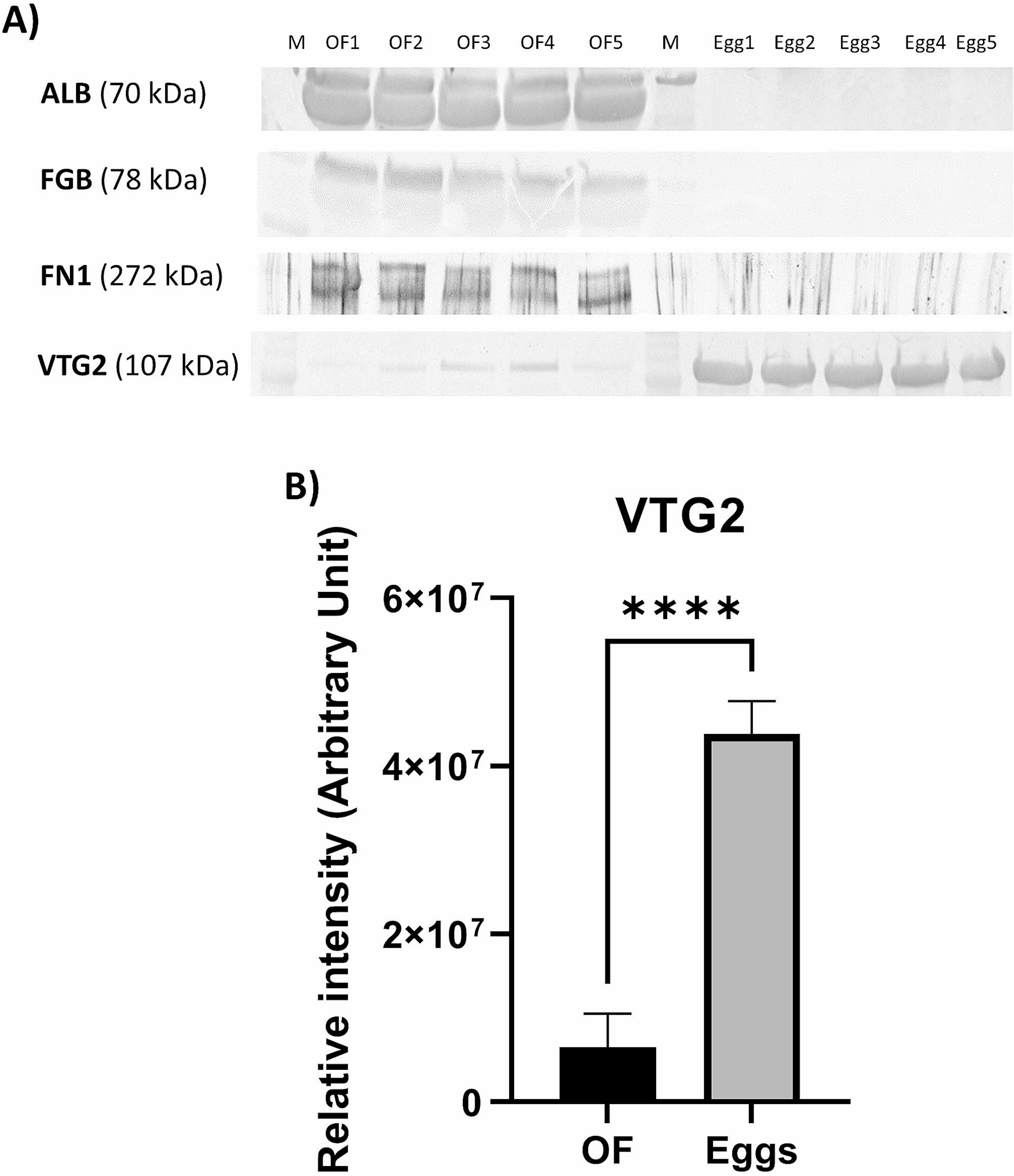


Fig. 5 Validation of proteins specific to ovarian fluid (OF) and eggs, such as albumin (ALB), fibrinogen (FGB), fibronectin-1 (FN1), and vitellogenin 2 (VTG2), using Western blot analysis (**A**). The full-length blots are presented in Supplementary Fig. S2. A quantitative comparison of VTG2 in the OF and eggs (**B**). The data are presented as the means ± S.D. ($n = 5$ in OF and eggs). Statistical analysis was performed using Student's t test; ****$p \leq 0.0001$. The intensity of the protein bands on the TGX Stain-Free gels was analyzed using Image Lab 6 software (Bio-Rad)

factor I (CFI), complement factor H (CFH), C4b-binding protein alpha chain (C4BP)), which tightly control complement system activity under normal and pathological conditions. The complement system interacts with the coagulation system identified in our study and serves as the "first line of defense" during infection to initiate opsonization, regulate adaptive immunity, enhance circulating immunoglobulins, promote immune cell recruitment, and facilitate direct cell membrane lysis [28]. We also identified positive (haptoglobin (HP), ceruloplasmin (CP), alpha-2-antiplasmin (SERPINF2), SERPINA1, heparin cofactor 2 (SERPIND1), fibrinogen alpha chain (FGA), fibrinogen gamma chain (FGG), FGB, and plasminogen (PLG)) and negative (interalpha-trypsin inhibitor heavy chain H2 and H3 (ITIH2, ITIH3), APOA1, alpha-2-HS-glycoprotein (AHSG), and histidine-rich glycoprotein (HRG)) APPs that are involved in defense-related mechanisms, including tissue repair, protection against ROS, metal chelation, activation of the complement system, enzyme neutralization and pathogen killing [29]. Proteins related to the coagulation cascade and acute inflammatory response in the OF may play a role in ovulation, which is considered a controlled inflammatory reaction in mammals and fish [30, 31]. Other humoral components identified in our study included lysozyme and cathelicidin, which are known antimicrobial peptides [32]. PRRs are part of the immune system of sturgeon OF. Our findings also suggest that proteins in sturgeon OF are involved in the activation of macrophages, neutrophil degradation and extracellular trap formation. These findings indicate that neutrophils, which are classified as heterophils in sturgeon [33], and macrophages are crucial elements of the cellular innate immune system of the OF. In summary, the complex immune system in sturgeon OF, which is composed of a variety of humoral and cellular components, strongly protects gametes against pathogens, scavenges free radicals, and may play important roles in ovulation and maintaining homeostasis following inflammation.

OF proteins involved in lipid metabolism

Our study identified several proteins involved in lipid metabolism in the OF, including lipoproteins (APOA1, apolipoprotein B-100 (APOB), apolipoprotein C-I (APOC1), apolipoprotein D (APOD), beta-2-glycoprotein 1 (APOH), apolipoprotein E (APOE)), lipoprotein and phospholipase receptors, phospholipase inhibitors and lipases. Lipoproteins, such as very low- and high-density lipoprotein (VLDL and HDL, respectively), are present in fish blood and the OF play a crucial role in lipid metabolism and transport, specifically cholesterol and triglycerides, to the ovary, where they are taken up by ovarian somatic cells and oocytes for energy production and processes related to oocyte maturation and development [34–36]. Our results suggest that the uptake of lipoproteins and VTG2 by eggs is facilitated by low-density lipoprotein receptor-related proteins (LRP1, LRP2, LRP6) and the very low-density lipoprotein receptor (VLDLR), which is similar to other fish species [37]. Although these receptors are typically found on the cell surface to mediate lipoprotein internalization, fragments or shed forms of these receptors may be detected in the OF. The presence of these receptors in the OF was detected in our study and other fish species [3, 8]. Our analysis also revealed the presence of lipoprotein lipase (LPL) in the OF, which is a key enzyme for hydrolyzing triglycerides from VLDL and LDL and generates essential free fatty acids and glycerol for lipid uptake and energy production in fish oocytes [38, 39]. The identification of a phospholipase A inhibitor suggests the presence of a regulatory mechanism that controls the activity of phospholipase A, which is responsible for the hydrolysis of phospholipids. The secretory phospholipase A2 receptor (PLA2R1) may further contribute to lipid metabolism by facilitating the binding and internalization of phospholipase A2 enzymes. Overall, our results highlight the tight regulation of lipid transport and metabolism in sturgeon OF, which involves a specialized set of proteins. These proteins play a significant role in lipid incorporation and accumulation, which may be important for prolonged oocyte maturation and development in sturgeon.

Extracellular matrix (ECM) proteins in the OF

ECM proteins play a crucial role by providing structural support, facilitating cell-matrix interactions, and regulating various processes involved in follicular development and oocyte maturation. The identification of specific ECM proteins in the OF, such as collagen alpha-1 (XI) chain (COL11A1), fibulin-1 (FBLN1), FN1, collagen alpha-1(V) chain (COL5A1), collagen alpha-3(VI) chain (COL6A3), lumican (LUM), prelamin-A/C (LMNA), proteoglycan 4 (PRG4), and chondroitin sulfate proteoglycan 4 (CSPG4), highlights their essential role in maintaining the integrity of ovarian tissue, particularly the basal lamina and theca. These structural proteins form an ECM framework that supports the growth and development of ovarian follicles in sturgeon, which is vital for housing oocytes [40]. In addition to structural proteins, other ECM components, such as cathepsins (pro-cathepsin H (CTSH), dipeptidyl peptidase 1 (CTSC), and cathepsin D (CTSD)), PLG, matrix metalloproteinase-2 and 9 (MMP2, MMP9), and their metalloproteinase inhibitor 4 (TIMP4), are involved in follicular remodeling and the breakdown of the follicle wall during ovulation [41–43]. Recent studies also suggested the involvement of the plat/plasmin system in ECM remodeling during ovulation in sturgeon [44], which further supports the dynamic nature of the ECM in the sturgeon ovary. ECM proteins, such as myocilin (MYOC), thrombospondin-4 (THBS4), cadherin 13 (CDH13), leucine-rich repeat-containing protein 15 (LRRC15), and tetranectin (CLEC3B), may contribute to cell adhesion and signaling within the ovary in sturgeon. They facilitate important cell−cell and cell−matrix interactions that regulate the signaling pathways necessary for oocyte maturation, ovulation, and tissue homeostasis [45]. We identified several ECM proteins, including scinderin (SCIN), vinculin (VCL), transforming protein RhoA (RHOA), ezrin (EZR), actin-related protein 2/3 complex subunit 3 (ARPC3), actin-related protein 2 and 3 (ACTR2, ACTR3), ras GTPase-activating-like protein IQGAP1 (IQGAP1), moesin (MSN), gelsolin (GSN), integrin beta-2 (ITGB2), cofilin-1 (CFL1), alpha-actinin-4 (ACTN4), and prothrombin (F2), which are involved in the regulation of the actin cytoskeleton. By modulating the actin cytoskeleton, these ECM proteins may contribute to various processes, including tissue remodeling and the successful release of mature oocytes [46]. Overall, the presence of these ECM proteins in sturgeon OF highlights their indispensable role in multiple processes within the ovary, including follicular structure and development, oocyte maturation, tissue remodeling, and the successful release of mature oocytes.

Egg proteins associated with transcription and translation processes

Translation and translation processes, specifically EIFs and ribosomal components, are highly important during oogenesis and early embryo development in metazoans [47]. Among the identified proteins, EIF2 signaling and the regulation of EIF4 and p70S6K signaling were the most significant canonical pathways in the sturgeon egg proteome. Notably, we identified the eukaryotic initiation factor 4 A-I and II (EIF4A1 and EIF4A2) proteins, which are responsible for unwinding mRNA secondary structures, facilitating ribosome binding, and initiating translation. We also detected eukaryotic translation initiation factor 4 gamma 3 (EIF4G3), which is an interacting scaffold protein with EIF4A and other initiation factors, and eukaryotic translation initiation factor 5 (EIF5), which facilitates the joining of ribosomal subunits during translation initiation. Other translation-associated proteins were detected, including 30 ribosomal proteins that actively contribute to the structure and function of the ribosome, polyadenylate-binding protein 1 (PABPC1), a poly(A)-binding protein, and phosphatidylinositol 4-phosphate 3-kinase C2A (PIK3C2A), which is a phosphoinositide kinase involved in intracellular signaling pathways, including the regulation of translation. These results further highlight the importance of these proteins in oocyte physiology in sturgeon. Notably, the presence of EIFs has been observed in the eggs of other fish species [8, 9]. Previous studies identified eukaryotic translation initiation factor 3 subunit D (EIF3D) and two ribosomal proteins (large ribosomal subunit protein eL36- RPL36-001, RPL36-002) as markers of high-quality eggs, and an upregulated form of ribosomal protein L22 (RPL22) was associated with poor-quality zebrafish eggs [9]. In summary, our findings highlight the significance of translation and transcription processes in the synthesis of maternal RNAs and proteins crucial for embryo development, oocyte maturation, and fertilization in sturgeon. Because the initial stages of embryo development are under maternal control, our results significantly contribute to this knowledge by revealing several proteins and their involvement in this process.

Egg metabolism-related proteins

Our study revealed the significance of the oxidative phosphorylation (OxPhos) and fatty acid β-oxidation pathways in sturgeon eggs, which are primarily represented by egg-specific proteins. OxPhos is the final biochemical pathway involved in ATP production within the mitochondria, and we identified proteins involved in the five multiprotein complexes I-V (CI-CV) of OxPhos, including several forms of NAD dehydrogenase (CI), succinate dehydrogenase (CII), cytochromes (CIII, CIV) and ATP synthase (CV) (Fig. 6A). Previous studies highlighted the impaired oxidative phosphorylation in low-quality eggs of rainbow trout, which highlights its importance in fish oocyte maturation [48]. Oxidative metabolism, including OxPhos, has been observed in unfertilized steelhead eggs and during early developmental stages [49]. We identified specific proteins in sturgeon eggs associated with the mitochondrial fatty acid β-oxidation I pathway, which is responsible for the degradation of fatty acids to produce energy [50]. These proteins participate in consecutive reactions, including dehydrogenation (catalyzed by medium-chain specific acyl-CoA dehydrogenase, mitochondrial (ACADM), hydroxylation (enoyl-CoA hydratase, mitochondrial (ECHS1), peroxisomal multifunctional enzyme type 2-HSD17B4), a second dehydrogenation (trifunctional enzyme subunit beta, mitochondrial (HADHB) and HSD17B4)) and thiolysis (3-ketoacyl-CoA-thiolase-ACAA1) (Fig. 6B). We identified other proteins involved in mitochondrial beta-oxidation, such as enoyl-CoA delta isomerase 1 and 2, mitochondrial (ECI1, ECI2), isovaleryl-CoA dehydrogenase, mitochondrial (IVD) and synaptonemal complex protein 2 (SCP2). Mitochondria in zebrafish embryos are active and utilize free fatty acids as substrates for oxidative phosphorylation to provide ATP [51]. Our results suggest that mitochondrial oxidative phosphorylation, in conjunction with fatty acid β-oxidation, plays a crucial role in oocyte maturation, metabolism, and energy homeostasis in chondrostean fish.

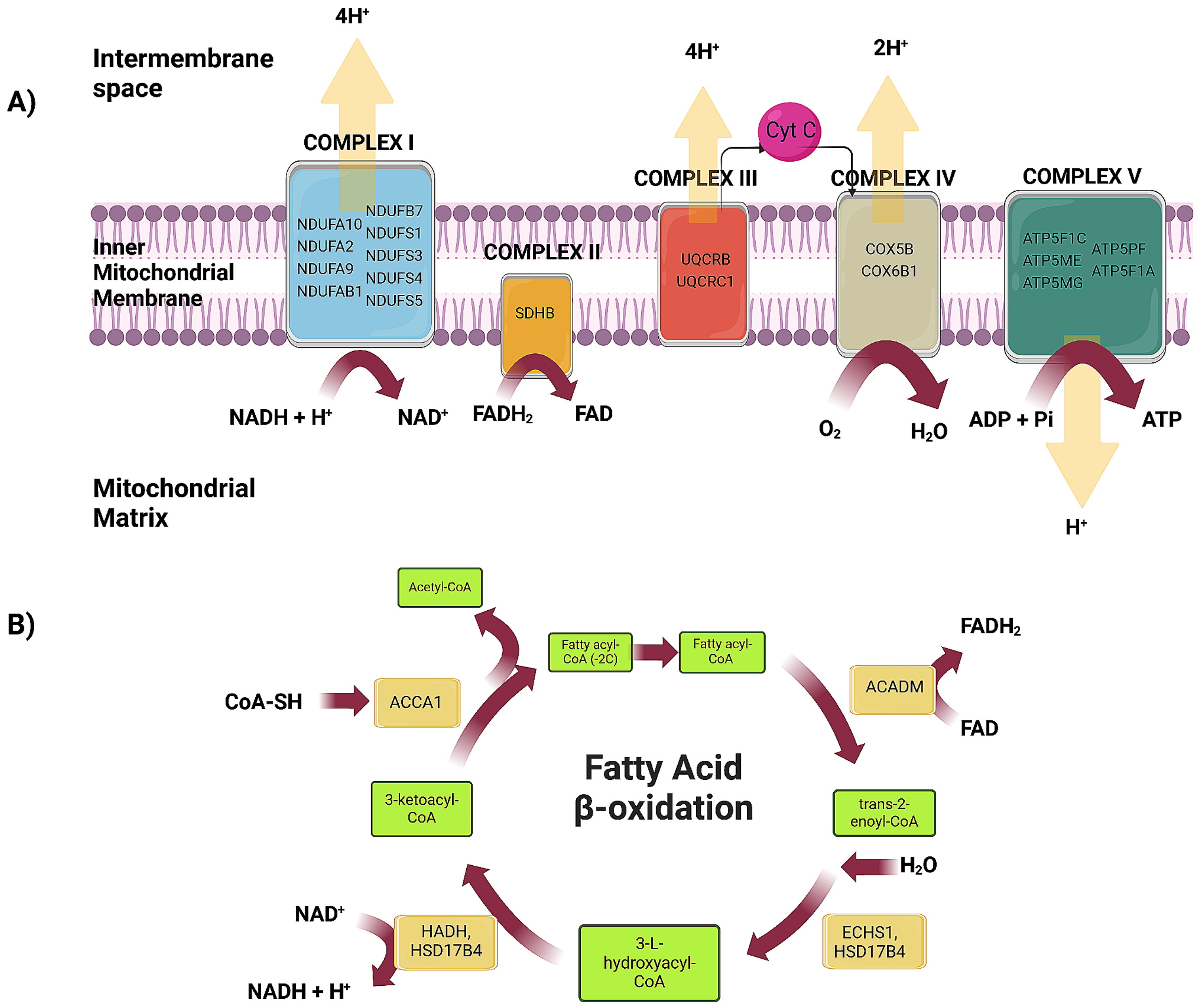


Fig. 6 Proteins identified in Siberian sturgeon eggs that participate in mitochondrial oxidative phosphorylation complexes I–V (**A**) and fatty acid β-oxidation I (**B**). The scheme was prepared using BioRender software. Descriptions of the gene/protein symbols are presented in Supplementary Table S2

Proteins involved in reproduction

We identified proteins involved in key reproduction processes in sturgeon OF and eggs, such as ovulation, oocyte maturation, prevention of polyspermy, fertilization, and sperm-egg activation. The eggs showed enrichment of common proteins, such as components of the ubiquitination cascade (UCHL1, UCHL3) and heat shock proteins (HSP5, HSP9 and HSP90AB1), which suggests their role in sturgeon oocyte development and regulation [52, 53]. ZP proteins (ZP1-4) were also increased in eggs, and these proteins are vital for oocyte growth, species-restricted fertilization, and preventing polyspermy [54]. Our investigation revealed 53 specific proteins in eggs that were primarily associated with early development processes. Among these proteins, we identified eight components of the 20 S and 26 S proteasomes that are essential for the ubiquitin-dependent protein degradation pathway and oocyte maturation in fish [55] and an oocyte-specific linker histone, protein B4, which is restricted to early development in Xenopus [56]. We also detected proteins, such as sperm acrosome associated 4 (SPACA4), zona pellucida sperm-binding protein 3 receptor (ZP3R) and glucosamine-6-phosphate isomerase 1 (GNPDA1), which are crucial for sperm-egg plasma membrane adhesion, fertilization, and early embryo development [57, 58]. In summary, our comprehensive analysis of sturgeon OF and egg protein profiles provides vital insights into the intricate mechanisms of female reproduction.

Comparison of OF and seminal plasma proteins

The comparison of Siberian sturgeon OF and seminal plasma (SP) proteins revealed similarities and specificities in their composition (Supplementary Figure S3, Supplementary Table S13). The identification of shared proteins in the OF and SP indicates the convergence of functional roles, particularly in the humoral response, metabolic processes, and cellular detoxification, which are sustained by common major proteins, such as ALB, TF, HPX, APOA1, and FEL [59], which are primarily associated with immune and stress responses. This finding reinforces the significance of the fluid milieu surrounding eggs and spermatozoa in providing a protective and supportive environment for their viability and functionality [8, 10, 11, 60]. The presence of OF-specific proteins involved in immune response processes, cell adhesion and metabolism reinforces its role in protection and oocyte maturation and highlights its potential nutritive contributions to gametes via the VTGs (see above). Conversely, proteins specific to seminal plasma, such as proteins related to developmental processes, metabolism, proteolysis, cell adhesion, and extracellular matrix organization, may be associated with spermatogenesis and sperm maturation. Significantly, our analysis identified specific proteins crucial for fertilization. We identified sperm-egg recognition proteins (ZP1, ZP2, ZP3, ZP4, T-complex protein 1 subunit epsilon (CCT5), folate receptor beta and gamma (FOLR2, FOLR3), voltage-dependent anion-selective channel protein 2 (VDAC2), and astacin-like metalloendopeptidase (ASTL)) in the OF. We identified proteins associated with the binding of sperm to the zona pellucida (acrosin (ACR), cAMP-dependent protein kinase type II-alpha regulatory subunit (PRKAR2A), zona pellucida-binding protein 2 (ZPBP2), T-complex protein 1 subunit gamma and eta (CCT3, CCT7), zonadhesin (ZAN), and arylsulfatase A (ARSA)) in seminal plasma. This finding highlights the distinct and complementary functions of the OF and SP proteins in facilitating successful sperm–egg interactions. The delineation of specific functions attributed to the OF and SP proteins provides valuable insights into their unique contributions to reproductive processes, which enhances our understanding of the complex interplay between these fluids and their impact on gamete function to ultimately influence fertilization success.

Conclusions

Our study presents a comprehensive characterization of the proteomic profiles of the OF and eggs from Siberian sturgeon and provides novel insights into their composition and functional significance. The identification of specific proteins and enrichment of distinct biological processes in each sample highlight the unique roles of the OF and eggs in sturgeon reproduction (Fig. 7).

Methods

Broodstock management, spawning and gamete collection

The Siberian sturgeons were maintained at the Department of Sturgeon Fish Breeding Inland Fisheries Institute in Pieczarki, Poland. The experiments were performed in the spawning season (May 2021) on five females (aged 9–14 years, body weight 16.5±2.7 kg). The sampling was performed using the reproduction protocol previously described by Szczepkowski [61]. Lighting for 24 h/0 h

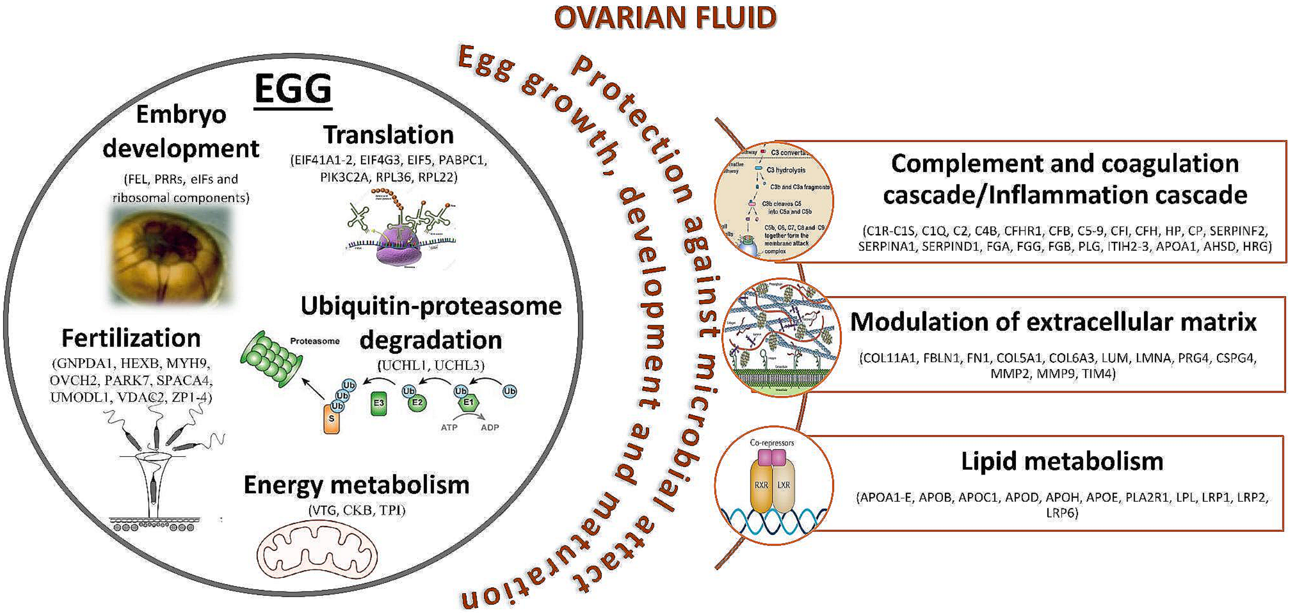


Fig. 7 Scheme presenting an overall summary of the unique roles of the identified proteins in the ovarian fluid (OF) and eggs of Siberian sturgeon. OF proteins were associated with the inflammatory cascade, modulation of the extracellular matrix and lipid metabolism, and proteins in the eggs were primarily involved in embryo development, fertilization, translation, ubiquitin - proteasome degradation and energy metabolism. Descriptions of the gene/protein symbols are presented in Supplementary Table S2

provided photostimulation, and the water temperature in the tanks was 16 °C. Females were hormonally stimulated with two doses of 0.1 mg kg^{-1} luteinizing hormone-releasing hormone agonist (LH-RHa) (Genscript, Piscataway, NJ, USA). The first injection was administered 36 h before planned sampling, and the second injection was given 24 h before sampling [62]. OF and eggs from the same female ($n=5$) were collected using a catheter [63] into separate dry plastic containers for each female. OF was collected from the surface of the egg using a pipette, taking care to avoid any contamination with blood. For fertilization, semen was collected from hormonally stimulated males using a catheter [64]. Before each handling, the fish were anesthetized via immersion in MS-222 solution (150 mg L^{-1}; Sigma–Aldrich, St. Louis., MO, USA). Ethics committee approval was not required for the experiments.

Gamete management and fertilization

Each OF ($n=5$) and egg ($n=5$) sample was first aliquoted into cryotubes and frozen in liquid nitrogen. The remaining eggs were used for fertilization. Each portion of eggs (app. 1.5 kg in portion) was fertilized with pooled milt collected from three males. Sperm motility was greater than 89%. The milt was diluted to 1:100 (20 mL per 1.5 kg of eggs) and incubated with the eggs for 3 min. The eggs were double-washed with hatchery water. Adhesiveness was removed from the eggs by double-washing with a tannin solution at a 1:2000 dilution then again in hatchery water [65]. After de-adhesion, the eggs were placed in McDonald jars. Fertilization success was determined at the second cleavage (4 h postfertilization) and hatching (after 7 days of incubation at 15 °C) stages.

Measurement of OF quality parameters

Osmolality was measured using a Minitübe Abfüll-u Labortechnik Löser apparatus (Tiefenbach, Germany). The protein concentration was measured by the Bradford method using a Coomassie Plus Kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as a standard. The pH of the OF was determined with a portable pH meter (HI9321 Hanna Instruments, USA).

Protein extraction from eggs

Proteins from eggs (100 mg) were extracted in lysis buffer (400 µl; 8 M urea, 2% CHAPS, 30 mM Tris, pH 8.5) containing 0.5% protease inhibitor cocktail (Sigma–Aldrich). Egg cells were homogenized for 4×30 s in 4 volumes of lysis buffer in a FastPrep-24 homogenizer and sonicated for 6×5 s on ice. The samples were incubated for 1 h on ice to facilitate protein extraction. Following incubation, the samples were centrifuged at 14 000×g for 15 min at 4 °C to separate the protein extract from the cellular debris. The protein concentration was measured using a Coomassie Plus Kit (Thermo Scientific) with bovine serum albumin as the standard. The protein extract (supernatant) was stored at -80 °C until analysis. The protein extracts were subjected to LC–MS/MS and Western blot analyses.

Liquid Chromatography–Mass Spectrometry

Protein digestion

Proteomic analysis was performed at the Mass Spectrometry Laboratory at the Institute of Biochemistry and Biophysics PAS, Warsaw.

Seventy micrograms of protein from each sample was digested according to the FASP protocol [66]. Cysteine groups were reduced by 1 h of incubation with 10 mM Tris (2-carboxyethyl) phosphine (TCEP) at 37 °C on a vortex. Protein solutions were transferred onto a Vivacon 30 kDa molecular weight cutoff filter (Sartorius Stedim). The filters were centrifuged at 14,500 × g for 15 min and washed with 200 µL of urea solution (8 M urea in 100 mM ammonium bicarbonate). Cysteines were alkylated via a 10-min incubation at room temperature with 25 mM methyl methanethiosulfonate (MMTS). The filters were washed three times with urea solution and 100 mM ammonium bicarbonate. After each addition, the samples were centrifuged until the cutoff filter was dry. Digestion was performed overnight using 2 µg of trypsin (Promega, WI, USA) at 37 °C (with an enzyme: protein ratio of 1:35). Peptides were eluted from spin filters by two 50 µL additions of 100 mM ammonium bicarbonate and one of 500 mM NaCl solution. The SpeedVac-dried samples were resuspended in 100 µL of 0.1% formic acid (FA) in water. Peptide concentrations were determined using a Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific).

Mass spectrometry

The samples were analyzed using an LC–MS/MS system composed of an Evosep One HPLC System (Evosep Biosystems, Odense, Danemark) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific). One microgram of peptide solution was loaded onto an Evotips C18 disposable trap column as previously described [67]. Peptides were fractionated using an 88 min (15 samples per day) predefined Evosep gradient at a flow rate of 220 nl/min on an analytical column (Dr Maisch C18 AQ, 1.9 µm beads, 150 µm ID, 15 cm long, Evosep Biosystems). The following data-dependent acquisition parameters were used: the top 40 precursors were selected for MS2 analysis, and a collisional induced fragmentation NCE of 30%, spray voltage of 2.1 kV, funnel RF level of 40, and heated capillary temperature of 275 °C. Full MS scans covered the mass range of 300–1600 m/z with a resolution of 60,000, a maximum injection time set to Auto and a normalized AGC target to 300%. MS2 scans were acquired at a resolution of 15,000, an automatic maximum injection time and a standard AGC target. The ion isolation window was set to 1.6 m/z, the dynamic exclusion time was set to 20 s, and the minimum intensity threshold was set to 5e3.

Data analysis

The raw data files were preprocessed with Mascot Distiller (version 2.7, Matrixscience, London, U.K) and searched against the *Acipenser* protein database derived from NCBI (version from 2021.12.12, 78.501 sequences) supplemented with popular contaminants (cRAP database, 115 sequences). Mass tolerance was set individually after offline mass recalibration (typical values for peptide mass tolerance: 5 ppm; for fragments– 0.01 Da). Other parameters were set as follows: enzyme– trypsin, fixed modifications– methylthio (C), variable modifications– oxidation (M), and missed cleavages– 1. The data were incorporated into the in-house data analysis software Mscan (http://proteom.ibb.waw.pl/mscan/). The FDR was calculated with the target/decoy strategy and kept under 1%. To obtain protein abundance estimates, a normalized spectral counting approach was used. For each sample, emPAI values were calculated using the numbers of unique precursors normalized to the total number of precursors to account for loading differences. The comparison of the main components of the two sample types was based on the fold-change values obtained by dividing the median emPAI from each sample group. These resulting values were used to approximate which proteins predominated in each type of sample.

Functional enrichment analysis of ovarian fluid and eggs

Gene Ontology (GO) annotations of identified proteins were obtained using the category 'biological process' (cut off at FDR = 0.05, ShinyGO v0.77: Gene Ontology Enrichment Analysis, http://bioinformatics.sdstate.edu/go/). The IPA (Qiagen, CA, US) of the identified proteins was used to interpret the identified proteins in the context of molecular and cellular functions and canonical pathways. The GI numbers of the identified proteins were matched to the UniProtKB database (www.UniProt.org). For protein–protein interaction network analysis, the differentially expressed proteins were analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (Search Tool for the Retrieval of Interacting Genes, http://string-db.org/). The search for interactions was restricted to *Homo sapiens* protein pairs. The reliability of the interactions between proteins was assessed using a combined score (edge score).

Western blot analysis

We used a Western blot procedure using stain-free gels (V3 stain-free workflow, Bio-Rad, Hercules, CA, USA) to validate the mass spectrometry results, as previously described [68], with some modifications. This method eliminates the need for the use of housekeeping proteins as loading controls for Western blots [69]. The expression of four proteins of interest, ALB, VTG2, FGB and FN1, was evaluated in the OF and eggs of sturgeon. The method of protein extraction from eggs was described above, and the OF was centrifuged at 3000 × g for 10 min at 4 °C. Equal amounts of protein (15 µg for FN1, 20 µg for VTG2 and ALB, 30 µg for FGB) were applied to Mini-Protean TGX Stain-Free 4–20% Gels (Bio-Rad). The quality of protein separation was checked after gel activation on a ChemiDoc instrument (Bio-Rad), and proteins were transferred to nitrocellulose membranes (0.22 µm) using a Mini Trans Biol Cell (Bio-Rad) in 20 mM Tris-HCl (pH 8.2), 150 mM glycine, and 10% methanol at 60 V for 90 min (4 °C). Nitrocellulose membranes were briefly rinsed in distilled water and blocked with 5% bovine serum albumin (Sigma‒Aldrich). The membranes were incubated overnight at 4 °C with primary polyclonal antibodies against ALB (1:1000), FGB (1:30000), FN1 (1:5000) and VTG2 (1:5000) (Supplementary Table S12). The membrane was rinsed to remove unbound primary antibodies and exposed to goat anti-rabbit antibodies (1:10000; Sigma‒Aldrich) linked to alkaline phosphatase. The products were visualized via incubation in a solution of alkaline phosphate buffer containing nitro blue tetrazolium (Sigma‒Aldrich) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma‒Aldrich) in the dark. The staining was stopped with 0.2 M EDTA. Antibody-bound proteins were detected via enhanced chemiluminescence using a ChemiDoc imaging system (Bio-Rad). The optical density of the protein bands detected on the membranes and the intensity of the protein bands on the TGX Stain-Free gels were analyzed using Image Lab 6 software (Bio-Rad). The image of the gel acquired before its transfer was used as a control for equal protein loading between samples. The volume density of each target protein band was normalized to its respective total protein content, and the total protein band density was normalized to the total protein loaded into each lane using stain-free technology. The data are expressed in arbitrary units according to the manufacturer's instructions (Bio-Rad) and Posch et al. [69]. To confirm the specificity of the antibodies used, protein bands corresponding to the detected bands were manually removed from the gels and prepared for digestion and protein identification using MALDI-TOF/TOF [70].

Experimental design and statistical rationale

To evaluate the protein composition and differences between the proteomes of the OF and eggs, the present study performed five biological replicates using LC‒MS/MS analysis. The resulting MS/MS data were processed using Mscan software. The FDR thresholds for proteins were specified at 1%. To estimate the protein abundance, emPAI was calculated using unique precursors and normalized by total abundance. The differentially abundant proteins were calculated (IBB, Warsaw) ($p < 0.05$, fold change > 3). Gene Ontology (GO) enrichment, KEGG pathways, and IPA (Ingenuity Pathway Analysis) were used to examine the functional significance of the identified proteins. Protein–protein interaction network analysis was performed using the STRING database with a medium confidence score cutoff of 0.4. The expression of four identified proteins was confirmed by Western blotting using specific antibodies. All of the details are described above.

Abbreviations

APPs	acute phase proteins
DAPs	differentially abundant proteins
ECM	extracellular matrix
emPAI	exponentially modified protein abundance index
EIFs	eukaryotic initiation factors
FA	formic acid
GO	Gene Ontology
HDL	high-density lipoprotein
IPA	Ingenuity Pathway Analysis
LH-RHa	luteinizing hormone releasing hormone agonist
OF	ovarian fluid
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
VLDL	very low density lipoprotein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10309-y.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8
Supplementary Material 9
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Supplementary Material 16

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Author contributions

N.K: Formal analysis, data curation, visualization, methodology, writing-original draft. A.C: Conceptualization, writing-original draft, writing-review & editing. B. S: Resources. A.M.: Formal analysis, data curation. M.A.D: Conceptualization, methodology, writing-original draft, writing - review & editing, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [71] partner repository with the dataset identifiers PXD044168 and https://doi.org/10.6019/PXD044168.

Declarations

Ethics approval and consent to participate

The Siberian sturgeon used for this study were sourced from a selectively bred broodstock at the Department of Sturgeon Fish Breeding in Pieczarki, Poland, in compliance with national aquaculture guidelines and veterinary requirements for activities in the aquaculture sector (veterinary identification number 28199201) and is under the supervision of the District Veterinary Officer. Our research adhered to the Polish Animal Protection Act (2023, Item 1580) and was exempt from additional ethics approval under the Polish Protection of Animals Used for Scientific Purposes Act (2015, Article 1.2, subparagraphs 1 and 5). All procedures followed the ARRIVE guidelines (https://arriveguidelines.org/).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Cryoprotectant-specific alterations in the proteome of Siberian sturgeon spermatozoa induced by cryopreservation

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Cryopreservation is crucial for conserving genetic diversity in endangered species including the critically endangered group of sturgeons (*Acipenseridae*), but it can compromise sperm quality and protein profiles. Although cryopreservation with dimethyl sulfoxide (DMSO) and methanol (MeOH) results in the recovery of good post-thaw motility, DMSO-preserved sperm show reduced fertilization ability. This study was conducted in Siberian sturgeon as a model for Acipenserid fishes to explore the effects of DMSO and MeOH on the proteome of semen using advanced proteomics methods—liquid chromatography—mass spectrometry and two-dimensional difference gel electrophoresis. We analyzed the proteomic profiles of fresh and cryopreserved spermatozoa and their extracellular medium and showed that cryopreservation decreases motility and viability and increases reactive oxygen species levels, membrane fluidity, and acrosome damage. Despite having similar post-thaw semen motility, sperm treated with DMSO had significantly lower fertilization success (6.2%) than those treated with MeOH (51.2%). A total of 224 and 118 differentially abundant proteins were identified in spermatozoa preserved with MeOH and DMSO, respectively. MeOH-related proteins were linked to chromosomal structure and mitochondrial functionality, while DMSO-related proteins impacted fertilization by altering the acrosome reaction and binding of sperm to the zona pellucida and nuclear organization. Additionally, cryopreservation led to alterations in the proacrosin/acrosin system in both cryoprotectants. This study provides the first comprehensive proteomic characterization of Siberian sturgeon sperm after cryopreservation, offering insights into how cryoprotectants impact fertilization ability.

Keywords *Acipenser baerii*, Semen, Extracellular medium, Cryopreservation, Proteome, Mass spectrometry

Sturgeons, which belong to the family *Acipenseridae*, are ancient fish of significant ecological, evolutionary, and economic interest which face a dramatic decline of their populations worldwide. As a result, is the most highly threatened family of all vertebrates (IUCN 2011)[1]. Sturgeon are characterized by unique reproductive biology, including atypical testicular morphology and a distinct sperm maturation process. In addition, compared to those of teleost fish, the spermatozoa of sturgeon exhibit a complex structure with an acrosome, extended motility duration, and lower sperm concentration. The seminal plasma also has unique characteristics, such as lower osmolality and protein concentration[2]. These factors pose additional challenges for the control of sturgeon reproduction. Given these complexities, deciphering and enhancing sturgeon reproductive success has emerged as a pivotal approach for conservation and sustainable aquaculture advancement.

The cryopreservation of spermatozoa is a critical technique for the ex situ conservation of genetic resources, facilitating the preservation of genetic diversity and supporting the reproduction of these endangered species. This technique, which involves freezing sperm cells at ultralow temperatures, effectively pauses biological time, safeguarding genetic material for future controlled fertilization endeavors. Semen cryopreservation in sturgeons, as detailed by Alavi et al.[3] and Kolyada et al.[4], often employs extenders containing sucrose with KCl buffered by

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Tris–HCl or saline solutions enriched with permeating cryoprotectants such as dimethyl sulfoxide (DMSO), N,N-dimethylacetamide (DMA), ethylene glycol (EG), methanol (MeOH), or the nonpermeating cryoprotectant egg yolk[5] to enhance post-thaw sperm viability and motility. However, the freeze−thaw cycle entails sudden osmotic and temperature shifts, which detrimentally impact semen quality, resulting in reduced sperm viability, diminished motility, and low fertilization capacity[3]. Furthermore, cryopreservation may alter spermatozoa molecular integrity, impacting the composition and functionality of sperm proteins that are crucial for effective fertilization and early embryonic development.

DMSO and MeOH are widely used as cryoprotectants due to their ability to freely permeate cell membranes, which is facilitated by their low hydrophilicity and molecular weight. These properties enable them to interrupt ice crystal nucleation and growth by forming hydrogen bonds with water[6]. Although cryopreservation with DMSO and MeOH resulted in the recovery of good post-thaw motility in sturgeon[7,8], the fertilization ability of DMSO-cryopreserved spermatozoa decreased, and these spermatozoa were characterized by higher amount of acrosome-reacted spermatozoa than MeOH-treated spermatozoa[9]. The exact nature of such disturbances is unknown and requires further investigation, especially regarding alteration in sturgeon-specific proteins. Such knowledge can potentially be important for developing recommendations concerning cryopreservation methods of sturgeon semen.

To explore the mechanism of cryodamage, proteomic techniques such as liquid chromatography−mass spectrometry (LC−MS) have been used to detect changes in sperm proteins of mammalian species[10–12]. In fish species, two-dimensional gel electrophoresis (2DE) and two-dimensional difference gel electrophoresis (2D-DIGE) have been used to analyze spermatozoa from common carp and sea bass[13,14], as well as the extracellular medium (EM) from common carp and rainbow trout[15,16], to monitor proteins released from sperm cells following cryopreservation. Only two studies have described the impact of cryopreservation on the sperm proteome in sterlet[17,18]. Despite the identification of a limited number of sturgeon proteins, these efforts underscore the need for further research to fully understand sturgeon sperm cryodamage mechanisms as a basis for the improvement of the method.

This study presents the hypothesis that cryopreservation with DMSO or MeOH differentially impacts the sperm proteome of the Siberian sturgeon, leading to specific molecular alterations. We also assumed that the DMSO variant would reveal changes in proteins critical for fertilization ability. The aim of this study was to bridge the gap in understanding sturgeon reproductive biology, the intricacies of sperm cryopreservation, and the roles of DMSO and MeOH as cryoprotectants. We used two complementary quantitative proteomic approaches, mass spectrometry-based (label-free LC−MS) and gel-based (2D-DIGE) coupled with matrix-assisted laser desorption/ionization time of flight (MALDI-TOF/TOF) mass spectrometry and performed simultaneous analysis of spermatozoa and the EM.

Methods

Ethics statement

The Siberian sturgeons used for this study were sourced from a selectively bred broodstock at the Department of Sturgeon Fish Breeding in Pieczarki, Poland, in compliance with national aquaculture guidelines and veterinary requirements for activities in the aquaculture sector (veterinary identification number 28199201) and were under the supervision of the District Veterinary Officer. Our research adhered to the Polish Animal Protection Act (2023, Item 1580) and was exempt from additional ethics approval under the Polish Protection of Animals Used for Scientific Purposes Act (2015, Article 1.2, subparagraphs 1 and 5) https://isap.sejm.gov.pl/isap.nsf/download.xsp/WDU20230000465/T/D20230465L.pdf and aligned with Directive 2010/63/EU (Article 1, Paragraph 5) on animal protection for scientific purposes. All procedures followed the ARRIVE guidelines (https://arriveguidelines.org/).

Fish maintenance and gamete collection

Semen was collected from seven mature Siberian sturgeons (weighing 9.5 ± 2.9 kg and aged 7–14 years) maintained in the Department of Sturgeon Fish Breeding at the National Inland Fisheries Research Institute in Pieczarki, Poland. Twenty-four hours before semen collection, males were hormonally stimulated by an injection of 0.03 mg kg^{-1} of luteinizing hormone-releasing hormone (LH-RHa, Genscript, USA)[19]. Semen samples were collected via a syringe attached to a rigid tube inserted into the urogenital opening, ensuring no blood contamination. The semen was stored individually in open glass beakers at 8 °C.

Eggs were collected from two mature female sturgeons (aged 9 and 14 years, body weight 16.5 ± 2.7 kg) maintained in the Department of Sturgeon Fish Breeding at the National Inland Fisheries Research Institute. The females were hormonally stimulated with two doses totalling 0.1 mg kg^{-1} of LH-RHa (GenScript), which were administered 36 and 24 h prior to ovulation and egg collection[20]. Eggs were collected via a catheter[21] in separate dry plastic containers for each female. Before gamete collection, the fish were anesthetized by spraying the gills with a Propiscin anesthetic (IFI, Poland) solution at a concentration of 15 mg L^{-1} (100 mL per fish).

Cryopreservation of semen

Semen samples were frozen individually (n = 7). The milt was diluted 1:1 with an extender containing 0.1 M glucose and 15% MeOH or 15% DMSO according to Judycka et al.[19]. This mixture was loaded into 250 µL plastic straws (IMV Technologies, L'Agile, France), which were placed on a floating rack (Minitübe GmbH, Tiefenbach, Germany). The straws were then frozen in liquid nitrogen vapor (3 cm above the liquid surface) for 5 min in a Styrofoam box with an isolating Neopor block (Minitübe GmbH) followed by placement in liquid nitrogen. Straws were thawed by immersion in a 40 °C water bath for 6 s and immediately used for the analysis of sperm quality parameters and proteomic analysis. The experimental design is shown in Fig. 1.

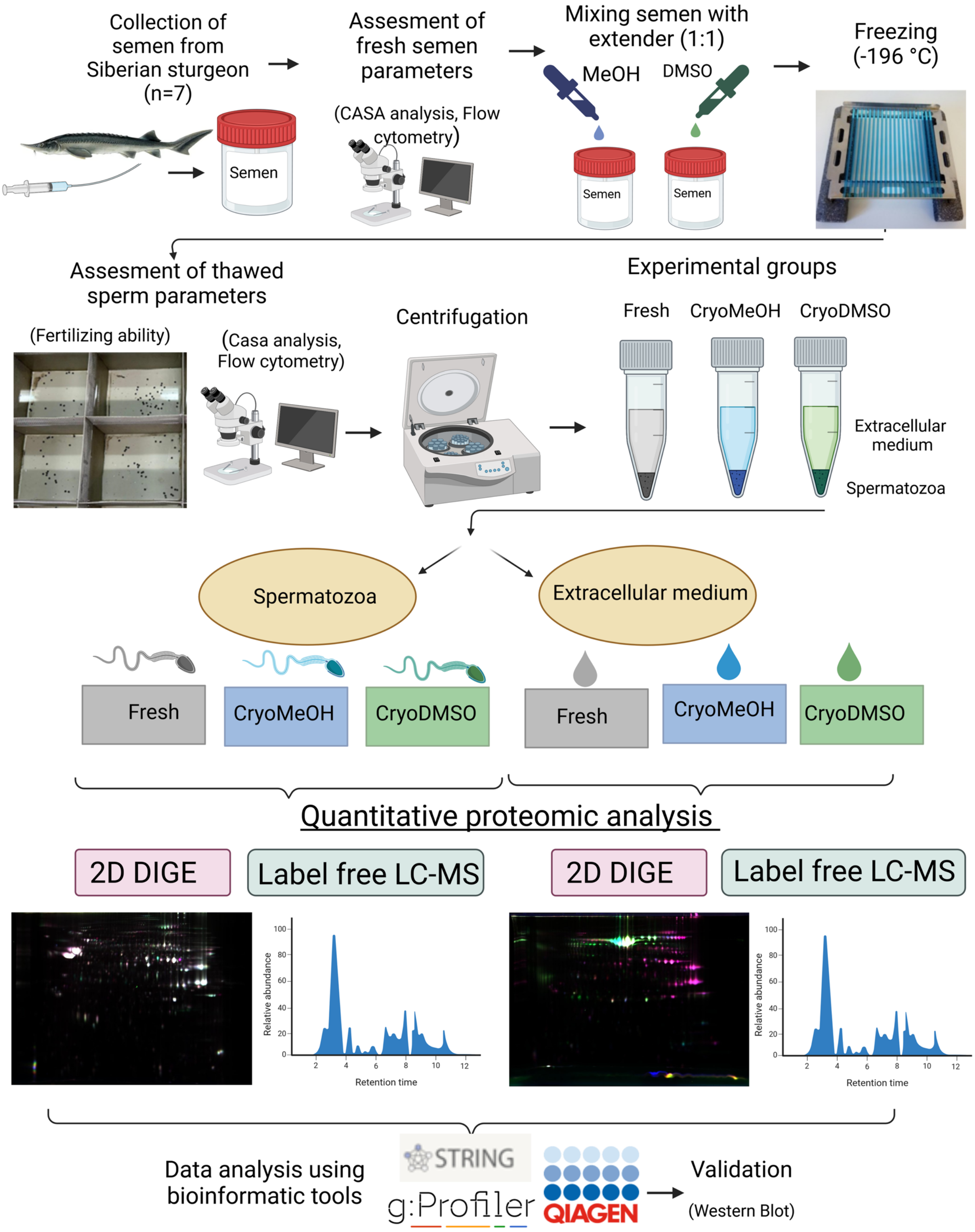


Figure 1. Experimental design and workflow for the proteomic analysis of cryopreserved Siberian sturgeon spermatozoa and extracellular medium using two complementary proteomic approaches: gel-based (2D-DIGE) and gel-free (label-free LC–MS) methods.

Measurement of semen quality parameters

Sperm motility parameters

The sperm movement characteristics were measured before and after cryopreservation via computer-assisted sperm analysis with the CEROS II system (Hamilton-Thorne, USA) using 12-well multitest slides (well diameter 4 mm, depth 30 µm; ICN Biomedicals Inc., Aurora, OH, USA). The semen was activated in 1:100 (for fresh semen) or 1:50 (for frozen/thawed semen) dilutions of activation solution (10 mM Tris, 20 mM NaCl and 2 mM

$CaCl_2$, 0.5% bovine serum albumin, pH 8.5)[22] at 8 °C. The value for each sample represents the mean of two separate measurements of motility parameters of 200 spermatozoa. Eight representative motility parameters were chosen for further analysis: straight-line velocity (VSL, $\mu m\ s^{-1}$), curvilinear velocity (VCL, $\mu m\ s^{-1}$), average path velocity (VAP, $\mu m\ s^{-1}$), linearity (LIN = 100 × VSL/VCL, %), amplitude of lateral head displacement (ALH, µm), percentage of motile sperm (MOT, %), beat-cross frequency (BCF, Hz) and progressive motility (PROG, %).

Flow cytometry analysis
Flow cytometry analyses of fresh and cryopreserved semen were performed using a Guava EasyCyte Flow Cytometer (Guava Technologies Inc., Luminex, Austin, TX, USA) cytometer. The data were acquired using GuavaSoft 3.2 software (Merck KGaA, Darmstadt, Germany). Sperm membrane fluidity, sperm viability, ROS^+ levels and acrosomal membrane integrity were assessed. Calibration was performed using the EasyCyte Check Kit (Merck KGaA) according to the manufacturer's instructions.

<u>Sperm concentration and viability.</u> For the evaluation of sperm concentration and viability, semen was diluted 1:100 (for fresh semen) or 1:50 (for frozen/thawed semen) with immobilizing solution. The diluted semen was rediluted with ViaCount Reagent to obtain a final concentration of 3×10^5 cells per mL and measured according to the Guava ViaCount protocol (Luminex, USA).

<u>Membrane fluidity.</u> The spermatozoa membrane fluidity was assessed using a flow cytometric method incorporating both the lipophilic dye merocyanine-540 (M540) and the impermeable nucleic acid stain Yo-Pro-1 (YP), adapted from Guthrie et al.[23]. A stock solution of M540 (5 mM) was prepared in DMSO, and for analysis, a working solution was created at a 1:100 dilution in immobilizing buffer (1 L: 1 g NaCl, 0.2 g KCl, 0.05 g $CaCl_2$, 0.05 g $MgSO_4$, 0.15 g $NaH_2PO\ 4 \times 2H_2O$, 0.38 g $Na_2HPO_4 \times 12H_2O$, 9.0 g glucose, pH 7.3–7.5)[24]. A 0.1 mM stock solution of YP in DMSO was diluted at a 1:10 ratio, and this diluted solution was added to 190 µL of the semen sample (1×10^6 cells per mL) and incubated for 10 min. at 37 °C. After this incubation, 5 µL of the M540 working solution was added to each sample, followed by a 2-min. incubation at 37 °C. The samples were then subjected to flow cytometry analysis, with green fluorescence (YP) detected using a 525-nm bandpass filter and red fluorescence (M540) detected using a 575-nm bandpass filter.

<u>Oxidative stress (ROS-positive cell content).</u> Oxidative stress was measured using a portable flow cytometer (Muse Cell Analyzer, Millipore, Billerica, MA, USA). The ROS in the samples were measured using a Muse Oxidative Stress kit according to Mostek et al.[25]. Briefly, semen was diluted to a concentration of 1×10^7 spermatozoa per mL; then, 10 µL of the semen suspension was mixed with 190 µL of Muse Oxidative Stress Working Solution and incubated at 37 °C for 30 min. A positive control containing completely oxidized sperm cells was prepared by incubating sperm cells in 3% H_2O_2. The data were generated using the Muse Count and Viability Software Module (Millipore).

<u>Acrosome integrity.</u> The sperm acrosome status was assessed with the lectin PNA from *Arachis hypogaea* conjugated with Alexa Fluor® 488 (Life Technologies Ltd., Grand Island, NY, USA). The samples (3×10^5 cells per mL) were combined with 1 µL of PNA working solution (1 mg mL^{-1}) and incubated for 5 min at room temperature in the dark. After incubation, 1 µL of propidium iodide (PI) (BD Biosciences, USA) was added before fluorescence analysis. The samples then underwent flow cytometry analysis, with green fluorescence (PNA) detected using a 515-nm bandpass filter, indicating acrosome-reacted spermatozoa and red fluorescence (PI) detected using a 617-nm bandpass filter, indicating dead spermatozoa.

Fluorescence microscopy analyses
The samples (1×10^6 cells per mL) were prepared as described above for the *Acrosome Integrity* Sect. "Acrosome integrity" with the same fluorochromes (PNA and PI). After staining, spermatozoa were fixed with 1% paraformaldehyde (PFA) for 5 min. Four microliters of the sperm suspension were placed on a clean glass slide and covered with a cover slip. The cells were observed under an Axio Observer Z1/7 fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany) equipped with ZEN 2.3 blue edition software (Carl Zeiss).

Measurement of seminal plasma osmolality and protein concentration
Seminal plasma was obtained by centrifugation of fresh semen at 9000×*g* (4 °C, 10 min), and then the supernatant was centrifuged again at 10,000×*g* (4 °C, 10 min). Osmolality was measured using a Minitübe Abfüll-u Labortechnik Loser apparatus (Tiefenbach, Germany). The protein concentration was measured by the Bradford method using a Coomassie Plus Kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as a standard.

Fertilization
Fertilization was performed one year after semen cryopreservation using eggs collected from two mature females. Each variant of cryopreserved semen (n = 7 for each cryoprotectant) was used separately for each female. Immediately before fertilization, the straws were thawed in a water bath at 40 °C for 5 s. The fertilization procedure was conducted according to Judycka et al.[19]. Briefly, an adequate volume of frozen/thawed milt (100,000 spermatozoa/egg) was added to approximately 100 eggs, and then 5 mL of sperm activation solution (10 mM Tris, 20 mM NaCl and 2 mM $CaCl_2$, pH 8.5) was added and left for 3 min. Next, the samples were washed with hatchery water. Egg samples were placed in separate baskets in an incubation apparatus. Fertilization rates were calculated as

the percentage of hatched larvae after 7 days of incubation at 15 °C. All trials were replicated twice. An excess of fresh semen collected from two males was used exclusively to test the quality of the eggs.

Preparation of spermatozoa and extracellular medium for proteomic analysis

EM, which is defined as a fluid surrounding extended semen[15,16], was obtained by centrifugation of fresh semen diluted 1:1 with immobilizing buffer (2.3.2.1) or semen cryopreserved with MeOH or DMSO at 9000×*g* (4 °C, 10 min) followed by a second centrifugation at 12,000×*g* (4 °C, 10 min).

The spermatozoa pellets were washed twice in immobilizing solution by centrifugation at 3000×*g* at 4 °C for 10 min. Spermatozoa proteins were extracted according to Dietrich et al.[26] using lysis buffer (8 M urea, 2 M thiourea, 4% [w/v] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]), 0.1% (w/v) Triton X-100, 100 mM dithiotreitol (DTT), 2% (v/v) immobilized pH gradient (IPG) buffer (3–10 NL), and 2.5% (v/v) protease inhibitor cocktail (Sigma–Aldrich, St. Louis, USA). Next, the samples were sonicated (5 s three times), kept on ice for 1 h, and centrifuged for 10 min at 14,000×*g* at 4 °C. The spermatozoa protein extracts were stored at − 80 °C until analysis.

Label-free LC–MS

Protein digestion

Spermatozoa. The sperm protein extracts were processed using a Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol, and the pellets were reconstituted in 100 µL of lysis buffer containing 1% SDS, 6% trifluoroethanol and 100 mM ammonium bicarbonate buffer (ABC). After 15 min of sonication in an ultrasonic bath followed by 15 min of vortexing, the proteins were digested according to the FASP method as described in a previous publication with minor modifications[27]. In brief, the samples were vortexed for 60 min in 10 mM tris(2-carboxyethyl)phosphine (TCEP) at 60 °C to reduce cysteine residues and then were transferred onto a Vivacon 30 kDa molecular weight cutoff filter (Sartorius Stedim Biotech). Proteins were rinsed with urea solution (8 M urea in 100 mM ABC) before cysteine blocking with 25 mM chloroacetamide (CAA) for 30 min at room temperature. The filters were further washed three times with urea buffer and three times with ABC. After each addition, the samples were centrifuged for 30 min at 14,500 × g until the cutoff filter was dry. Digestion was performed overnight using 2.4 µg of trypsin (Promega GmbH) at 37 °C. Peptides were eluted from spin filters by two washes with ABC. Peptide concentrations were measured using a Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific). For analysis, 10 µg of peptide was suspended in 100 µL of Evosep solvent A (0.1% formic acid (FA)) in water.

EM. Three hundred microliters of each sample were subjected to protein precipitation and digestion. Proteins were precipitated by vortexing for 40 min with 80 µL of 90% trichloroacetic acid and 620 µL of acetonitrile (ACN). After centrifugation (30 min, 21,000×*g*, 4 °C), the protein pellets were washed three times with 1 mL of ice-cold ACN. Proteins were reconstituted in 40 µL of urea buffer (7.5 M urea, 10 mM tris(2-carboxyethyl) phosphine, 100 mM ABC) and vortexed for 60 min at 37 °C. Reduced cysteines were blocked by the addition of 30 mM methyl methanethiosulfonate (MMTS). Digestion was performed using 3 µg of trypsin/Lys-C mix (Promega GmbH) in two steps—30 min at 60 °C, followed by the addition of 250 µL of ABC buffer and overnight incubation at 37 °C. Peptide samples were acidified with FA to a final concentration of 0.1%.

Mass spectrometry

Spermatozoa and EM samples (n = 7 for each group) were measured using LC–MS system composed of an Evosep One instrument (Evosep Biosystems) directly coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). One-third of the final sample volume was loaded onto disposable Evotips C18 trap columns (Evosep Biosystems) according to the manufacturer's protocol. Chromatography was conducted at a flow rate of 220 nL min^{-1} using an 88 min (15 samples per day) gradient on an EV1106 analytical column (Dr Maisch C18 AQ, 1.9 m beads, 150 m ID, 15 cm long, Bruker). The data were acquired in positive mode with a data-dependent method using the following parameters. The MS1 resolution was set at 60,000 with a normalized AGC target of 300%, an auto maximum injection time and a scan range of 300 to 1600 mz. For MS2, the resolution was set at 15 000 with a standard normalized AGC target, auto maximum injection time and top 40 precursors within an isolation window of 1.6 mz considered for MS/MS analysis. Dynamic exclusion was set at 20 s with an allowed mass tolerance of 10 ppm, and the precursor intensity threshold was set at 5e3. Precursors were fragmented in HCD mode with a normalized collision energy of 30%. The spray voltage was set to 2.1 kV, the funnel RF level was 40, and the capillary temperature was 275 °C.

Data analysis

Two sets of calculations in Andromeda MaxQuant (version 2.1.1.0) were performed using *Acipenser* proteins derived from NCBI (version 13042023 for spermatozoa and version 10012023 for EM) supplemented with common contaminants. Label-free quantification (LFQ), along with the match between runs option, was enabled, and a target decoy strategy was used to determine the false discovery rate (FDR). Other search parameters were as follows: variable modification oxidation (M), fixed modification methylthio (C), enzyme trypsin, missed cleavages 2, PSM and protein FDR 0.01. The quantitative results from the global analysis were further analyzed in Perseus (version 1.6.15). Proteins identified only by site, contaminants and proteins from the reverse database were removed from the analysis. The intensity values were log2-transformed. Proteins with one or no quantitative value in one or more groups were treated separately.

2D-DIGE and protein identification by MALDI TOF/TOF

2D-DIGE

Two independent 2D-DIGE analyses were performed to compare the protein profiles of spermatozoa and EM before and after cryopreservation with different cryoprotectants (DMSO or MeOH; n = 7 for each group). Before analysis, the EM was concentrated using an Amicon ultracentrifuge filter with a cutoff of 3 kDa (Millipore). Aliquots containing approximately 800 µg of spermatozoa and EM proteins were processed using a Clean-Up Kit (GE Healthcare) according to the manufacturer's protocol. For each biological replicate, 50 µg of protein extract of each sample type (spermatozoa and EM from fresh and cryopreserved samples with MeOH or DMSO) was labeled with 400 pmol of Cy3 or Cy5 (GE Healthcare), with dye swaps to exclude dye bias. An internal standard was created by mixing equal amounts of each sample within the experiment and was labeled with Cy2. After incubation on ice and in the dark for 30 min, the reaction was terminated by the addition of 10 mM lysine. The three labeled samples were then combined within each experiment and diluted with rehydration buffer (8 M urea, 2% CHAPS, 18 mM dithiothreitol (DTT), 0.5% carrier ampholyte, pH 3–10 NL) to 340 µL. The combined samples were loaded on a pH gradient strip (24 cm, pH 3–10 NL) for isoelectric focusing (IEF) on an Ettan IPGphor system (Amersham Biosciences) as described by Dietrich et al.[28]. After IEF, the strips were first equilibrated in equilibration solution (50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, traces of bromophenol blue, and 1% (w/v) DTT) for 15 min and later in the same solution except that DTT was replaced by 4% (w/v) iodoacetamide for an additional 15 min. IPG strips were transferred onto 12.5% vertical polyacrylamide gels (with a gel size of 25.5 × 19.6 cm and a thickness of 1 mm) cast on low-fluorescence glass plates using an Ettan DALTsix system (GE Healthcare, Uppsala, Sweden). Electrophoresis was conducted overnight at a constant current of 1.5 W per gel. The Cy2-, Cy3-, and Cy5-labeled images were acquired on a Typhoon 9400 scanner (Amersham Biosciences) at excitation and emission wavelengths of 488/520, 532/580, and 633/670 nm, respectively. Intragel spot detection and quantification and Intergel matching and quantification were performed using differential in-gel analysis (DIA) and biological variation analysis (BVA) modules of DeCyder software version 6.5 (Amersham Biosciences) according to Dietrich et al.[15].

MALDI-TOF/TOF protein identification

After 2D-DIGE analysis, the differentially abundant spots were manually cut off from the gels and prepared for digestion and protein identification using MALDI-TOF/TOF. Spots were digested and identified using a protocol described previously[18]. Briefly, MS and MS/MS spectra were searched using MascotServer (Matrix Sciences) in the *Acipenser* database, created on 05.07.2022 with the following MASCOT settings: cleavage enzyme, trypsin; max missed cleavages, 2; fragment ion mass tolerance, 0.7 Da; parent ion mass tolerance, 50 ppm; alkylation of cysteine by carbamidomethylation as a fixed modification; and oxidation of methionine as a variable modification. For the peptide mass fingerprinting (PMF) and MS/MS ion searches, statistically significant ($p \le 0.05$) matches identified by MASCOT were regarded as correct hits.

Functional classification of differentially abundant proteins (DAPs)

Ingenuity pathway analysis (IPA) (Qiagen, CA, US) of the identified proteins was used to interpret the identified proteins in the context of molecular and cellular functions and canonical pathways. The functional profiling of the identified proteins into three Gene Ontology (GO) categories, biological processes, cellular components, and molecular functions, was performed using ShinyGO (v. 0.8; http://bioinformatics.sdstate.edu/go/) and g:Profiler (https://biit.cs.ut.ee/gprofiler/gost, v. e111_eg58_p18_30541362). Protein–protein interaction network analysis of proteins associated with reproductive processes was performed using the Search Tool for the Retrieval of Interacting Genes (STRING) database (v. 12.0; http://string-db.org/) with a medium confidence score cutoff of 0.4. The search for interactions was restricted to *Homo sapiens* protein pairs. The reliability of the interactions between proteins was assessed by a combined score (edge score). Functional diagrams were visualized using SRplot tools (http://www.bioinformatics.com.cn/SRplot).

Western blot analysis

We used a western blot procedure using stain-free gels (V3 stain-free workflow, Bio-Rad, Hercules, CA, USA) to validate the mass spectrometry results, as previously described by Kodzik et al.[29], with some modifications. This method eliminates the need for housekeeping proteins as loading controls for western blots[30]. The expression of seven proteins of interest (L-lactate dehydrogenase A chain (LDHA), creatine kinase B-type (CKB), enolase 3 (ENO3), fructose-bisphosphate aldolase A (ALDOA), histone (H2A.Z), mitochondrial fumarate hydratase (FH) and acrosin (ACR)) were evaluated. Equal amounts of protein (10 µg for LDHA, CKB, ENO3, ALDOA, and FH and 20 µg for H2A.Z and ACR in spermatozoa and 7 µg for LDHA, ENO3, and ALDOA, 10 µg for CKB, H2A.Z, and FH and 9 µg for ACR in EM) were applied to Mini-Protean TGX Stain-Free 4–20% gels (Bio-Rad, Hercules, CA, USA). The quality of protein separation was checked after gel activation on a ChemiDoc imager (Bio-Rad), and proteins were then transferred to nitrocellulose membranes (0.22 µm) using a Mini Trans Biol Cell (Bio-Rad) in 20 mM Tris–HCl (pH 8.2), 150 mM glycine, and 10% MeOH at 60 V for 90 min (4 °C). Nitrocellulose membranes were briefly rinsed in distilled water and blocked with 5% nonfat dried milk. Next, the membranes were incubated overnight at 4 °C with primary polyclonal antibodies against LDHA, CKB, ENO3, ALDOA, FH, H2A.Z and ACR. Details of the dilution of the tested antibodies are provided in Supplementary Table S1. After the membranes were rinsed to remove unbound primary antibodies, they were exposed to goat anti-rabbit antibodies (Sigma—Aldrich) linked to alkaline phosphatase. The products were visualized by incubation in a solution of alkaline phosphate buffer containing nitro blue tetrazolium (Sigma—Aldrich) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma—Aldrich) in the dark. The staining was stopped with 0.2 M EDTA. Antibody-bound proteins were detected by enhanced chemiluminescence using a ChemiDoc imaging system (Bio-Rad). The optical density of the protein bands detected on the membranes and the intensity of the protein bands on the TGX Stain-Free gels were analyzed using Image Lab 6 software (Bio-Rad). The image of the gel acquired before its transfer was used as a control for equal protein loading. The volume density of each target protein band was normalized to its respective total protein content, whereas the total protein band density was normalized to the total protein loaded into each lane using stain-free technology, with the data expressed in arbitrary units according to the manufacturer's instructions (Bio-Rad) and Posch et al.[30].

Statistical analysis

One-way ANOVA followed by Tukey's test for post hoc comparison of means was used for the analysis of sperm quality parameters and western blotting. To analyze the differences in the hatching rates of semen cryopreserved with MeOH and DMSO for each female, two-way ANOVA with a Šidák post hoc test was used. The percentage data were normalized using an arcsine square root transformation prior to the statistical procedures. The results are presented as the mean ± standard deviation (SD). All the statistical analyses were performed at a significance level of $p < 0.05$ using GraphPad Prism software 9.0.0 (GraphPad Software Inc., San Diego, CA, USA).

Statistical analysis of changes in protein abundance after 2D-DIGE and label-free LC—MS was performed using the BVA module of DeCyder Differential In-Gel Analysis v. 5.02 and Perseus software (version 1.6.15.0), respectively. Protein spots with a p value < 0.05 according to one-way ANOVA, which indicated an increase or decrease in relative intensity, were considered DAPs. For LC—MS analysis of spermatozoa, paired t tests with Benjamini—Hochberg correction for multiple testing were performed to compare the groups, with significance assigned to protein peptides with q values less than 0.05. For EM, two-sample t tests with permutation-based FDR were performed on proteins with at least three valid values in both tested groups.

Principal component analysis (PCA) and heatmaps for 2D-DIGE data of the protein abundance change between samples were generated using DeCyder Differential Analysis Software (v 5.0; GE Healthcare) in the BVA module, whereas LC—MS data were generated using Perseus software (version 1.6.15).

Results

Parameters of fresh semen

The quality parameters of individual Siberian sturgeon semen samples, detailed in Supplementary Table 2, demonstrated high sperm motility (> 80%) and viability (> 96%). The sperm concentration varied between 0.85 and 2.50×10^9 spermatozoa mL^{-1}, with seminal plasma osmolality ranging from 81 to 102 mOsm kg^{-1} and a protein concentration of 219.35 ± 72.80 μg mL^{-1}.

Impact of cryopreservation with MeOH and DMSO on sperm quality parameters

Sperm movement characteristics

Cryopreservation significantly reduced MOT, VCL and PROG, regardless of the cryoprotectant used (Fig. 2). A decrease in ALH was observed for DMSO-cryopreserved semen, while the BCF increased in MeOH-cryopreserved semen compared to fresh semen. These changes in ALH and BCF were significantly different when comparing semen cryopreserved with MeOH to that cryopreserved with DMSO. Other motility parameters did not differ between MeOH- and DMSO-cryopreserved semen.

Flow cytometry parameters

Membrane fluidity. Flow cytometry analysis using M540 and YP staining revealed significant changes in sperm membrane fluidity between fresh and cryopreserved semen with DMSO and MeOH (Fig. 3A). The proportion of live sperm with destabilized membranes ($YP^-/M540^+$) increased from 1.5 ± 0.4% in fresh semen to 10.1 ± 4.1% and 12.3 ± 5.2% in semen cryopreserved with MeOH and DMSO, respectively, with DMSO treatment showing a more pronounced increase. Similarly, the percentage of live sperm with intact membranes ($YP^-/M540^-$) decreased from 90.7 ± 5.3% in the fresh samples to 29.8 ± 7.3% and 35.4 ± 8.3% in the MeOH- and DMSO-cryopreserved samples, respectively.

Sperm viability. Cryopreservation resulted in a significant reduction in sperm viability, decreasing from 97.9% ± 1.3% in fresh semen to 61.1% ± 3.8% and 70% ± 3.2% in cryopreserved semen with MeOH and DMSO, respectively (Fig. 3B). Notably, the viability of sperm cryopreserved in DMSO was significantly greater than that of those cryopreserved in MeOH.

$ROS^{\pm}$ level. Cryopreservation increased the percentage of sperm undergoing oxidative stress, with the proportion increasing from 10.7% ± 2.5% in fresh semen to 23.1% ± 8.6% and 23.7% ± 7.7% in cryopreserved semen with MeOH and DMSO, respectively (Fig. 3C). No significant differences in ROS production were detected between MeOH- and DMSO-cryopreserved semen.

Acrosome integrity. Flow cytometric analysis of acrosome membrane integrity for fresh and cryopreserved semen is shown in Fig. 4. Briefly, the proportion of live sperm with intact acrosomes (PI^-/PNA^-) decreased from 96.5 ± 2.3% in fresh semen to 57.5 ± 4.1% and 68.8 ± 7.5% in semen cryopreserved with MeOH and DMSO, respectively. Similarly, the percentage of live sperm with damaged acrosomes (PI^-/PNA^+) increased from 0.3 ± 0.5% in the fresh samples to 0.65 ± 0.54% and 1.00 ± 0.66% in the MeOH– and DMSO-cryopreserved samples, respectively (Fig. 4C). The percentage of dead spermatozoa with damaged acrosomes (PI^+/PNA^+) significantly increased from 0.3 ± 0.2% in the fresh samples to 24.8 ± 3.4% and 21.8 ± 4.7% in the MeOH- and DMSO-cryopreserved samples, respectively. No significant changes were observed between cryopreserved samples in

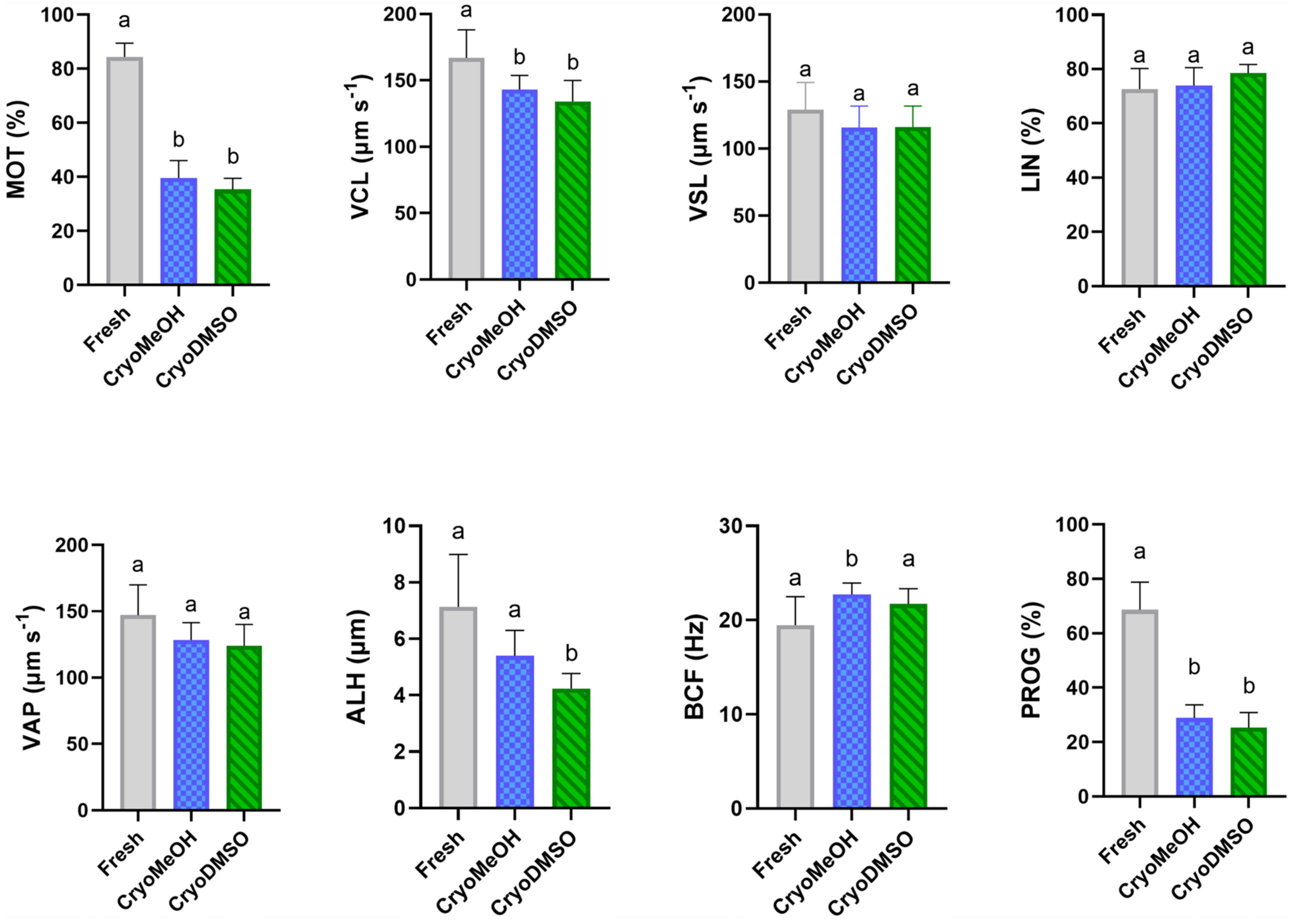


Figure 2. Impact of cryopreservation with the use of an extender containing either MeOH or DMSO on sperm movement parameters, including motility (MOT), curvilinear velocity (VCL), straight-line velocity (VSL), linearity (LIN), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and progressive motility (PROG). The results are expressed as the mean ± SDs. Different superscripts indicate significant differences ($p < 0.05$) between groups.

any group (Fig. 4C). Representative flow cytometry plots and fluorescence microscopy images are shown in Fig. 4A and B, respectively. The number of spermatozoa with reacted acrosomes was negatively correlated with the variability of fresh and cryopreserved spermatozoa (Fig. 4D).

Hatching rate

The use of semen cryopreserved with DMSO resulted in a significantly lower hatching rate (0.8 ± 1.6% for female #1 and 11.6 ± 13.3% for female #2) compared to semen cryopreserved with MeOH (35.5 ± 8.5% for female #1 and 66.9 ± 18.1% for female #2) for both females ($p < 0.0001$; Fig. 5). Significant differences in hatching rates were also observed among females under the same cryoprotectant conditions ($p < 0.05$). Fresh semen, used as a control to assess egg quality, achieved a hatching rate of 57% for female #1 and 75% for female #2.

Label-free LC—MS and 2D-DIGE identification of DAPs in spermatozoa and EM of fresh and cryopreserved semen with MeOH or DMSO

Spermatozoa

Using label-free LC—MS, we identified 1109 proteins from at least four biological replicates across the fresh, CryoMeOH, and CryoDMSO groups. Among these, 160 proteins exhibited differential abundance in the comparison of MeOH vs. fresh groups, and 43 proteins were differentially abundant when comparing between CryoDMSO and fresh groups. A comparison between CryoMeOH and CryoDMSO spermatozoa identified four DAPs. The putative methyltransferase DDB_G0268948, which does not have specific human homologs, was identified in the MeOH group compared with the fresh group. These findings are detailed in Supplementary Table S3.

Further analysis employing the 2D-DIGE approach revealed significant changes in the abundance of spermatozoa proteins, with 163 and 144 protein spots altered following cryopreservation in the MeOH and DMSO groups, respectively, out of 2,469 matched spots. These corresponded to 93 and 86 unique proteins, with 35 and 28 proteins identified in multiple protein spots, likely representing proteoforms varying by isoelectric point (pI) and/or molecular weight (MW). Notable examples include pyruvate kinase PKM (PKM) (identified in 4 spots for MeOH and 8 for DMSO), LDHA (3 spots for MeOH and 4 for DMSO), glyceraldehyde-3-phosphate

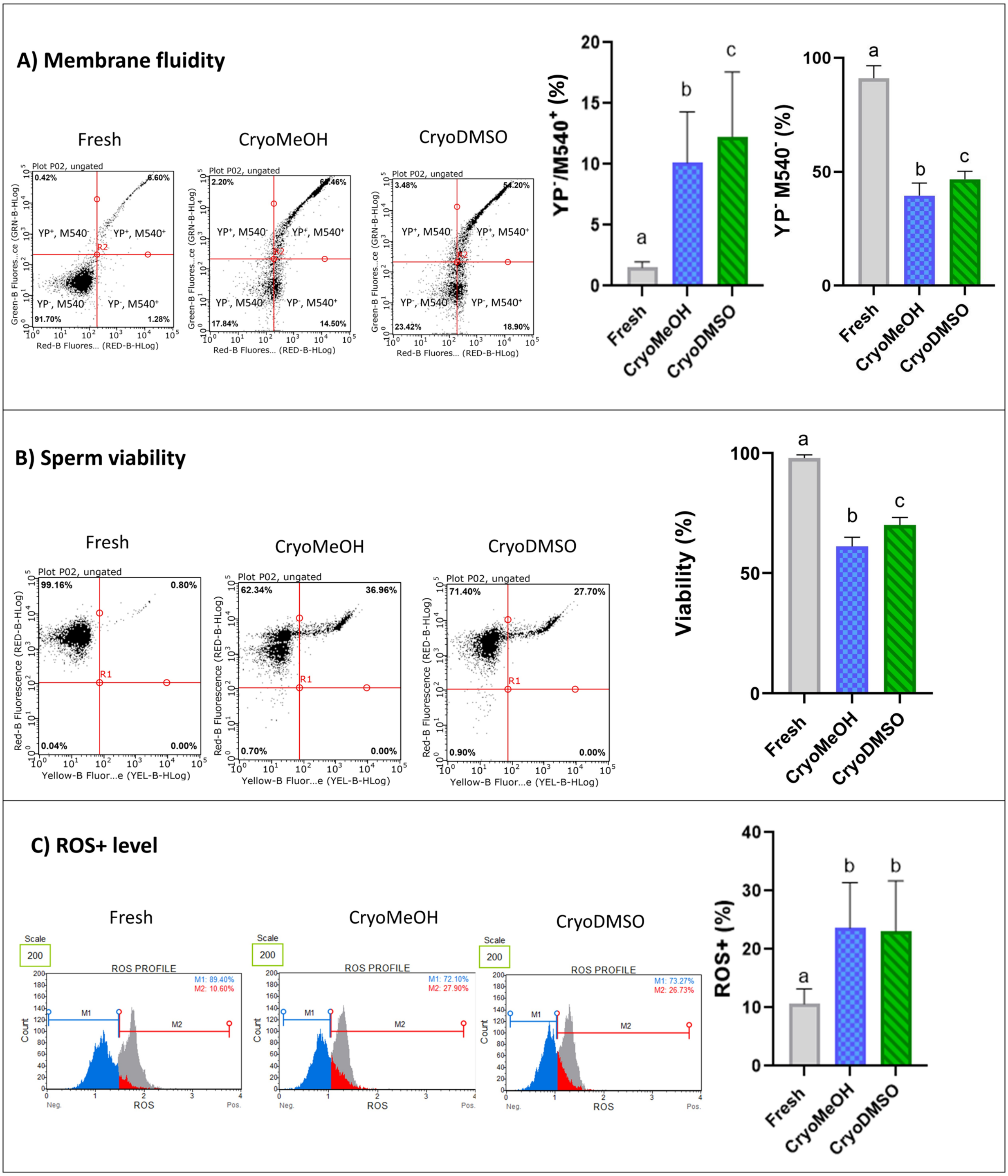


Figure 3. Flow cytometry evaluation of sperm membrane fluidity (M540/YP staining) (**A**), viability (Guava ViaCount reagent) (**B**) and ROS$^+$ levels (**C**) in fresh and cryopreserved semen with DMSO and MeOH. (**A**) Representative plots showing viable sperm with intact membranes (YP$^-$/M54$^-$, lower left quadrant), viable sperm with destabilized membranes (YP$^-$/M54$^+$, lower right quadrant), dead sperm with intact membranes (YP$^+$/M54$^-$, upper left quadrant) and dead sperm with destabilized membranes (YP$^+$/M54$^+$, upper right quadrant). (**B**) Plots showing live cells in the upper-left quadrant of the first plot and in the lower-right corner of the second plot. (**C**) Histogram showing spermatozoa in the ROS$^-$ (marker M1) and ROS$^+$ (marker M2) groups. Different superscripts indicate significant differences ($p < 0.05$) among groups. The data are presented as the means ± SDs. YP-Yo-Pro-1, M540- merocyanine 540.

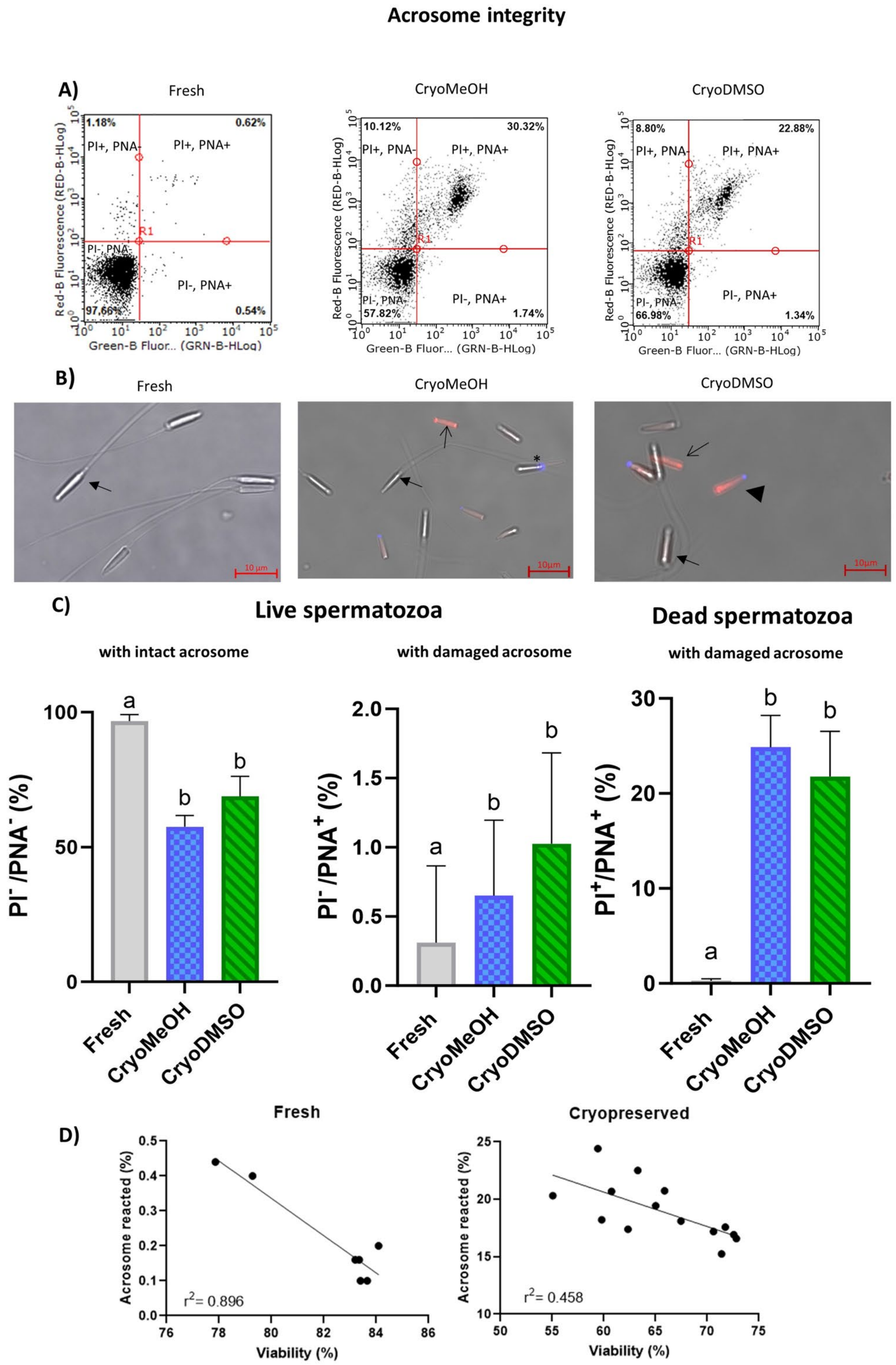


Figure 4. Flow cytometry analysis (PNA/PI labeling) of acrosome membrane integrity in fresh and cryopreserved spermatozoa treated with MeOH and DMSO. (**A**) Representative flow cytometry plots showing viable acrosome intact (PI^-/PNA^-, lower left quadrant), viable acrosome ruptured (PI^-/PNA^+, lower right quadrant), dead acrosome intact (PI^+/PNA^-, upper left quadrant) and dead acrosome-ruptured (PI^+/PNA^+, upper right quadrant) spermatozoa. (**B**) Fluorescence microscopy images showing unstained live sperm (indicated by bold-headed arrows for spermatozoa with intact acrosomes and asterisks for spermatozoa with damaged acrosomes) and dead sperm stained in red (indicated by fine-headed arrows for spermatozoa with intact acrosomes and arrowheads for spermatozoa with damaged acrosomes). (**C**) Graphs and (**D**) correlation between sperm viability and acrosome-reacted sperm in fresh and cryopreserved semen. Different superscripts indicate significant differences ($p < 0.05$) among groups ($n = 7$ in each group). The data are presented as the means ± SDs. *PI* propidium iodide; *PNA* peanut agglutinin.

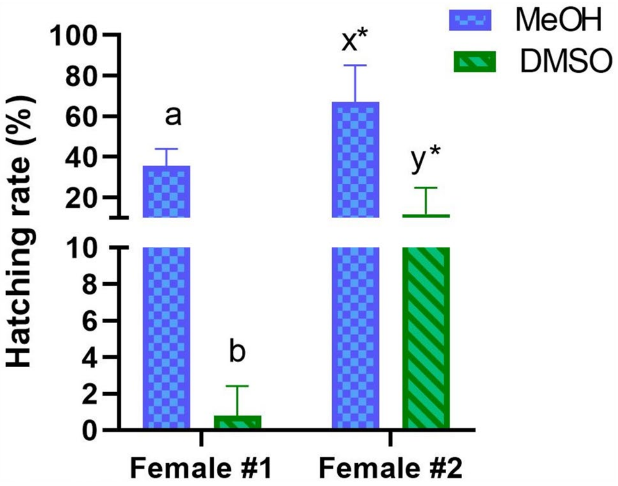


Figure 5. Effects of the cryoprotectants MeOH and DMSO on the hatching rate of two females. The data are presented as the means ± SDs (n = 7). Different superscripts indicate significant differences ($p < 0.0001$) among the cryoprotectants for female #1 (a, b) and female #2 (x, y). The hatching rates for fresh semen were 57% for female #1 and 75% for female #2. Asterisks (*) indicate significant differences ($p = 0.0052$) between females under the same cryoprotectant conditions.

dehydrogenase, testis-specific (GAPDHS) (4 spots across both groups), phosphoglucomutase-1 (PGM1) (5 spots for MeOH and 3 for DMSO), ENO3 (4 spots for MeOH and 2 for DMSO) and puromycin-sensitive aminopeptidase (NPEPPS) (4 spots for MeOH and 3 for DMSO). Comparison of CryoDMSO and CryoMeOH groups revealed 19 DAPs. These findings are detailed in Supplementary Table S4, with representative 2D-DIGE images provided in Supplementary Figs. S1 and S2.

Additionally, 8 proteins (actin, cytoplasmic 1 (ACTB), actin-related protein T3 (ACTRT3), mitochondrial hydroxyacyl-coenzyme A dehydrogenase (HADH), muscle glycogen phosphorylase (PYGM), ropporin-1A (ROPN1), septin-11 (SEPTIN11), sperm acrosome-associated protein 9 (SPACA9) and tubulin beta-4B chain (TUBB4B)) in MeOH vs. fresh samples and 6 proteins (ACTB, isoaspartyl peptidase/L-asparaginase (ASRGL1), calcium-binding tyrosine phosphorylation-regulated protein (CABYR), PYGM, ROPN1L and SEPTIN11) in DMSO vs. fresh samples were identified as both up- and downregulated. These findings are detailed in Supplementary Fig. S3, with representative 2D-DIGE images.

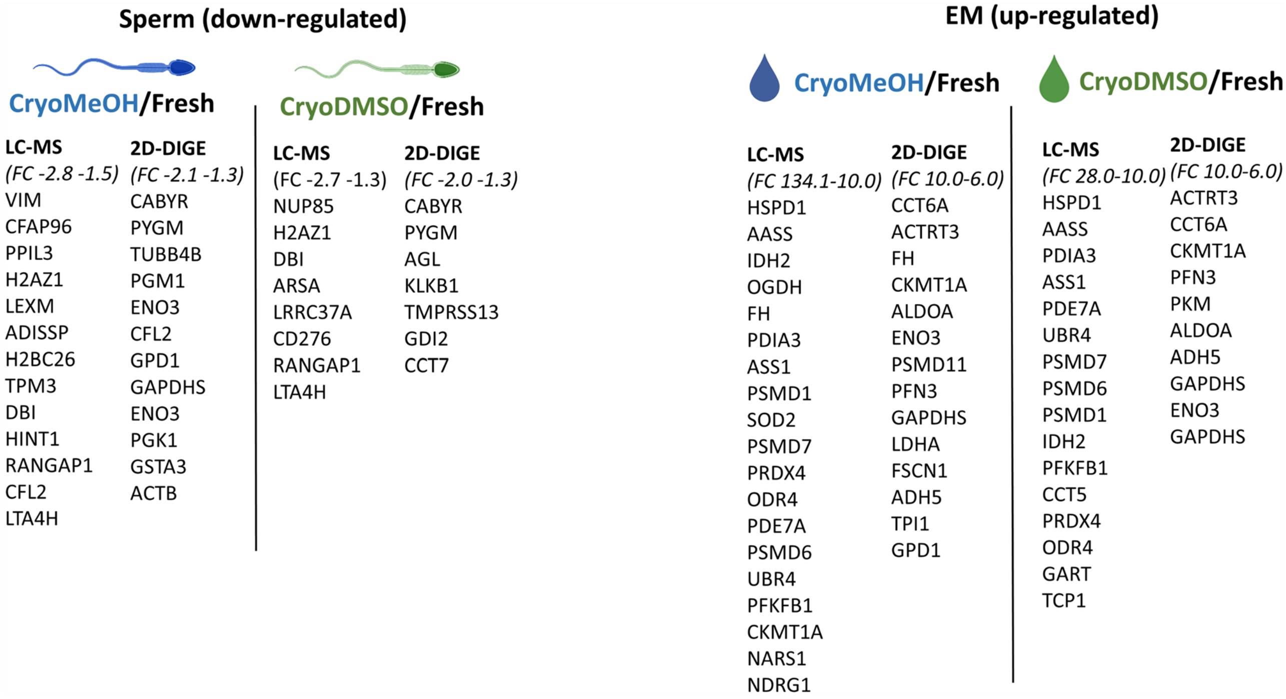
The top downregulated proteins following cryopreservation with MeOH included vimentin (VIM), cilia- and flagella-associated protein 96 (CFAP96), peptidyl-prolyl cis–trans isomerase-like 3 (PPIL3), histone H2A.Z (H2A.Z) and ciliary microtubule-associated protein 2 (LEXM) via LC—MS and CABYR, PYGM, TUBB4B, phosphoglucomutase-1 (PGM1) and ENO3 via 2D-DIGE, whereas nucleoporin nup85 (NUP85), H2A.Z1, acyl-CoA-binding protein (DBI), arylsulfatase A (ARSA) and leucine-rich repeat-containing protein 37A (LRRC37A) (LC—MS method) and CABYR, PYGM, glycogen debranching enzyme (AGL), plasma kallikrein (KLKB1) and transmembrane protease serine 13 (TMPRSS13) (2D-DIGE method) were predominantly altered after cryopreservation with DMSO. A summary of the top significantly altered proteins is presented in Fig. 6.

Combining the results from 2D-DIGE and LC–MS/MS yielded 224 and 118 DAPs in cryopreserved semen with MeOH and DMSO, respectively, compared to fresh semen, highlighting the proteomic alterations induced by cryopreservation (Fig. 7A–C).

Extracellular medium

Label-free LC—MS analysis of the EM revealed 1,900 proteins, with 330 and 349 proteins significantly upregulated following cryopreservation with MeOH and DMSO, respectively. Comparison of CryoDMSO and CryoMeOH samples revealed 11 DAPs. The detailed identifications are provided in Supplementary Table S5. The upregulation of proteins in the EM suggested protein leakage from the post-cryopreservation sperm. The 2D-DIGE analysis corroborated these findings, identifying 116 and 131 upregulated protein spots following cryopreservation in the MeOH and DMSO groups, respectively, out of 1,529 matched spots. These spots corresponded to 56 and 47 unique proteins, with 24 and 23 identified in multiple spots, implying proteoform diversity. Prominent among them were ENO3 (identified in 9 spots for MeOH and 10 for DMSO), LDHA (9 spots in both groups), PGK1 (6 spots for MeOH and 9 for DMSO), GAPDHS (8 spots in both groups), PKM (7 spots for MeOH and 8 for DMSO) and ALDOA (7 spots in both groups). Comparison of CryoDMSO and CryoMeOH groups revealed 4 DAPs. Representative 2D-DIGE images and detailed spot identifications are provided in Supplementary Figs. S4 and S5 and Supplementary Table S6, respectively.

The top upregulated proteins following cryopreservation in the EM included 60 kDa mitochondrial heat shock protein (HSPD1), mitochondrial alpha-aminoadipic semialdehyde synthase (AASS), mitochondrial isocitrate dehydrogenase [NADP] (IDH2), 2-oxoglutarate dehydrogenase complex component E1 (OGDH) and FH (LC—MS method) and T-complex protein 1 subunit zeta (CCT6); ACTRT3, FH, mitochondrial creatine kinase



Sperm (down-regulated)

CryoMeOH/Fresh

LC-MS (FC -2.8 -1.5)	2D-DIGE (FC -2.1 -1.3)
VIM	CABYR
CFAP96	PYGM
PPIL3	TUBB4B
H2AZ1	PGM1
LEXM	ENO3
ADISSP	CFL2
H2BC26	GPD1
TPM3	GAPDHS
DBI	ENO3
HINT1	PGK1
RANGAP1	GSTA3
CFL2	ACTB
LTA4H	

CryoDMSO/Fresh

LC-MS (FC -2.7 -1.3)	2D-DIGE (FC -2.0 -1.3)
NUP85	CABYR
H2AZ1	PYGM
DBI	AGL
ARSA	KLKB1
LRRC37A	TMPRSS13
CD276	GDI2
RANGAP1	CCT7
LTA4H	

EM (up-regulated)

CryoMeOH/Fresh

LC-MS (FC 134.1-10.0)	2D-DIGE (FC 10.0-6.0)
HSPD1	CCT6A
AASS	ACTRT3
IDH2	FH
OGDH	CKMT1A
FH	ALDOA
PDIA3	ENO3
ASS1	PSMD11
PSMD1	PFN3
SOD2	GAPDHS
PSMD7	LDHA
PRDX4	FSCN1
ODR4	ADH5
PDE7A	TPI1
PSMD6	GPD1
UBR4	
PFKFB1	
CKMT1A	
NARS1	
NDRG1	

CryoDMSO/Fresh

LC-MS (FC 28.0-10.0)	2D-DIGE (FC 10.0-6.0)
HSPD1	ACTRT3
AASS	CCT6A
PDIA3	CKMT1A
ASS1	PFN3
PDE7A	PKM
UBR4	ALDOA
PSMD7	ADH5
PSMD6	GAPDHS
PSMD1	ENO3
IDH2	GAPDHS
PFKFB1	
CCT5	
PRDX4	
ODR4	
GART	
TCP1	

Figure 6. List of the top proteins identified as downregulated in spermatozoa and upregulated in the EM for MeOH and DMSO, as determined by LC/MS and 2D DIGE analysis.

U-type (CKMT1A) and ALDOA (2D-DIGE method) for the MeOH group; and HSPD1, AASS, protein disulfide-isomerase A3 (PDIA3), argininosuccinate synthase (ASS1), high affinity 3',5'-cyclic-AMP phosphodiesterase 7A (PDE7A) (LC—MS method) and ACTRT3, CCT6, CKMT1A, profilin-3 (PFN3), and PKM (2D-DIGE method) for the DMSO group. Figure 6 presents a summary of the top significantly altered proteins identified in EM.

Overall, the integration of 2D-DIGE and LC–MS/MS facilitated the identification of 342 and 363 upregulated proteins in the EM from cryopreserved semen with MeOH and DMSO, respectively, compared to those in fresh semen (Fig. 7D–F).

Overlap of sperm leakage proteins identified by analysis of spermatozoa and EM following cryopreservation

The comparative analysis between downregulated proteins in spermatozoa and upregulated proteins in the EM after cryopreservation revealed a subset of overlapping proteins that were concurrently downregulated in spermatozoa and upregulated in the EM. Specifically, 77 proteins in the MeOH group and 53 proteins in the DMSO group exhibited this pattern, indicating an overlap of sperm leakage proteins due to cryopreservation effects (Fig. 7G,H).

Principal component analysis

PCA and hierarchical clustering of spermatozoa and the EM proteome after 2D-DIGE analysis revealed distinct clustering patterns, with fresh samples segregating from those cryopreserved with MeOH and DMSO. Specifically, PC1 accounted for 50.9% of the variance in the spermatozoa and 68.2% in the EM, whereas PC2 explained an additional 13.5% and 12.6% of the variation in the spermatozoa and EM, respectively (Fig. 8A,C). Heatmaps further illustrate the differences in the proteomes of fresh and cryopreserved semen (Fig. 8B,D). Additional PCA score plots and heatmaps derived from the LC—MS data are provided in Supplementary Fig. S6.

Functional insights into spermatozoa proteins altered by cryopreservation with MeOH and DMSO

For the combined set of proteins associated with sperm leakage (downregulated proteins in spermatozoa and upregulated proteins in the EM, totaling 376 proteins in the MeOH group and 379 in the DMSO group), key biological processes included metabolic processes, protein folding, positive regulation of the establishment of protein localization to telomeres, binding of sperm to the zona pellucida, and cellular detoxification (Fig. 9A) for both groups. In addition, proteins altered by MeOH were involved in protein export from the nucleus, whereas DMSO-altered proteins participated in ubiquitin protein ligase binding, nucleocytoplasmic transport, and nuclear pore organization. The molecular function annotation confirmed a strong association with binding and catalytic activity for both groups (Fig. 9A). IPA further revealed a significant association between cell viability, glycolysis and gluconeogenesis and the levels of sperm leakage proteins in both the MeOH and DMSO groups. The top ten IPA biological processes related to leakage proteins are shown in Table 1.

The sperm leakage proteins, regardless of the cryoprotectant used, were localized to extracellular vesicles, the proteasome complex, mitochondria, nuclear pores, sperm flagella and the ZP receptor complex (Fig. 9A).

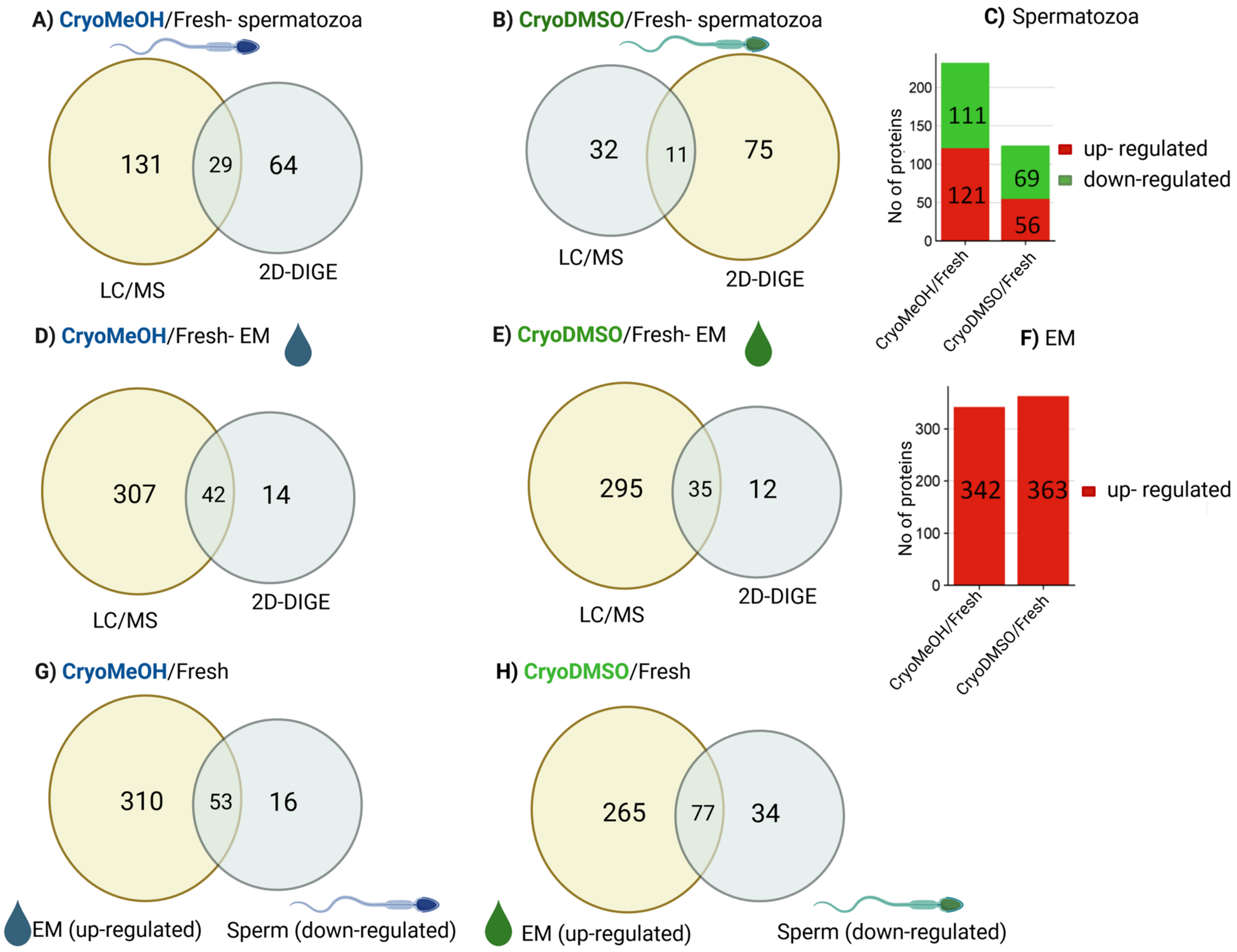


Figure 7. Diagrams representing DAPs of spermatozoa (**A**–**C**) and upregulated proteins in the extracellular medium (EM) (**D**–**F**) following cryopreservation with MeOH or DMSO identified by two complementary approaches: LC–MS and 2D-DIGE. Venn diagram illustrating the overlap of sperm leakage proteins identified as downregulated proteins in spermatozoa and upregulated proteins in the EM following cryopreservation with MeOH (**G**) and DMSO (**H**).

DMSO-altered proteins also localized to the cAMP-dependent protein kinase complex. All functional analyses are shown in Supplementary Table S7.

GO analysis revealed that the upregulated sperm proteins, regardless of the cryoprotectant used, were involved in biological processes such as microtubule-based movement, nucleoside triphosphate biosynthetic processes and cilium organization. In the MeOH group, proteins involved in energy derivation by the oxidation of organic compounds, left/right pattern formation, transmembrane transport and ventricular system development were identified. Conversely, upregulated proteins in spermatozoa following cryopreservation with DMSO were enriched in cellular respiration, regulation of brood size, spermatid development and the carboxylic acid metabolic process. GO molecular function annotations indicated that most proteins were associated with microtubule motor activity and metabolism in both groups (Fig. 9B). Localization analysis revealed that the upregulated sperm proteins in the DMSO group were primarily located in cilia and mitochondria, whereas in the MeOH group, they were localized to the axoneme, motile cilia, the microtubule cytoskeleton and the inner mitochondrial membrane protein complex. Functional analysis of the upregulated proteins in cryopreserved spermatozoa treated with MeOH or DMSO is shown in Supplementary Table S8.

Functional annotation of sperm leakage proteins uniquely altered by cryopreservation with MeOH and DMSO

A comparison of sperm leakage proteins following cryopreservation with MeOH and DMSO revealed 36 proteins uniquely altered by MeOH and 39 proteins uniquely altered by DMSO, with a shared group of 340 proteins commonly affected by both cryoprotectants (Fig. 10).

ShinyGO analysis revealed that sperm leakage proteins unique to MeOH were predominantly involved in small molecule metabolic processes, organic acid catabolic processes, fatty acid catabolic processes, cytoskeleton

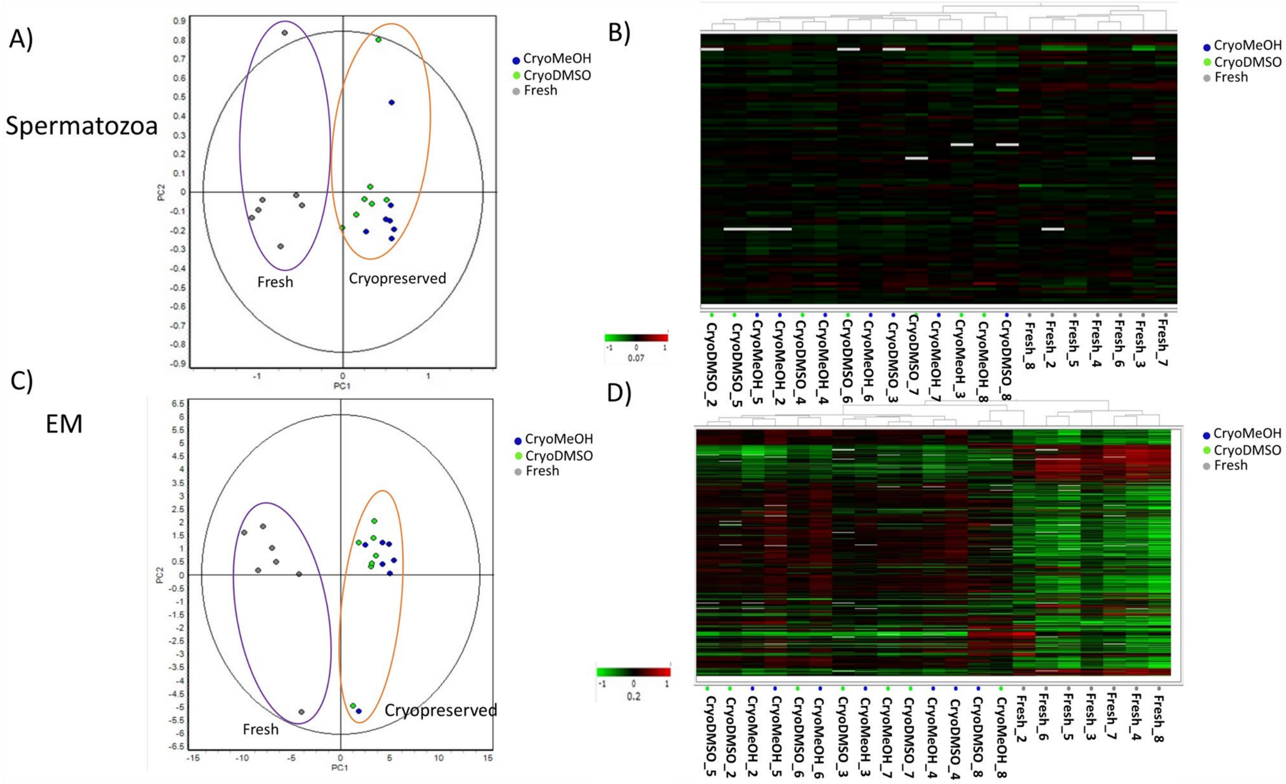


Figure 8. PCA score plots and heatmaps of the 2D-DIGE data acquired for fresh (in gray) and cryopreserved (in blue; CryoMeOH, in green; CryoDMSO) spermatozoa (**A**,**B**) and extracellular medium (EM, **C**,**D**). PC1 (x-axis) was 50.9%; PC2 (y-axis) was 13.5% for spermatozoa. PC1 (x-axis) was 68.2%, and PC2 (y-axis) was 12.6% for the EM. The heatmap code is presented, with red areas representing greater amounts of protein and green areas representing less protein.

organization and microtubule-based processes. In contrast, DMSO-specific alterations involved mRNA transport and fertilization processes, among others. Functional categorization revealed that the MeOH-specific proteins were largely related to oxidoreductase activity, hydrolase activity acting on acid anhydrides and magnesium ion binding, while the DMSO-specific proteins included structural constituents of the nuclear pore, arylsulfatase activity, calmodulin-dependent protein kinase activity, and ATP-dependent protein folding chaperones. Localization analysis revealed the mitochondrial matrix and microtubules for MeOH-specific proteins, whereas DMSO-specific proteins were primarily found in the BBSome, secretory granules, primary lysosomes and nuclear pores (Fig. 10).

The protein−protein interaction networks identified via the STRING database illustrated distinct clusters related to metabolic processes and the microtubule cytoskeleton for MeOH (Fig. 11A) and transport processes, fertilization, and kinase activity for DMSO (Fig. 11B). Functional analysis of sperm leakage proteins uniquely altered by cryopreservation in MeOH or DMSO is shown in Supplementary Table S9.

Validation of proteomic results by western blotting

To confirm the proteomic findings, seven proteins (LDHA, CKB, ENO3, ALDOA, H2A.Z, FH and ACR) were selected for validation by western blotting in spermatozoa and EM. In details, in spermatozoa, LDHA and H2A.Z decreased in abundance following cryopreservation with MeOH and DMSO, while CKB abundance decreased only in the MeOH group (Fig. 12A). Although no significant differences in protein levels were found for ENO3, ALDOA or FH, the changes in protein expression agreed with the 2D-DIGE results. On the other hand, the abundance of all proteins increased in the EM after cryopreservation in the MeOH and DMSO groups (Fig. 12B). In fresh and cryopreserved spermatozoa, anti-acrosin antibodies identified two protein bands: a predominant band at 38 kDa and a less pronounced band at 24 kDa (see Fig. 13A). Cryopreservation did not alter the intensity of these bands (Fig. 13B). On the other hand, in fresh EM samples, one predominant band of 73 kDa was recognized using anti-acrosin antibodies. After cryopreservation, 3–5 additional bands with molecular weights ranging from 53 to 24 kDa were detected, which varied among individuals (Fig. 13A). A decrease in the 73 kDa band corresponding in our opinion to proacrosin was observed following cryopreservation with DMSO and MeOH. In contrast, an increase in bands of lower molecular mass (38, 33, 24 kDa) was observed after cryopreservation (Fig. 13C). The band intensities at 73 and 38 kDa differed between the DMSO and MeOH groups.

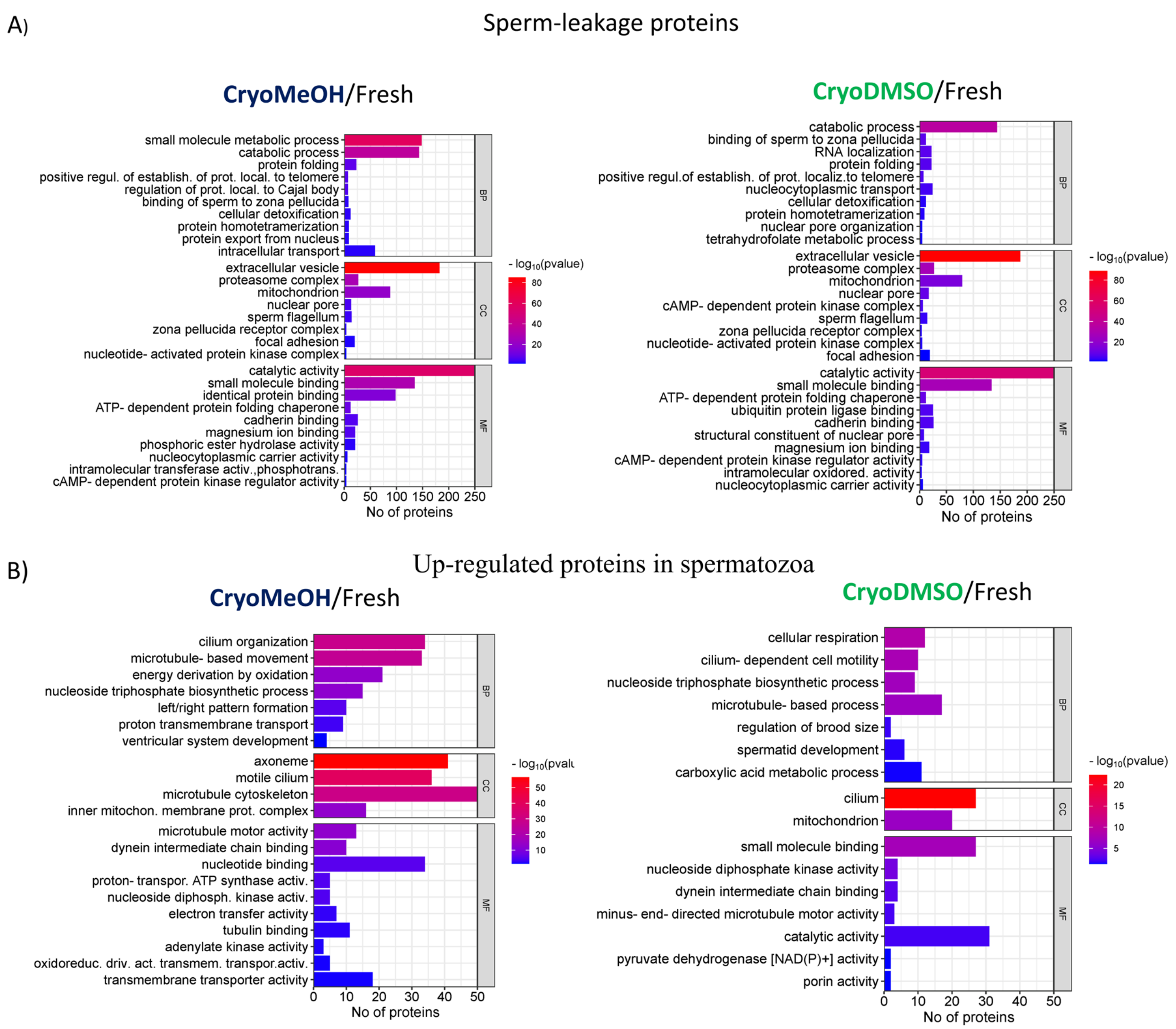


Figure 9. Gene ontology (GO) analysis of biological process (BP), cellular component (CC) and molecular function (MF) for sperm leakage proteins (downregulated proteins in spermatozoa and upregulated proteins in the EM (**A**) and upregulated proteins in spermatozoa (**B**)) after cryopreservation with MeOH and DMSO.

Diseases or functions annotation	p-value	Number of molecules	Diseases or functions annotation	p-value	Number of molecules
CryoMeOH/fresh			CryoDMSO/fresh		
Metabolism of amino acids	1.09E−28	40	Metabolism of amino acids	2.33E−25	37
Metabolism of nucleic acid component or derivative	4.52E−22	57	Metabolism of nucleic acid component or derivative	1.37E−20	55
Metabolism of nucleotide	1.50E−17	47	Metabolism of nucleotide	1.50E−17	47
Metabolism of dicarboxylic acid	8.82E−15	14	Cell viability	4.16E−16	100
Cell viability	6.98E−14	95	Metabolism of carbohydrate	6.38E−14	53
Metabolism of carbohydrate	2.37E−13	52	Metabolism of dicarboxylic acid	2.36E−13	13
Folding of protein	3.79E−13	17	Folding of protein	3.79E−13	17
Glycolysis	2.17E−12	29	Binding of zona pellucida	1.35E−12	12
Gluconeogenesis	3.00E−11	18	Glycolysis	2.17E−12	29
Metabolism of NADH	5.72E−10	7	Gluconeogenesis	3.18E−12	19

Table 1. The top ten canonical pathways for sperm-leakage proteins indicated by IPA analysis.

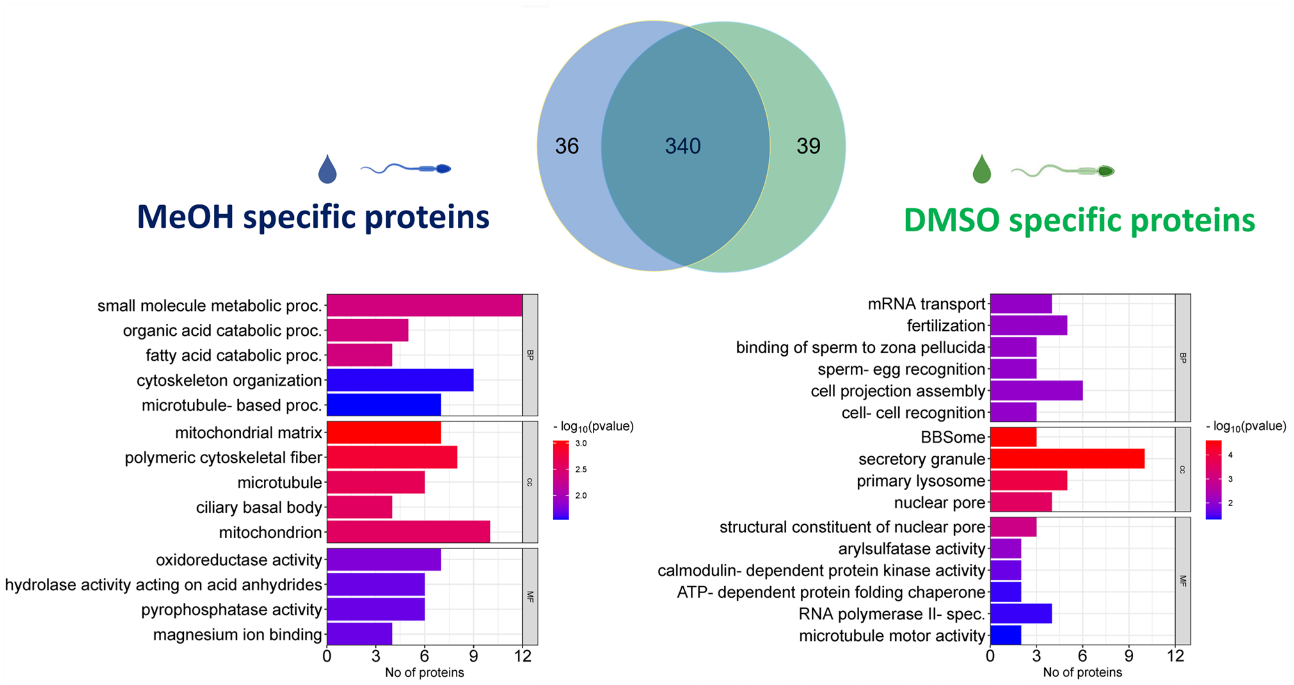
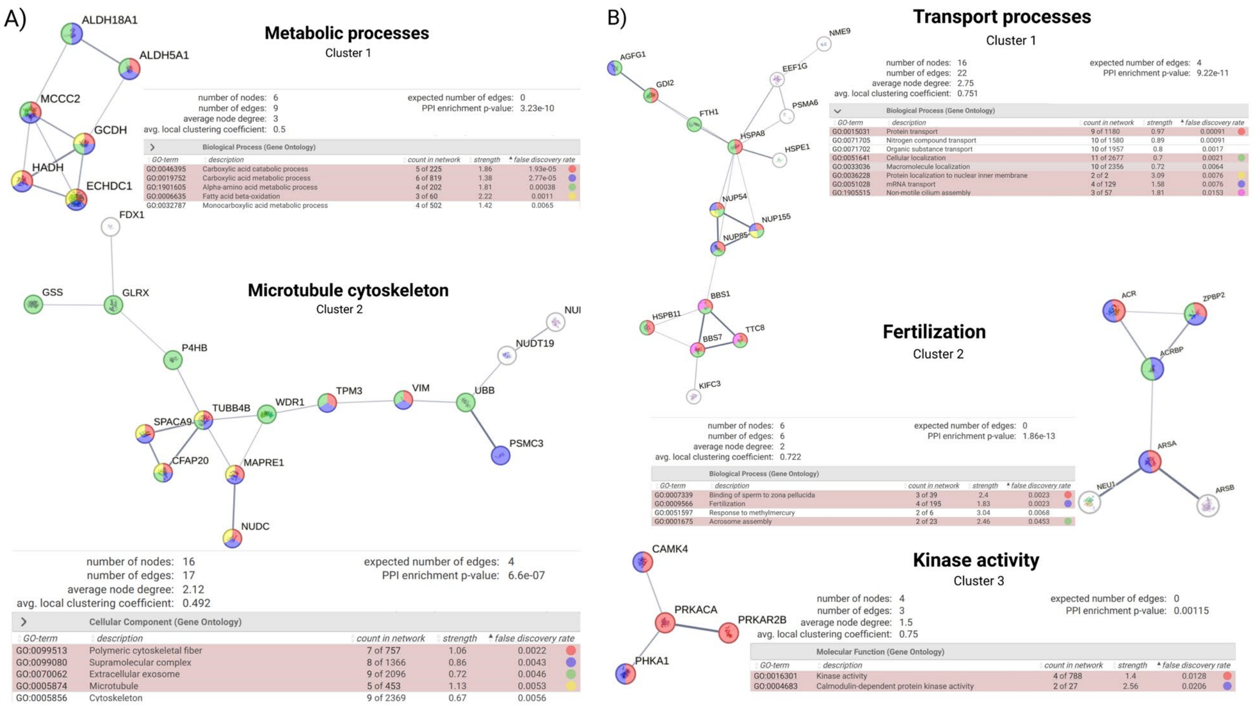


Figure 10. Venn diagram illustrating the common and distinct sperm leakage proteins following cryopreservation with MeOH and DMSO, along with functional annotation of proteins uniquely altered by each cryoprotectant. Functional analysis was performed using ShinyGO (v0.80; http://bioinformatics.sdstate.edu/go/).



Metabolic processes

Cluster 1

number of nodes: 6
number of edges: 9
average node degree: 3
avg. local clustering coefficient: 0.5
expected number of edges: 0
PPI enrichment p-value: 3.23e-10

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate	
GO:0046395	Carboxylic acid catabolic process	5 of 225	1.86	1.93e-05	🔴
GO:0019752	Carboxylic acid metabolic process	6 of 819	1.38	2.77e-05	🔵
GO:1901605	Alpha-amino acid metabolic process	4 of 202	1.81	0.00038	🟢
GO:0006635	Fatty acid beta-oxidation	3 of 60	2.22	0.0011	🟡
GO:0032787	Monocarboxylic acid metabolic process	4 of 502	1.42	0.0065	

Microtubule cytoskeleton

Cluster 2

number of nodes: 16
number of edges: 17
average node degree: 2.12
avg. local clustering coefficient: 0.492
expected number of edges: 4
PPI enrichment p-value: 6.6e-07

Cellular Component (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate	
GO:0099513	Polymeric cytoskeletal fiber	7 of 757	1.06	0.0022	🔴
GO:0099080	Supramolecular complex	8 of 1366	0.86	0.0043	🔵
GO:0070062	Extracellular exosome	9 of 2096	0.72	0.0046	🟢
GO:0005874	Microtubule	5 of 453	1.13	0.0053	🟡
GO:0005856	Cytoskeleton	9 of 2369	0.67	0.0056	

Transport processes

Cluster 1

number of nodes: 16
number of edges: 22
average node degree: 2.75
avg. local clustering coefficient: 0.751
expected number of edges: 4
PPI enrichment p-value: 9.22e-11

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate	
GO:0015031	Protein transport	9 of 1180	0.97	0.00091	🔴
GO:0071705	Nitrogen compound transport	10 of 1580	0.89	0.00091	
GO:0071702	Organic substance transport	10 of 1957	0.8	0.0017	
GO:0051641	Cellular localization	11 of 2677	0.7	0.0021	🟢
GO:0033036	Macromolecule localization	10 of 2356	0.72	0.0064	
GO:0036228	Protein localization to nuclear inner membrane	2 of 2	3.09	0.0076	🟡
GO:0051028	mRNA transport	4 of 129	1.58	0.0076	🔵
GO:1905515	Non-motile cilium assembly	3 of 57	1.81	0.0153	🟣

Fertilization

Cluster 2

number of nodes: 6
number of edges: 6
average node degree: 2
avg. local clustering coefficient: 0.722
expected number of edges: 0
PPI enrichment p-value: 1.86e-13

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate	
GO:0007339	Binding of sperm to zona pellucida	3 of 39	2.4	0.0023	🔴
GO:0009566	Fertilization	4 of 195	1.83	0.0023	🔵
GO:0051597	Response to methylmercury	2 of 6	3.04	0.0068	
GO:0001675	Acrosome assembly	2 of 23	2.46	0.0453	🟢

Kinase activity

Cluster 3

number of nodes: 4
number of edges: 3
average node degree: 1.5
avg. local clustering coefficient: 0.75
expected number of edges: 0
PPI enrichment p-value: 0.00115

Molecular Function (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate	
GO:0016301	Kinase activity	4 of 788	1.4	0.0128	🔴
GO:0004683	Calmodulin-dependent protein kinase activity	2 of 27	2.56	0.0206	🔵

Figure 11. Protein–protein interaction networks for sperm leakage proteins uniquely altered by cryopreservation with MeOH and DMSO. The nodes correspond to the proteins, and the edges represent the interactions (thick lines indicate a high score > 0.9; thin lines indicate a medium score > 0.7). Model statistics and an explanation of edge colors are presented on the left.

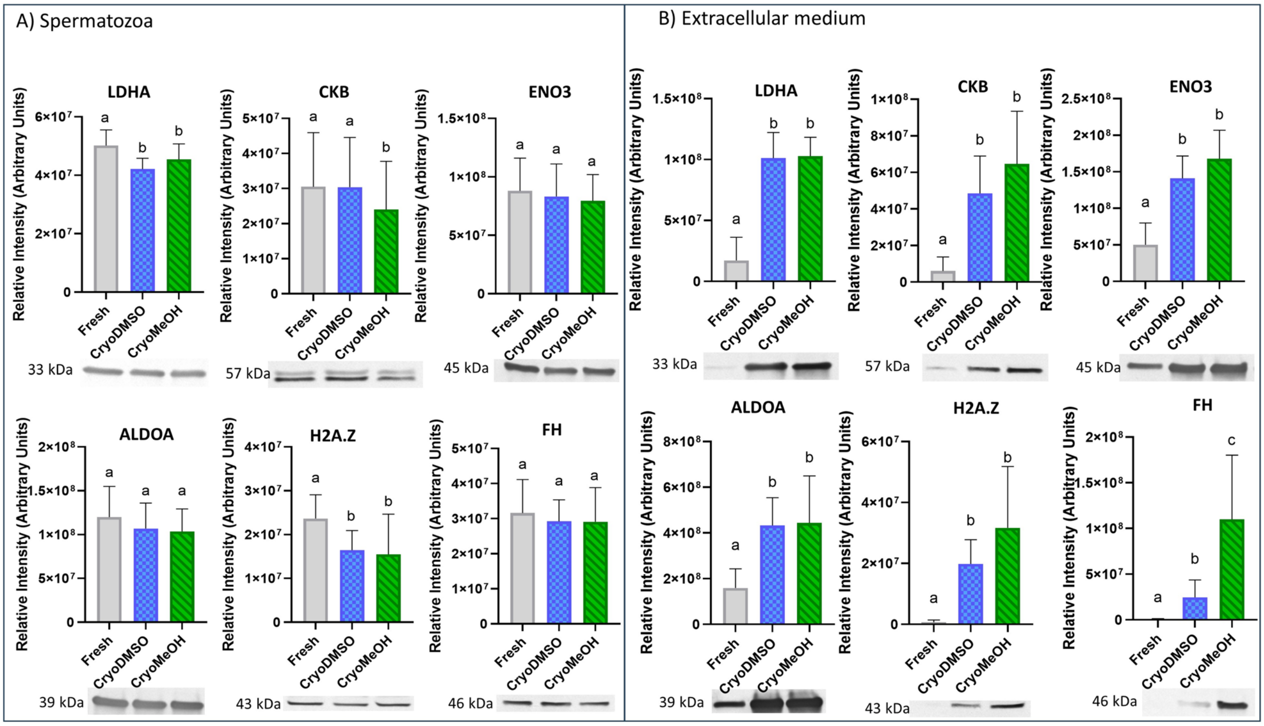


Figure 12. Immunoblotting validation of L-lactate dehydrogenase A chain (LDHA), creatine kinase B-type (CKB), enolase 3 (ENO3), fructose-bisphosphate aldolase A (ALDOA), histone (H2A.Z) and mitochondrial fumarate hydratase (FH) and acrosin (ACR) in fresh and cryopreserved spermatozoa (**A**) and the extracellular medium (**B**). The full-length blots are presented in Supplementary Figs. S7 and S8. The intensity of the protein bands on the TGX Stain-Free gels was analyzed using Image Lab 6 software (Bio-Rad).

Discussion

This study represents the first comprehensive examination of the alterations in the proteome of Siberian sturgeon spermatozoa resulting from cryopreservation, delineating the distinct effects of DMSO and MeOH cryoprotectants on sperm fertilization. Our findings contribute to a deeper understanding of the molecular mechanisms leading to a decrease in sperm quality following cryopreservation, particularly by identifying significant alterations in proteins essential for flagellar motility, energy metabolism, nuclear pore organization and fertilization processes. Notably, we identified proteins distinctly affected by DMSO and MeOH, underscoring the cryoprotectant-specific mechanisms potentially responsible for the observed differences in sperm fertilization ability (Fig. 14).

Consistent with previous studies on sturgeons, we observed a decrease in sperm motility parameters (MOT, VCL, ALH, and PROG) and viability following cryopreservation[9,19], which was further corroborated by increased ROS production, a known factor in semen quality degradation[31]. Using flow cytometry, we observed a significant increase in the percentage of dead spermatozoa with reacted acrosomes following cryopreservation, reaching 22–24%, without notable differences between the cryoprotectants used. This increase correlated with elevated percentages of nonviable sperm. The proportion of viable spermatozoa with reacted acrosomes, while remaining low (0.3% in fresh samples), exhibited notable changes due to cryopreservation (approximately 1%), irrespective of the cryoprotectant used. In contrast, Lahnsteiner et al.[32] reported no change in acrosome-reacted sperm after cryopreservation in sterlets, ranging from 15 to 19% for both fresh and cryopreserved semen with MeOH and DMSO, as measured under a phase contrast microscope through acrosomal filament determination. On the other hand, Psenicka et al.[9] reported that DMSO specifically induced acrosome damage (12%) in sterlet semen, whereas MeOH maintained acrosome integrity comparable to that of fresh semen (5–8%), as determined by microscopic observation with SBTI-Alexa488 staining. These disparities underscore the variability in cryopreservation outcomes, likely due to variations in species and methods used for assessing acrosome status. Our results indicated that despite similar motility parameters, membrane fluidity, and acrosome status between the two cryoprotectants and greater viability in the DMSO group, we observed a marked decrease in the fertilization success of semen cryopreserved with DMSO compared to MeOH, which aligns with the findings of a previous study on sterlet and paddlefish[32–34] and was explained by acrosomal reaction activation after cryopreservation. Our results suggest that standard sperm quality parameters may not adequately predict fertilization potential after cryopreservation, highlighting the significant role of molecular factors, such as protein composition, in determining fertilization success.

We utilized two complementary methods, gel-based and MS-based methods, to maximize the number of proteins altered due to cryopreservation. This combined approach identified more proteins than did LC−MS or 2D-DIGE alone. The complementary nature of 2D-DIGE and LC-based methods has been shown in other proteomics studies[35]. The observed low overlap of differential proteins between the methods could result from

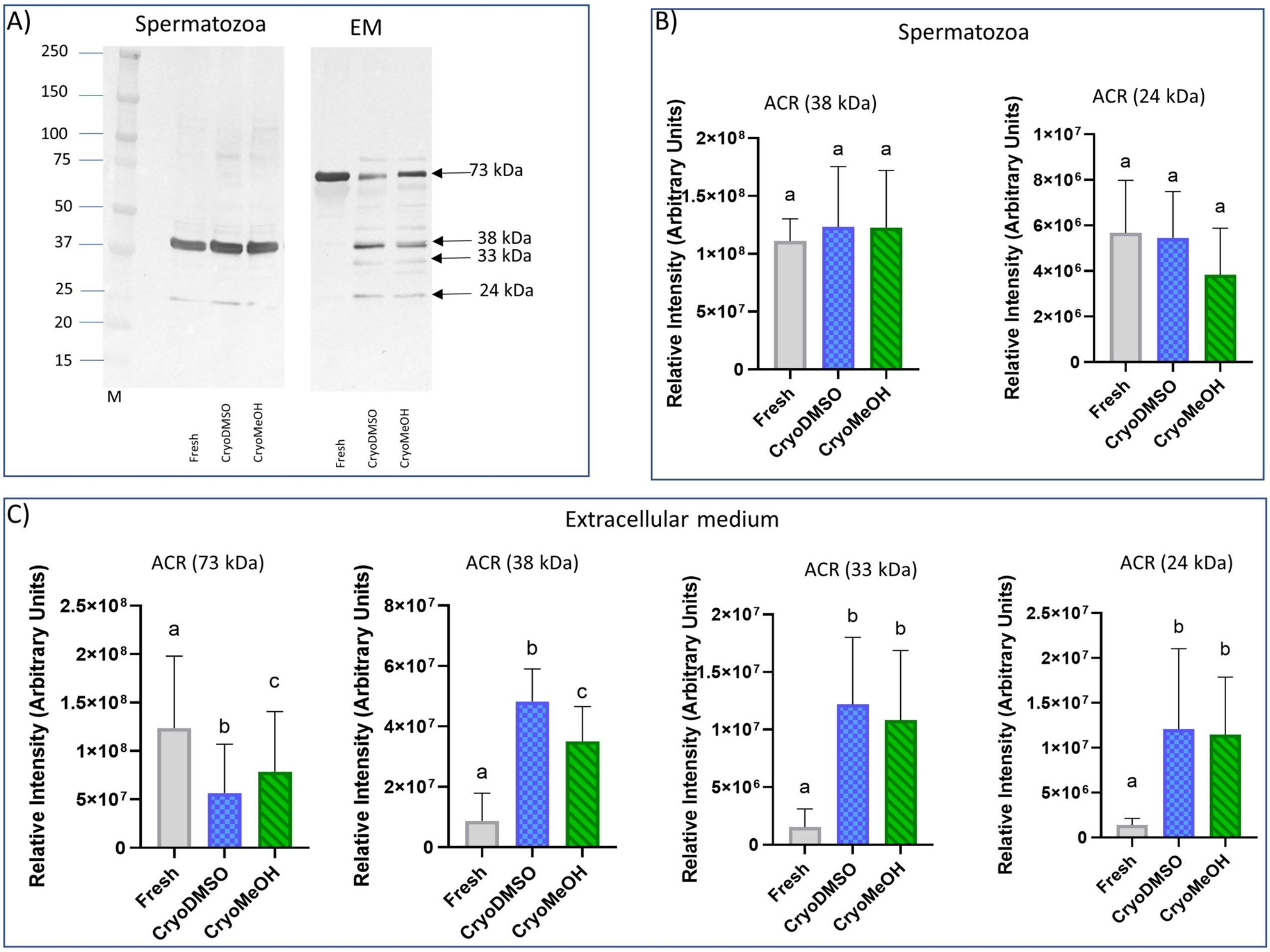


Figure 13. Validation of acrosin (ACR) immunoblotting between fresh and cryopreserved spermatozoa with DMSO and MeOH. Western blot profile (**A**), graph of the relative intensity of acrosin bands of different molecular masses in spermatozoa (**B**) and extracellular medium (**C**). The results are expressed as the means ± SDs. Different letters indicate statistical significance at a p value ≤ 0.05, n = 7 per group. The full-length blots are presented in Supplementary Figs. S7 and S8.

their inherent strengths and analytical bases[36]. 2D-DIGE separates intact proteins based on pI and Mw, allowing visualization of isoforms and posttranslational modifications (PTMs). This method excels in distinguishing subtle protein variations but overall has lower sensitivity and a limited dynamic range. MS-based methods separate peptides based on hydrophobicity, thus detecting low-abundance proteins more effectively. However, MS-based techniques may struggle to differentiate between isoforms and modifications without additional targeted analyses. These fundamental differences in separation principles and detection capabilities have led to the identification of distinct sets of differentially expressed proteins by each approach.

Our results revealed that, unlike MeOH, DMSO selectively altered 39 sturgeon sperm proteins during cryopreservation. This unique subset of proteins, which diverges from the 340 proteins altered by both cryoprotectants, highlights the distinct impact of DMSO on sperm function and can be vital for understanding the loss of sperm fertilization ability when DMSO was used. Remarkably, a substantial majority of these proteins (33 proteins) were involved in fertilization processes, especially in the acrosome reaction, binding of sperm to the *zona pellucida,* nuclear pore organization, and the flagellum/centrosome structure. Most importantly, DMSO-induced changes shed new light on proteins important for sperm fertilization ability. This knowledge is new for sturgeons and is potentially important for better understanding how cryoprotectants influence sperm functionality and fertilization success.

Cryopreservation with DMSO led to alterations in acrosin (ACR), acrosin binding protein (ACRBP) and other proteins essential for the acrosome reaction (Arf-GAP domain and FG repeat-containing protein 1 (AGFG1), Rab GDP dissociation inhibitor beta (GDI2) and lysosomal Pro-X carboxypeptidase (PRCP)), which highlights the unique effect of DMSO on this crucial fertilization step. Unique to sturgeons and lampreys, the acrosome reaction involves the formation of actin-based filaments which occurs within the micropylar canal[37]. ACR, a serine protease found in the sturgeon acrosome, is associated with the acrosome reaction[38,39] and may be facilitated by ACRBP, which regulates its activity. The presence of the acrosin/proacrosin system was identified previously in Siberian sturgeon spermatozoa[40], and alterations of this system in seminal plasma following cryopreservation were detected in the present study. AGFG1 is involved in the fusion of proacrosomal vesicles and facilitates

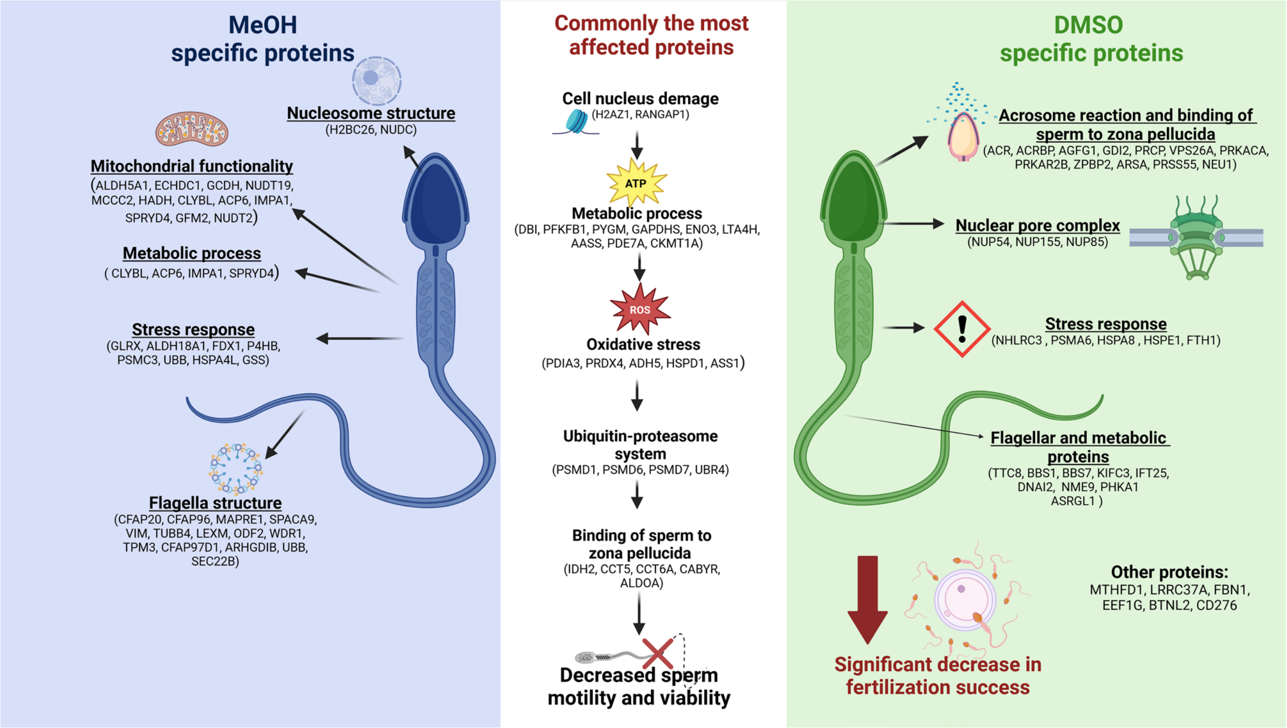


Figure 14. Overall summary of the unique roles of the sperm-leakage proteins of Siberian sturgeons, which depend on the cryoprotectant used. Descriptions of the gene/protein symbols are presented in Supplementary Tables S3–S6.

the structural rearrangements necessary for enzyme release during the acrosome reaction[41], while GDI2 plays a key role in activating Rab proteins essential for this process[42]. Moreover, proteins such as vacuolar protein sorting-associated protein 26A (VPS26A), which is involved in vesicular trafficking, likely influence the acrosome reaction[43], suggesting that extracellular vesicles participate in the acrosome reaction, aligning with their general role in the maturation of fish spermatozoa. Moreover, alterations in signaling proteins such as calcium/calmodulin-dependent protein kinase type IV (CAMK4), cAMP-dependent protein kinase catalytic subunit alpha (PRKACA), cAMP-dependent protein kinase type II-beta regulatory subunit (PRKAR2B), and LOC117435672, similar to calcium-binding and tyrosine phosphorylation-regulated proteins, highlight the effect of DMSO on sperm motility and the acrosome reaction. Taken together, our results provide the first evidence supporting the role of the acrosome reaction in sturgeon fertilization.

The uniqueness of sturgeons is further exemplified by their eggs being in possession of numerous micropyles at the animal pole, necessitating potentially more intricate sperm−egg interactions than those in teleost species with a single micropyle. The identified changes in proteins crucial for sperm-egg recognition and binding uniquely altered by DMSO, such as zona pellucida-binding protein 2 (ZPBP2), ARSA, a vital enzyme in sturgeon sperm[44], serine protease 55 (PRSS55), a chymotrypsin-like serine protease, and sialidase-1 (NEU1), which are responsible for the desialylation of sperm surface glycoproteins, underscore their role in the modification of the sperm surface to enable proper acrosome reactions and subsequent attachment to the *zona pellucida*[45]. In addition, PRSS55 was identified as a sperm-borne marker crucial for oocyte fertilization and early embryo development in mammals[46]. Notably, the functions of *zona pellucida*-binding proteins have been established in mammals, but no information on the role of these proteins in sturgeons is available. Nevertheless, our results clearly indicate the presence of these proteins in sturgeon sperm with a possible role in sperm-oocyte interactions. Further studies are needed to unravel their specific role in fertilization.

Cryopreservation with DMSO distinctively altered nuclear pore complexes (NPCs) in spermatozoa, especially affecting nucleoporins such as the nuclear pore complex proteins Nup54 (NUP54), Nup155 (NUP155), and Nup 85 (NUP85), which are key for NPC integrity and function. Nucleoporins are involved in nucleocytoplasmic transport and are crucial for cell functions such as proliferation, DNA repair, and cell cycle regulation, with significant roles in spermatogenesis, including aiding chromatin compaction, flagellar biogenesis, nuclear elongation and epigenetic regulation affecting embryonic development[47–49]. NUP54, a central channel protein, is crucial for nucleocytoplasmic transport. Mirroring the role of NUP54 in oocyte pronucleus formation[50], NUP54 in spermatozoa could aid chromatin decondensation and nuclear envelope reassembly, which are vital for zygote formation. Alterations in NUP54 have been associated with increased cell death, mitotic disruption, and prolonged cell cycle phases, which exacerbate chromosomal aberrations in mammals[51]. NUP155 is crucial for nuclear envelope integrity, and its depletion causes embryonic lethality, underscoring its role in embryogenesis[52]. The importance of NUP85 in NPC assembly suggests that changes in this protein could impact genomic stability and transcriptional regulation, which are essential for embryo development[53]. While DMSO and MeOH affect various nucleoporins, the alteration of these nucleoporins by DMSO may impair fertilization outcomes by impacting genomic stability, nucleocytoplasmic transport, gamete fusion, and transcriptional activities, which are crucial for successful fertilization and early embryo development.

DMSO-induced alterations in flagellar and metabolic proteins significantly affected sperm functionality, potentially decreasing fertility. The BBSome complex, which includes tetratricopeptide repeat protein 8 (TTC8), Bardet-Biedl syndrome 1 protein (BBS1), and Bardet-Biedl syndrome 7 protein (BBS7), plays significant role in intraciliary transport, cell membrane protein trafficking and mitochondrial activity[54,55]. Alterations to these proteins cause flagellar defects and reduced motility[56,57]. IFT25, which is involved in intraflagellar transport, is vital for flagellar assembly[58,59], while the kinesin-like protein KIFC3 (KIFC3), a motor protein, has been implicated in cellular transport and mitotic spindle assembly[60]. These proteins are also categorized as centrosome sperm proteins[61]. In addition to its roles in spermatogenic cell division and sperm formation, the centrosome is essential for regulating sperm tail beating and organizing zygote centrosomes after fertilization[62,63]. This finding underscores the novel insight that fertilization failure in DMSO-cryopreserved spermatozoa may also stem from disturbances to centrosome function. Our study also revealed a decrease in dynein axonemal intermediate chain 2 (DNAI2) and thioredoxin domain-containing protein 6 (NME9), which are crucial components for flagellar movement and axonemal organization, respectively, suggesting compromised flagellar integrity and potentially decreased fertility[64,65]. The significance of sperm tail morphology for fertilization capacity, particularly during the early postfertilization stages, has been emphasized[66]. Additionally, reductions in phosphorylase b kinase regulatory subunit alpha, skeletal muscle isoform (PHKA1) and ASRGL1, which are necessary for glycogen and asparagine metabolism, respectively, may affect energy production, impacting motility. These alterations could explain differences in motility parameters, such as ALH and BCF, between sperm cryopreserved with MeOH and those cryopreserved with DMSO. These parameters, while not related to major movement characteristics, such as the percentage of moving spermatozoa or their velocity, may reflect specific alterations to flagellar morphology. Overall, our study highlights a set of proteins that play a specific role in flagellar structure maintenance, centrosome function and metabolic processes, affecting both sperm movement patterns and fertilization ability, particularly in early embryo development. Further research is needed to explore the roles of these proteins in fish fertilization.

Among the DMSO-treated proteins, stress response proteins were altered. NHL repeat-containing protein 3 (NHLRC3) is critical for the ubiquitination process, and its reduction may disrupt the regulatory mechanisms of sperm essential for protein quality control. Proteasome subunit alpha type-6 (PSMA6), which supports protein degradation and turnover, is essential for cellular functions such as cell cycle regulation and DNA repair. The ubiquitin—proteasome system (UPS) is instrumental in the targeted degradation of proteins, including the removal of misfolded proteins, thus ensuring cellular homeostasis. In spermatozoa, the UPS plays a role in fertilization, particularly in the degradation of sperm mitochondrial components[67]. Heat shock proteins (HSPs), such as HSPA8 and HSPE1, are vital for protecting spermatozoa against stress-induced damage. They act as molecular chaperones, ensuring proper protein folding and assembly, which is crucial for maintaining the structural and functional integrity of sperm[68]. FTH1, which is involved in scavenging free radicals, is crucial for shielding spermatozoa from oxidative damage. The alterations observed in these proteins due to DMSO cryopreservation suggest a compromised ubiquitin—proteasome system and heat shock protein response; this may affect the proper folding of sperm proteins and the degradation of mitochondria following fertilization, leading to embryo death.

We identified several DMSO-specific alterations in sperm proteins whose roles in sperm functionality are not well understood. These proteins are involved in various biological processes, including nucleotide production, protein synthesis and immune response, suggesting a multifaceted impact on sperm functionality and fertility. Cytoplasmic C-1-tetrahydrofolate synthase (MTHFD1) has emerged as a key player in folate metabolism and is essential for nucleotide production and homocysteine methylation. Elevated homocysteine levels lead to recurrent miscarriage and compromised embryo quality[69–71]. Characterized by its leucine-rich repeat motifs, LRRC37A was predominantly expressed in the testis[72]. Leucine-rich repeat motifs, which are involved in protein—protein interactions, suggest that LRRC37A may play a role in spermatogenesis or other testicular functions, although its precise role has not been fully elucidated. Although the specific function of fibrillin-1 (FBN1) in spermatozoa is unknown, it is known to be critical during embryonic development, and mutations or dysfunction of this protein can lead to significant developmental disorders[73]. Furthermore, elongation factor 1-gamma (EEF1G) is involved in the elongation step of protein synthesis, which is fundamental for cell growth, division, and function. We also observed alterations in butyrophilin-like protein 2 (BTNL2) and CD276 antigen (CD276), proteins involved in immune regulation[74–76]. In summary, our findings underscore the intricate roles of DMSO-specific protein alterations in sperm functionality and fertility, highlighting the necessity for further research to unravel the complexities of their contributions to fertilization ability.

MeOH specifically influenced proteins associated with mitochondrial functionality, including those involved in energy production, as well as flagellar structure. Key mitochondrial enzymes, such as mitochondrial succinate-semialdehyde dehydrogenase (ALDH5A1), ethylmalonyl-CoA decarboxylase (ECHDC1), mitochondrial glutaryl-CoA dehydrogenase (GCDH), acyl-coenzyme A diphosphatase NUDT19 (NUDT19), mitochondrial methylcrotonoyl-CoA carboxylase beta chain (MCCC2), and HADH, which are involved in carboxylic and fatty acid catabolic processes, are crucial for ATP synthesis and maintaining controlled ROS levels, thereby ensuring cellular energy and reducing oxidative stress[77]. Additionally, alteration in mitochondrial citramalyl-CoA lyase, (CLYBL), mitochondrial ribosome-releasing factor 2 (GFM2) and bis(5'-nucleosyl)-tetraphosphatase [asymmetrical] (NUDT2) suggest potential disruption in mitochondrial integrity and function. Metabolic and regulatory proteins, including lysophosphatidic acid phosphatase type 6 (ACP6), inositol monophosphatase 1 (IMPA1), and SPRY domain-containing protein 4 (SPRYD4), which are involved in lipid metabolism, myo-inositol synthesis, osmoregulation and signaling pathways, respectively, also show significant changes[78]. Notable changes in proteins related to the stress response, such as oxidoreductase activities (glutaredoxin-1 (GLRX), delta-1-pyrroline-5-carboxylate synthase (ALDH18A1), mitochondrial adrenodoxin (FDX1), protein disulfide-isomerase (P4HB)), the ubiquitin−proteasome system (polyubiquitin-B (UBB) and 26S proteasome regulatory subunit 6A (PSMC3)), heat shock 70 kDa protein 4 L (HSPA4) and glutathione synthetase (GSS)), further suggest an elevated risk of oxidative damage, exacerbating susceptibility to oxidative stress, which is a common consequence of cryopreservation[79,80]. Alterations in axonemal proteins, including cilia- and flagella-associated protein 20 (CFAP20), CFAP96, microtubule-associated protein RP/EB family member 1 (MAPRE1), SPACA9, VIM, TUBB4, LEXM, and outer dense fiber protein 2 (ODF2), as well as the actin dynamics regulators WD repeat-containing protein 1 (WDR1) and tropomyosin alpha-3 chain (TPM3), may be indicative of decreased sperm motility due to compromised structural integrity and reduced tail flexibility[81]. Additionally, MeOH-induced modifications in the sperm axonemal maintenance protein CFAP97D1, Rho GDP-dissociation inhibitor 2 (ARHGDIB), and the SNARE protein vesicle-trafficking protein SEC22b (SEC22B), which is critical for vesicle trafficking, likely contribute to nuanced reductions in fertilization capability. Alterations in chromosomal structure-related proteins such as histone H2B (H2BC26) and the nuclear migration protein NudC (NUDC) suggest disturbances in the nucleosome structure of the chromosomal fiber, which may impact genetic integrity and expression. Collectively, these findings underscore the profound impact of MeOH on sperm functionality, particularly highlighting the decreased sperm motility and potential reductions in fertilization capacity following freeze−thaw cycles. Nevertheless, these changes in the proteome seem to be sublethal, as they did not produce a dramatic decrease in sperm fertilization ability compared to that of DMSO.

Our findings align with the literature on mammalian and fish species, demonstrating that sperm cryopreservation induces significant proteomic changes, impacting proteins essential for flagellar structure maintenance, membrane fluidity, sperm motility, energy production, antioxidative defense mechanisms and fertilization ability[10–18,82–86]. We identified major alterations in proteins involved in metabolic pathways (DBI, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1 (PFKFB1), PYGM, GAPDHS, ENO3, leukotriene A-4 hydrolase (LTA4H), AASS, PDE7A, and CKMT1A), suggesting a potential correlation with decreased semen quality after cryopreservation. Additionally, our findings revealed disruptions in oxidative and immune stress defenses (PDIA3, peroxiredoxin-4 (PRDX4), S-(hydroxymethyl)glutathione dehydrogenase (ADH5), HSPD1, ASS1) and protein degradation pathways (26S proteasome non-ATPase regulatory subunits 1, 6 and 7 (PSMD1, PSMD6, PSMD7), E3 ubiquitin-protein ligase UBR4 (UBR4)) that are critical for sperm motility, suggesting vulnerabilities in cellular integrity and fertilization processes. Additionally, as the most affected proteins, we identified nuclear proteins such as histones (H2A.Z) and Ran GTPase-activating protein 1 (RANGAP1), which belong to the nuclear complex family, and those involved in reproductive processes, including binding to the zona pellucida (IDH2, T-complex protein 1 subunit epsilon and subunit zeta (CCT5, CCT6A), CABYR, and ALDOA), which suggest disturbances in chromatin integrity, nucleocytoplasmic transport and fertilization ability. We observed that some proteins showed more significant alterations in response to different cryoprotectants. Moreover, this study revealed species-specific impacts, with only a few of the most altered proteins being commonly affected across sturgeons and carp (PDIA3)[26], rainbow trout (CCT6A and GAPDHS)[16], and sterlets (ENO3)[17,18], underscoring the nuanced effects of cryopreservation across species.

Interestingly, our study identified proteins with significantly increased abundance in sperm, despite sperm cells being transcriptionally and translationally inactive. This finding aligns with observations from previous proteomic studies examining the impact of cryopreservation on sperm proteomes in aquatic and mammalian species[26,82]. These prior studies suggest that the observed increase could be the result of stress-induced responses, potentially leading to selective protein exclusion from the membrane or the induction of PTMs, such as phosphorylation. This explanation was supported in our study by the identification of both up- and downregulated proteins that exhibited shifts in their pI, altering their positions on 2D-DIGE gels (Supplementary Fig. S3). Moreover, structural alterations to proteins have been posited as another potential mechanism for the observed increase in protein levels following cryopreservation[11]. However, further research could help to fully understand this issue using different methodological approaches.

The underlying mechanisms through which DMSO affects the fertilization capabilities of sturgeon remain largely unclear; however, recent studies have highlighted its substantial impact on cellular processes. The use of DMSO in cryopreservation has been associated with substantial modifications in mitochondrial function, including increased ROS levels and reduced ATP production in spermatozoa[87]. Additionally, the broader cytotoxic effects of DMSO, as evidenced by extensive DNA methylation disruptions in human cells[88], may explain the adverse effects observed in sturgeon spermatozoa. The proteomic alterations in sturgeon sperm following cryopreservation with DMSO likely reflect secondary damage rather than direct effects of the cryoprotectant, suggesting deeper implications of cellular disturbances linked to the mechanisms of DMSO action. Interestingly, while DMSO decreased fertilization efficacy in our studies, contrasting findings in other species, such as the Indian red jungle fowl, have shown enhanced fertilization potential when sperm were cryopreserved with 8% DMSO compared to glycerol[89]. This variation underscores the species-specific responses to cryoprotectants.

Conclusions

In summary, we unravel several significant changes in the sperm proteome specific to sturgeon in response to cryopreservation. Among the DMSO-specific proteins, those involved in the acrosome reaction, zona pellucida binding, flagellar structure and nuclear pore organization could explain why spermatozoa cryopreserved with DMSO had low hatching rate. These changes were not as pronounced when semen was cryopreserved with MeOH. The unique structure of sturgeon sperm and its specialized fertilization process, underscore the importance of tailored cryopreservation methods. Our research supports the earlier recommendation that methanol is the most effective cryoprotectant for sturgeon semen. This work also lays the groundwork for future research aimed at refining cryopreservation techniques, which is crucial for the preservation and successful reproduction of this ecologically and economically valuable species.

Data availability

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE[90] partner repository with the dataset identifiers PXD052170 and https://doi.org/https://doi.org/10.6019/PXD052170. Additional data that support the findings of this study are available from the corresponding author (M.A.D.) upon reasonable request.

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N.K.: Formal analysis, data curation, visualization, methodology, writing-original draft, writing-review & editing. A.C.: Conceptualization, writing-original draft, writing-review & editing. S.J.: Formal analysis and data curation. M.S.: Methodology. B.S.: Resources. B.Ś.: Formal analysis and data curation. M.A.D.: Conceptualization, methodology, writing-original draft, writing-review & editing, supervision, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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