Instytut Rozrodu Zwierząt i Badań Żywności

Polskiej Akademii Nauk w Olsztynie

PRACA DOKTORSKA

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Analiza transkryptomu oraz ekspresja markerów funkcji mitochondriów w blastocystach wyhodowanych in vitro z komórek jajowych pobranych od jałówek niedojrzałych płciowo

Praca doktorska wykonana
w Zakładzie Biologii Gamet i Zarodka
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Polskiej Akademii Nauk w Olsztynie
pod kierunkiem
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Pragnę podziękować osobom, które umożliwiły powstanie niniejszej rozprawy doktorskiej.

Pani prof. dr hab. Izabeli Wocławek- Potockiej

za wszechstronną pomoc udzieloną w trakcie przygotowywania pracy doktorskiej, nieocenione wsparcie merytoryczne, cenne rady, liczne konsultacje naukowe oraz nieustającą inspirację do krytycznego spojrzenia na problematykę badawczą

Pani dr inż. Ilonie Kowalczyk-Zięba

za okazaną pomoc udzieloną w trakcie przygotowywania pracy doktorskiej, istotne wskazówki w przeprowadzeniu analiz laboratoryjnych, wsparcie merytoryczne, oraz nieustającą motywację do wnikliwego spojrzenia na problematykę badawczą.

Serdeczne podziękowania składam także

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Słowa podziękowania składam na ręce moich

Rodziców

za wychowanie, motywację, cierpliwość, oraz wiarę we mnie

W sposób szczególny pragnę podziękować

Siostrze

na którą zawsze mogłam liczyć, za troskę, dobrą radę oraz wspieranie mnie w dążeniu do celu.

Dziękuję również wszystkim tym, których nie wymieniłam z Imienia i Nazwiska, za życzliwość i służenie pomocą.

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Spis treści

Streszczenie

Introdukcja do praktyki hodowlanej w najbardziej rozwiniętych gospodarstwach hodujących bydło, metody przyżyciowego pozyskiwania komórek jajowych (ovum pick up-OPU), szczególnie od zwierząt niedojrzałych płciowo, wraz z produkcją zarodków *in vitro* daje największe możliwości przyspieszenia postępu hodowlanego. Pomimo licznych doniesień świadczących, iż komórki jajowe pobrane od jałówek niedojrzałych płciowo cechują się niższą jakością oraz niższą kompetencją rozwojową, co bezpośrednio skutkuje niższym odsetkiem wyhodowanych *in vitro* blastocyst, nie było danych literaturowych dotyczących potencjalnych różnic w profilach transkryptomicznych zarodków wyhodowanych z komórek pobranych od zwierząt dojrzałych i niedojrzałych. Celem niniejszej rozprawy doktorskiej było zatem określenie współczynników rozwojowych oraz jakości blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo. Ponadto, w dysertacji porównano profile transkryptomiczne, oznaczono ekspresję genów związanych z regulacją fosforylacji oksydacyjnej (OXPHOS), z funkcją mitochondriów oraz liczbę kopii mitochondrialnego DNA (mtDNA), w blastocystach pozyskanych od jałówek dojrzałych i niedojrzałych płciowo. Dodatkowo w rozprawie doktorskiej określono liczbę komórek apoptotycznych w blastocystach bydlęcych wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo.

W ramach przeprowadzonych badań wykazano wyższy odsetek blastocyst pozyskanych od jałówek dojrzałych w porównaniu do niedojrzałych płciowo, nie stwierdzając różnic w jakości pozyskanych blastocyst. Ponadto, wykazano różnice w poziomie ekspresji genów biorących udział w szlaku OXPHOS, w zależności od wieku dawczyń komórek jajowych. Dodatkowo wykazano, iż blastocysty od jałówek niedojrzałych płciowo wykazują mniejszą możliwość efektywnego transportu elektronów podczas OXPHOS oraz znacznie mniej efektywną produkcję ATP, prowadząc do wyższego poziomu ROS. Dodatkowo,

zaobserwowane zmiany markerów molekularnych związanych z funkcją mitochondriów, ekspresja *TFAM* oraz wyższa liczba kopii mtDNA w zarodkach od niedojrzałych jałówek dowodzi o ich niższej zdolności do implantacji w porównaniu do zarodków pozyskanych od zwierząt dojrzałych płciowo. Jednakże, wyższy poziom glutationu w blastocystach od niedojrzałych jałówek, może chronić przed nadmierną akumulacją reaktywnych form tlenu obniżając liczbę komórek apoptotycznych w tych blastocystach.

Reasumując, uzyskane w niniejszej rozprawie doktorskiej wyniki są pierwszymi identyfikującymi różnice w ekspresji genów biorących udział w OXPHOS w blastocystach od jałówek niedojrzałych i dojrzałych płciowo. Ponadto, uzyskane w dysertacji wyniki wykazały, iż profil transkryptomiczny, ze szczególnym uwzględnieniem różnic przebiegu szlaku OXPHOS blastocyst wyhodowanych *in vitro*, ekspresja molekularnych markerów związanych z funkcją mitochondriów w blastocystach oraz liczba kopii mtDNA odzwierciedlają kompetencje rozwojowe zarodków. Dodatkowo, stworzona w ramach badań rozprawy doktorskiej baza profili transkryptomicznych umożliwia dalsze poszukiwanie molekularnych markerów kompetencji blastocyst zdolnych do transferu. Jednakże, w celu jednoznacznego potwierdzenia uzyskanego w niniejszej rozprawie wniosku, potrzebne jest przeprowadzenie dalszych doświadczeń *in vivo* i transfer wyhodowanych *in vitro* zarodków.

Summary

Introduction to the breeding practice, in the most developed cattle farms, the method of ovum pick up (OPU) for *in vitro* embryo culture, from prepubetral heifers, gives the best possibilities for the fastest genetic progress. Despite many scientific reports concerning that oocytes collected from prepubertal animals have much lower quality and developmental competence, resulting in much lower blastocyst rate comparing to oocytes collected from pubertal heifers, nobody has ever examined the differences between transcriptomic profiles of blastocysts derived from prepubertal and pubertal animals. Therefore, the first aim of the presented study was to establish developmental rates and quality of blastocysts derived *in vitro* from oocytes collected from prepubertal and pubertal animals. Moreover, in the dissertation we compared: the transcriptomic profiles, the expression of the genes connected with oxidative phosphorylation (OXPHOS) and mitochondrial function as well as the copy number of mitochondrial DNA (mtDNA) in the blastocysts derived from prepubertal and pubertal animals. Additionally, we determined the number of apoptotic cells in the blastocysts cultured *in vitro* from oocytes derived from prepubertal and pubertal animals.

In the conducted study, we documented higher blastocyst rate derived *in vitro* from oocytes from pubertal compared to those from the prepubertal group, finding no differences in the quality of the obtained blastocysts. Moreover, the differences in the expression levels of genes involved in the OXPHOS pathway were demonstrated, in accordance with the age of the donor of oocytes. In addition, blastocysts derived from prepubertal heifers oocytes showed to exhibit less ability of the effective electron transport during the OXPHOS and much lower effective production of ATP, leading to higher level of ROS, respectively. Additionally, observed changes of the expression of molecular markers related to mitochondrial function, the expression of *TFAM* and higher mtDNA copy number in blastocysts derived from prepubertal heifers oocytes, demonstrate their lower implantation ability compared to the ability obtained

from pubertal heifers. However, the higher intracellular levels of GSH in blastocysts derived from prepubertal heifers could probably protect cells against excessive accumulation of ROS and lead to lower number of apoptotic cells in those blastocysts.

To conclude, the results obtained in this dissertation are the first to identify differences in the expression of genes involved in OXPHOS in blastocysts from prepubertal and pubertal heifers oocytes. Furthermore, the results obtained in the dissertation indicate that the transcriptomic profile, with significant regard to the OXPHOS pathway of blastocyst cultured *in vitro*, the expression of molecular markers related to mitochondrial function in blastocysts and the number of mtDNA copies reflect the developmental competence of embryos. Additionally, the created database of the transcriptomic profiles, as the part of the dissertation reseach, enable further searching for the molecular markers of developmental competence of blastocysts designated for transfer. However, in order to unequivocally confirm the conclusion obtained in this dissertation, further *in vivo* experiments concerning transfer of *in vitro* cultured embryos are needed.

Wykaz skrótów

3PG ang. 3-Phosphoglyceric acid, kwas 3-fosfoglicerynowy

Acetyl-Coa ang. acetyl coenzyme A, acetylokoenzym A

ADP ang. adenosine diphosphate , adenozyno-5′-difosforan

AQP3 ang. Aquaporin 3, akwaporyna 3

ATP ang. adenosine triphosphate, adenozyno-5′-trifosforan

ATP12A ang. ATPase H^+/K^+ transporting non - gastric alpha2 subunit, ATPaza transportująca jony H^+, K^+

ATP5F1A ang. mitochondrial ATP synthase, mitochondrialna syntaza ATP

ATP5MF ang. ATP synthase membrane subunit f, błonowa podjednostka f syntazy ATP

ATP5PD ang. ATP synthase peripheral stalk subunit, podjednostka d obwodowej syntazy ATP

BP ang. biological process, proces biologiczny

CC ang. cellular component, składnik komórkowy

cDNA ang. complementary DNA, komplementarny DNA

CL- ang. corpus luteum, ciałko żółte

COX17 ang. cytochrome c oxidase copper chaperone, białko oksydazy cytochromu c

CS2 ang. citrate synthase, syntaza cytrynianowa

CYCS ang. cytochrome c somatic , cytochrom c somatyczny

CYTB ang. cytochrome b, region cytochromu B

DAVID ang. Database for Annotation, Visualization and Integrated Discovery, Baza danych do adnotacji, wizualizacji i zintegrowanego odkrywania

DCHFDA ang. 2’7’ dichlorodihydrofluorescein diacetate, dioctan 2', 7' dichlorodihydrofluoresceiny

DHAP ang. dihydroxyacetone phosphate, fosfodihydroksyaceton, fosforan dihydroksyacetonu

DNMT3A ang. DNA methyltransferase 3 alpha, metylotransferaza DNA 3 alfa

DSC2 ang. desmocollin 2, desmokolina 2

e^- ang. electron, jony elektronów

EGA ang. embryonic genome activation, aktywacja genomu zarodkowego

ET ang. embryo transfer, embriotransfer

FAD ang. flavin adenine dinucleotide, oxidized form , dinukleotyd flawinoadeninowy, forma utleniona

$FADH_2$ ang. flavin adenine dinucleotide, reduced form , dinukleotyd flawinoadeninowy, forma zredukowana

G6PD ang. glucose-6-phosphate dehydrogenase, dehydrogenaza glukozo-6-fosforanowa

GA3P ang. glyceraldehyde 3-phosphate, triose phosphate, 3-phosphoglyceraldehyde, aldehyd-3-fosfoglicerynowy

GAPDH ang. glyceraldehyde-3-phosphate dehydrogenase, dehydrogenaza aldehydu 3-fosfo-glicerynowego

GO ang. gene ontology, ontologia genów

GPX1 ang. glutathione peroxidase 1, peroksydaza glutationowa

GSH ang. glutathione, glutation

GV ang. germinal vesicle, stadium pęcherzyka zarodkowego

H^+ ang. cationic form of atomic electron, kationowa forma wodoru atomowego

H_2O-ang. hydrogen oxide, tlenek wodoru

IETS ang. International Embryo Technology Society, Międzynarodowe Towarzystwo Technologii Zarodków

IGF1R ang. insulin-like growth factor receptors 1, receptory insulinopodobnego czynnika wzrostu 1

IGF2R ang. insulin-like growth factor receptors 2, receptory insulinopodobnego czynnika wzrostu 2

KEGG ang. Kyoto Encyclopedia of Genes and Genomes, Kyoto encyklopedia genów i genomów – baza danych

LDHA2 ang. enzyme lactate dehydrogenase A, dehydrogenaza mleczanowa

MF ang. molecular function, funkcja molekularna

mRNA ang. messenger RNA, informacyjny RNA, matrycowy RNA

mtDNA ang. mitochondrial DNA, mitochondrialny DNA

mTOR ang. mammalian target of rapamycin, szlak kinazy serynowo-treoninowej

NAD^+ ang. Nicotinamide adenine dinucleotide, oxidized form, dinukleotyd nikotynoamidoadeninowy, forma utleniona

NADH ang. nicotinamide adenine dinucleotide, reduced form, dinukleotyd nikotynoamidoadeninowy, forma zredukowana

NAM+o-ADPR ang. nicotinamide adenosine diphosphate ribose (ADPR), nikotynamid adenozynodifosforanu rybozy

NANOG ang. homeobox nanog, czynnik transkrypcyjny pluripotencji

ND2 ang. NADH dehydrogenase 2, podjednostka II dehydrogenazy NADH

NDUFA13 ang. NADH: ubiquinone oxidoreductase subunit A13, podjednostka A13 oksydoreduktazy ubichinonowej

NDUFA3 ang. NADH: ubiquinone oxidoreductase subunit A3, NADH: podjednostka A3 oksydoreduktazy ubichinonu

NDUFS3 ang. NADH: ubiquinone oxidoreductase core subunit S3, podjednostka S3 rdzenia oksydoreduktazy ubichinonowej NADH

NGS ang. next generation sequencing, sekwencjonowanie nowej generacji

NRF2 ang. nuclear factor erythroid 2-related factor 2, jądrowy czynnik transkrypcyjny pochodzenia erytroidalnego typu 2

O_2 ang. molecular oxygen, dioxygen, wzór dwuatomowej cząsteczki tlenu

OAA ang. Oxaloacetic acid, Kwas szczawiowooctowy

OCT4 ang. octamer-binding transcription factor 4, czynnik transkrypcyjny 4 wiążący oktamer

OPU ang. ovum pick up, metody przyżyciowego pozyskiwania komórek jajowych

OXPHOS ang. oxidative phoshorylation, fosforylacja oksydacyjna

PEP ang. phosphoenolpyruvate, kwas fosfoenolopirogronowy

PEPCK ang. phosphoenolpyruvate carboxykinase, karboksykinaza fosfoenolopirogronianowa

PLAC8 ang. placenta-specific 8 gene, gen swoisty dla łożyska 8

PLAU ang. plasminogen activator urokinase, urokinaza aktywatora plazminogenu

POU5F1 ang. POU Class 5 Homeobox 1, domena POU klasy 5 homeobox 1

PPAR ang. peroxisome proliferator-activated receptors, receptory aktywowane przez proliferatory peroksysomów

qPCR ang. Quantitative PCR, ilościowa reakcja łańcuchowej polimerazy w czasie rzeczywistym

real-time PCR ang. real-time polymerase chain reaction, ilościowa reakcja łańcuchowa polimerazy, reakcja łańcuchowa polimerazy w czasie rzeczywistym

RNA- seq ang. RNA sequencing, sekwencjonowanie RNA

ROS ang. reactive oxygen species, reaktywne formy tlenu

SD ang. standard deviation, odchylenie standardowe

SIRT2 ang. mitochondrial NAD-dependent deacetylase, mitochondrialna deacetylaza zależna od NAD

SLC2A1 ang. solute carrier family 2 facilitated glucose transporter member 1, transporter glukozy typu 1

SLC2A5 ang. solute carrier family 2 facilitated glucose transporter member 5, transporter glukozy typu 5

SOX2 ang. sex determining region Y-box 2, region determinujący płeć

SSLP1 ang. secreted vesicle seminal protein 1, białko pęcherzyka nasiennego 1

SucCoa ang. Succinyl-coenzyme A, succinyl-CoA, bursztynylo-koenzym A

TFAM ang. mitochondrial transcription factor A, mitochondrialny czynnik transkrypcyjny A

TUNEL ang. terminal-uridine nick-end labeling, metoda do oceny stopnia fragmentacji DNA

UQCRC1 ang. ubiquinol-cytochrome C reductase core protein I, białko rdzeniowe reduktazy ubichinolu-cytochromu C I

WebGestalt ang. WEB-based Gene SeT Analysis Toolkit

α -KG ang. α-Ketoglutaric acid, Kwas α-ketoglutarowy

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I. Traut M, Kowalczyk-Zieba I, Boruszewska D, Jaworska J, Lukaszuk K, Woclawek-Potocka I. Mitochondrial DNA content and developmental competence of blastocysts derived from pre-pubertal heifer oocytes. Theriogenology. 2022 Oct 1;191:207-220. doi: 10.1016/j.theriogenology.2022.07.017. Epub 2022 Aug 8. PMID: 35998404.

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II. Traut M, Kowalczyk-Zieba I, Boruszewska D, Jaworska J, Gąsiorowska S, Lukaszuk K, Ropka-Molik K, Piórkowska K, Szmatoła T, Woclawek-Potocka I. Deregulation of oxidative phosphorylation pathways in embryos derived in vitro from prepubertal and pubertal heifers based on whole-transcriptome sequencing. BMC Genomics. 2024 Jun 24;25(1):632. doi: 10.1186/s12864-024-10532-7. PMID: 38914933; PMCID: PMC11197288.

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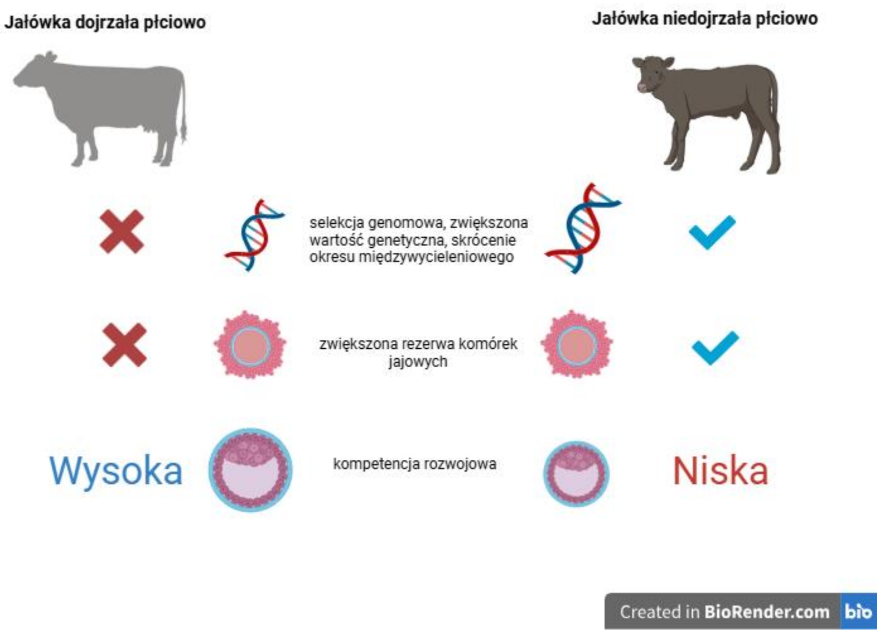
Publikacje przedstawiono w formie załączników do rozprawy doktorskiej.

Wstęp

Wśród metod wspomaganego rozrodu stosowanych w rozrodzie bydła, embriotransfer (ET, ang. embryo transfer) daje największe możliwości przyśpieszenia postępu hodowlanego [Ferré i wsp. 2020]. Metoda ta znalazła zastosowanie w nowoczesnej hodowli bydła ze względu na możliwość uzyskania w szybkim czasie wielu osobników, o wyselekcjonowanych cechach, pochodzących od cennych genetycznie matek i buhajów. Powszechnie stosowana metoda inseminacji umożliwia uzyskanie średnio jednego cielęcia od krowy w ciągu roku, z transferu zarodków wypłukanych od samic dawczyń możemy uzyskać nawet ponad 20 cieląt [Lonergan, 2007], a z transferu zarodków wyprodukowanych *in vitro* aż 80-100 cieląt od krowy w ciągu roku [Van Wagtendonk-de Leeuw, 2006]. Introdukcja metody przyżyciowego pozyskania komórek jajowych (OPU, ang. ovum pick up) na skalę przemysłową, w celu produkcji zarodków *in vitro*, umożliwia ponadto znaczne przyśpieszenie postępu hodowlanego, również dzięki możliwości wykorzystania do produkcji zarodków, gamet, pobieranych od coraz młodszych zwierząt ze stada, w tym również od osobników niedojrzałych płciowo [Faber i wsp. 2003; Galli i wsp. 2003; Currin i wsp. 2021]. Bezsprzeczną zaletą wykorzystania zwierząt niedojrzałych płciowo w technologii produkcji zarodków *in vitro* jest fakt, że pula dostępnych u tych zwierząt komórek jajowych jest dużo większa w porównaniu do puli komórek u zwierząt dojrzałych [Revel i wsp. 1995; Tervit, 1996]. Biorąc powyższe okoliczności pod uwagę, w praktyce hodowlanej produkcja zarodków *in vitro*, również od zwierząt niedojrzałych płciowo, stale zyskuje na popularności z uwagi na możliwość znacznego przyspieszenia postępu hodowlanego poprzez skrócenie okresu międzywycieleniowego [Duby i wsp. 1996; Khatir i wsp. 1996; Kasinathan i wsp. 2015] oraz zwiększenia wydajności selekcji zwierząt (selekcja genomowa pod kątem pożądanych cech hodowlanych) [Wiggans i wsp. 2017; Moore & Hasler, 2017].

Pomimo stale zwiększającego się zainteresowania dużych ferm produkcyjnych pozyskiwaniem zarodków od jałówek niedojrzałych płciowo, istnieją ograniczenia i trudności w uzyskaniu podobnej, do zwierząt dojrzałych, skuteczności uzyskania blastocyst, wynikające w głównej mierze z braku oddziaływania hormonów płciowych na gonady. Przed osiągnięciem dojrzałości płciowej samicy, oś podwzgórze-przysadka-jajnik jest niedojrzała, co może prowadzić do zaburzeń procesu folikulogenezy, steroidogenezy oraz owulacji [Apter, 1997; Atkins i wsp. 2013; Currin i wsp. 2021]. Udowodniono, że komórki jajowe pobrane od jałówek niedojrzałych płciowo cechują się niższą jakością oraz niższą kompetencją rozwojową, co bezpośrednio skutkuje niższym odsetkiem wyhodowanych *in vitro* blastocyst [Seidel i wsp. 1971; Lévesque & Sirard, 1994; Revel i wsp. 1995]. Na schemacie poniżej (Ryc. 1) przedstawiono porównanie wykorzystania jałówek dojrzałych i niedojrzałych płciowo w technologii produkcji zarodków *in vitro*.



Rycina 1. Wykorzystanie jałówek dojrzałych i niedojrzałych płciowo w produkcji blastocyst w warunkach in vitro (Opracowanie własne wykonane z wykorzystaniem programu Biorender na podstawie Currin i wsp. 2021).

Biorąc powyższe pod uwagę, nieustającym wyzwaniem dla wydajności produkcji zarodków *in vitro,* zarówno od zwierząt dojrzałych jak i niedojrzałych płciowo, jest duże zróżnicowanie jakości komórek jajowych. Potencjał rozwojowy oocytów pobranych przyżyciowo lub pośmiertnie jest wciąż głównym czynnikiem limitującym uzyskanie właściwej kompetencji rozwojowej blastocyst, co skutkuje pozyskiwaniem trudnej do oszacowania liczby blastocyst nadających się do transferu, a w dalszej kolejności implantacji w macicy i powstania ciąży [Revel i wsp. 1995; Kauffold i wsp. 2005; Rizos i wsp. 2005; Zaraza i wsp. 2010; Currin i wsp. 2017]. Już w 1995 roku Revel i wsp. opisali model dobrej i złej jakości oocytów bydła, na podstawie wieku i dojrzałości płciowej samic od których zostały pozyskane komórki. Wyniki przeprowadzonych przez Revel'a i wsp. [1995] badań, wskazują na statycznie niższy odsetek blastocyst wyhodowanych *in vitro* od cieląt w porównaniu do krów. Pomimo licznych doniesień o niższej jakości komórek jajowych pochodzących od zwierząt niedojrzałych płciowo [Revel i wsp. 1995; Armstrong, 2001; Kauffold i wsp. 2005; Rizos i wsp. 2005; Warzych i wsp. 2017; Currin i wsp. 2021] oraz związanej z tym większej trudności w uzyskaniu zarodków *in vitro*, nie ma danych literaturowych dotyczących istniejących różnic w profilach transkryptomicznych zarodków wyhodowanych *in vitro* pomiędzy tymi dwiema grupami jałówek. Określenie różnic w globalnej ekspresji genów, wyznaczenie szlaków metabolicznych potencjalnie istotnych dla funkcjonowania wyhodowanych *in vitro* zarodków, oraz scharakteryzowanie biologicznie nowych jednostek transkrypcyjnych, pozwoli zrozumieć różnice w przemianach metabolicznych w obydwu badanych grupach zarodków i potencjalnie powiązać konkretne z nich z jakością komórek jajowych. Zdobycie nowej wiedzy w tym zakresie, może w przyszłości przyczynić się do rozwoju technik selekcji genomowej zarodków przed ich transferem do biorczyń, a także umożliwi podjęcie próby identyfikacji markerów jakości gamet, które mogłyby być celem terapii genowych, umożliwiających zwiększenie wydajności produkcji *in vitro* zarodków od zwierząt niedojrzałych.

Pośród wielu szlaków metabolicznych mających kluczowe znaczenie dla funkcjonowania wyhodowanych *in vitro* zarodków, fosforylacja oksydacyjna (OXPHOS, ang. oxidative phoshorylation) znalazła się w kręgu szczególnych zainteresowań podjętych badań w ramach pracy doktorskiej. Fosforylacja oksydacyjna jest głównym procesem umożliwiającym powstanie adenozynotrifosforanu (ATP, ang. adenosine triphosphate), czyli cząsteczek energii niezbędnych do prawidłowego przebiegu większości reakcji komórkowych. Zjawisko OXPHOS zachodzi w mitochondriach - centrach energetycznych komórki. Podczas OXPHOS, 33 cząsteczki ATP są wytwarzane przez łańcuch transportu elektronów w błonie mitochondrialnej [Berg i wsp. 2002; Mookerjee i wsp. 2017]. Mitochondria są niewielkimi organellami występującymi we wszystkich komórkach eukariotycznych [May-Panloup i wsp. 2021]. Uważa się, że wyewoluowały w wyniku procesu endosymbiozy między pierwotną komórką eukariotyczną, a α-proteobakterią zdolną do metabolizowania tlenu [Gray, 1999]. Udowodniono, że również w komórkach jajowych mitochondria odpowiedzialne są za produkcję ponad 90% ATP jako głównego źródła energii poprzez proces OXPHOS z wykorzystaniem glukozy [Van Blerkom, 2009; Van Blerkom, 2011; Harvey, 2019], co z kolei niezbędne jest do podtrzymania prawidłowych kompetencji rozwojowych zarodka [Van Blerkom i wsp. 1995; Herst i wsp. 2017]. Jakkolwiek, znaczenie mitochondriów podczas przedimplantacyjnego rozwoju zarodka nie odnosi się tylko do wytwarzania ATP [Harvey, 2019] lecz również regulacji biosyntezy wielu związków organicznych, apoptozy, homeostazy wapnia w komórce, termogenezy, a także regulacji ekspresji, istotnych dla samych mitochondriów genów [Gut & Verdin, 2013; Chandel, 2014; Shadel & Horvath, 2015]. Ponadto, z literatury wiadomo, że geny kodujące syntezę czynników transkrypcyjnych, regulujących syntezę mitochondrialnego DNA (mtDNA, ang. mitochondrial DNA) mogą również, dzięki kontroli tych procesów, być uznane za markery jakości zarówno gamet jak i zarodków [May-Panloup i wsp. 2007; Fragouli i wsp. 2015; Viotti wsp. 2017; Victor i wsp. 2017; Hoshino, 2018]. Jednym z takich czynników jest mitochondrialny czynnik transkrypcyjny A (*TFAM*, ang. mitochondrial transcription factor A). Jest to białko będące kluczowym aktywatorem replikacji i transkrypcji mtDNA [Parisi i wsp. 1993], niezbędne dla regulacji liczby kopii mtDNA [Larsson i wsp. 1998]. May-Panloup i wsp. [2005] udowodnili, że liczba kopii mtDNA potrzebnego do prawidłowego rozwoju embrionalnego jest bezpośrednio skorelowana z ekspresją *TFAM*. Znane są również inne potencjalne markery związane z funkcją mitochondriów takie jak: *SLC2A1* (solute carrier family 2 facilitated glucose transporter member 1-transporter glukozy typu 1), *SLC2A5* (solute carrier family 2 facilitated glucose transporter member 5- transporter glukozy typu 5), *LDHA2* (enzyme lactate dehydrogenase A-dehydrogenaza mleczanowa), *G6PD* (glucose-6-phosphate dehydrogenase-dehydrogenaza glukozo-6-fosforanowa), *SIRT2* (mitochondrial NAD-dependent deacetylase-mitochondrialna deacetylaza zależna od NAD), *CS2* (citrate synthase-syntaza cytrynianowa), *ATP5F1A* (mitochondrial ATP synthase- mitochondrialna syntaza ATP), oraz *GPX1* (glutathione peroxidase 1- peroksydaza glutationowa) [Augustin i wsp. 2001; Cetica i wsp. 2002; Krisher i wsp. 2007; Bermejo-Alvarez i wsp. 2010; Goovaerts i wsp. 2011; Wang i wsp. 2012; Wang i wsp. 2014], których poziom ekspresji bezpośrednio wiąże się z zachowaniem prawidłowej funkcji różnych typów komórek oraz samych mitochondriów.

Reasumując, niewyjaśniony pozostaje fakt, czy profil transkryptomiczny, ze szczególnym uwzględnieniem szlaku OXPHOS blastocyst wyhodowanych *in vitro* odzwierciedla jakość komórek jajowych. Dodatkowo, znalezienie markerów związanych z funkcją mitochondriów oraz jakością blastocyst mogłoby być alternatywą dla stosowanej dotychczas subiektywnej oceny morfologicznej, podczas selekcji zarodków do transferu. Ponadto, wiedza na temat fizjologii oocytów oraz blastocyst mogłaby przyczynić się do rozwoju technik selekcji genomowej zarodków przedimplantacyjnych. Brak jest również danych literaturowych dotyczących różnic w liczbie kopii mtDNA w blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo, a także czy liczba kopii mtDNA w obu badanych grupach blastocyst związana jest z jakością komórki jajowej. Zaplanowane w pracy doktorskiej badania nad główną funkcją mitochondriów mają na celu znalezienie markerów molekularnych mogących odzwierciedlać jakość komórek jajowych oraz kompetencje rozwojowe zarodków.

Hipoteza i cele badawcze:

W oparciu o badania własne oraz dane literaturowe, w ramach niniejszej pracy doktorskiej, postawiono nadrzędną hipotezę badawczą:

Profil transkryptomiczny, ze szczególnym uwzględnieniem szlaku fosforylacji oksydacyjnej, blastocyst wyhodowanych in vitro, ekspresja molekularnych markerów związanych z funkcją mitochondriów w blastocystach oraz liczba kopii mtDNA, odzwierciedlają jakość komórek jajowych oraz kompetencję rozwojową zarodków.

CELE:

1. Określenie współczynników rozwojowych blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo;
2. Porównanie profili transkryptomicznych blastocyst bydlęcych wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo;
3. Porównanie ekspresji molekularnych markerów związanych z funkcją mitochondriów w blastocystach bydlęcych wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo;
4. Porównanie liczby kopii mtDNA w blastocystach bydlęcych wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo;
5. Określenie liczby komórek apoptotycznych w blastocystach bydlęcych wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo.

Materiał i metody

Doświadczenia z wykorzystaniem zwierząt zostały pozytywnie zaopiniowane przez Lokalną Komisję Etyczną ds. Doświadczeń na Zwierzętach w Olsztynie (Decyzje nr 76/2014/DTN oraz 55/2023).

Do doświadczeń wykorzystano jałówki rasy polskiej holsztyńsko-fryzyjskiej odmiany czarno-białej, wolne od chorób. Dojrzałość płciową jałówek oceniono poprzez badanie USG przy użyciu skanera DRAMIŃSKI ANIMAL profi Scanner (DRAMIŃSKI, Olsztyn, Polska). Zwierzęta zostały podzielone na dwie grupy: jałówki dojrzałe płciowo (≥15 miesięcy; n=15) oraz niedojrzałe płciowo (≤12 miesięcy, brak obecności CL- ang. corpus luteum, ciałko żółte i/lub pęcherzyka dominującego na jajniku; n=15). Jałówki dojrzałe płciowo stanowiły grupę kontrolną [Traut i wsp. 2022, **I;** Traut i wsp. 2024, **II**].

Komórki jajowe pobierano przyżyciowo metodą OPU wg metody opisanej przez Cavalieri i wsp. [2018]. Oocyty do badań pozyskano łącznie w trakcie 54 sesji OPU w grupach liczących jednorazowo 15 jałówek dojrzałych i 15 jałówek niedojrzałych płciowo. Uzyskane kompleksy oocyt-komórki wzgórka jajonośnego oceniano morfologicznie pod względem jakości (jednorodności cytoplazmy oraz liczby warstw komórek ziarnistych). Następnie, kompleksy oocyt-komórki wzgórka jajonośnego (oddzielnie od jałówek dojrzałych i niedojrzałych płciowo) dojrzewano *in vitro* przez 23 godziny. Dojrzałe oocyty zapładniano *in vitro* z wykorzystaniem kriokonserwowanego, zakupionego komercyjnie nasienia buhaja Ouled (Sexing Technologies, USA). Po 24 godzinach od zapłodnienia usunięto komórki wzgórka a potencjalne zygoty hodowano *in vitro* do osiągnięcia stadium blastocysty (Dzień 7). Współczynniki rozwojowe uzyskanych blastocyst i ich jakość określono zgodnie z normami przyjętymi przez IETS (International Embryo Technology Society, Międzynarodowe Towarzystwo Technologii Zarodków).

Do analiz przeprowadzonych w ramach niniejszej rozprawy doktorskiej wykorzystano blastocysty wyhodowane *in vitro* z oocytów pobranych od zwierząt niedojrzałych płciowo w liczbie: n=52 [Traut i wsp. 2022, **I**] oraz n=45 [Traut i wsp. 2024, **II**] oraz blastocysty wyhodowane *in vitro* z oocytów pobranych od zwierząt dojrzałych płciowo w liczbie: n=63 [Traut i wsp. 2022, **I**] i n=45 [Traut i wsp. 2024, **II**].

Metoda Real-Time PCR została wykorzystana w celu oznaczenia ekspresji mRNA markerów związanych z funkcją mitochondriów dla: *TFAM*, *SLC2A1*, *SLC2A5*, *LDHA2*, *G6PD*, *SIRT2*, *CS2*, *ATP5F1A*, oraz *GPX1* [Traut i wsp. 2022, **I**] oraz markerów jakości blastocyst: *OCT4*, *SOX2*, *NANOG*, *PLAC8*, *IGF1R*, *IGF2R*, *PLAU*, *SSLP1*, *DSC2*, *DNMT3A*, *AQP3* oraz *GAPDH* jako kontroli wewnętrznej [Traut i wsp. 2022, **I**]. Metoda Real-Time PCR posłużyła także do walidacji wyników uzyskanych w ramach analizy NGS w celu potwierdzenia różnic w ekspresji mRNA dla genów: *ATP5MF*, *ATP5PD*, *ATP12A*, *NDUFA13*, *NDUFA3*, *NDUFS3*, *CYCS*, *COX17*, *UQCRC1* oraz *GAPDH* jako kontroli wewnętrznej [Traut i wsp. 2024, **II**].

Metoda Sekwencjonowania Nowej Generacji (NGS; RNA-seq) została wykorzystana w celu analizy całego transkryptomu blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo [Traut i wsp. 2024, **II**]. Dla analizowanych blastocyst przygotowano biblioteki cDNA, wg zestawu SMARTer Stranded Total RNA Seq Kit v3- Pico Input Mammalian (Takara Bio, USA, Inc) zgodnie z instrukcją producenta [Traut i wsp. 2024, **II**]. Geny różniące się poziomem ekspresji zostały zakwalifikowane do odpowiedniej kategorii ontologii genów oraz szlaków biologicznych z wykorzystaniem oprogramowania DAVID 6.8 i baz danych: KEGG (zgodnie z odniesieniem Bos Taurus) oraz WebGestalt (WEB-based Gene SeT Analysis Toolkit) [Traut i wsp. 2024, **II**].

W blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo określono liczbę kopii mitochondrialnego DNA. Do izolacji całkowitego DNA wykorzystano zestaw Sherlock AX (#095-100, A&A Biotechnology, Polska), zgodnie z protokołem producenta [Traut i wsp. 2022, **I**]. Ilość mitochondrialnego DNA oznaczono stosując specyficzne startery dla regionu cytochromu B: *CYTB* oraz podjednostki dehydrogenazy NADH-*ND2* [Traut i wsp. 2022, **I**]. Ilość DNA jądrowego oznaczono stosując startery specyficzne dla genu *POU5F1* [Traut i wsp. 2022, **I**]. Ilość mitochondrialnego DNA mierzono za pomocą ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym (qPCR) i wyrażono jako względny stosunek ilości mitochondrialnego DNA do jądrowego DNA (dzieląc przez połowę liczby kopii genu *POU5F1*) [Traut i wsp. 2022, **I**].

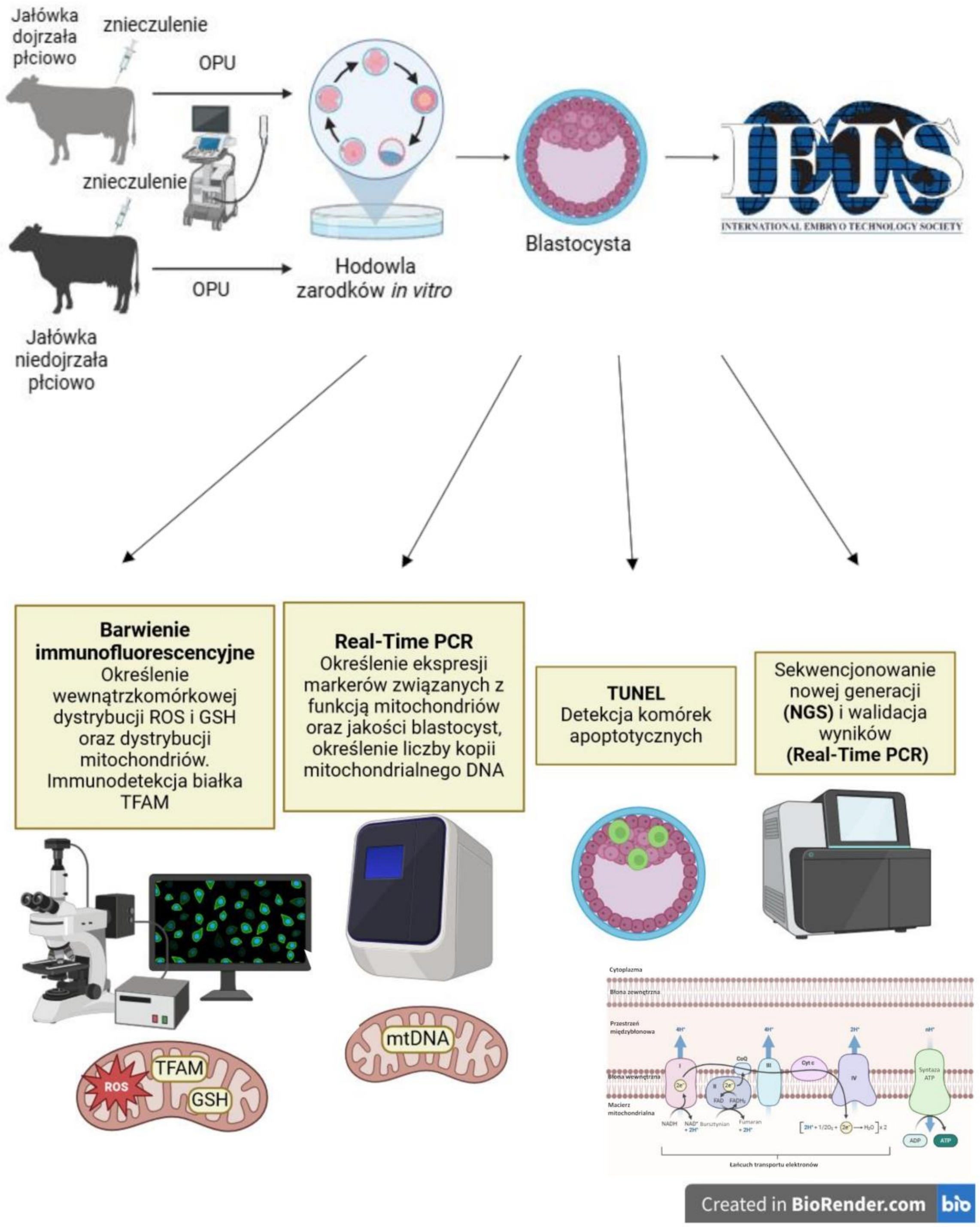
Wewnątrzkomórkową dystrybucję ROS i GSH oraz dystrybucję mitochondriów określono metodą barwień immunofluorescencyjnych z wykorzystaniem komercyjnych zestawów, odpowiednio: (DCHFDA) (#D6665; Merck, Niemcy), ThiolTracker™ Violet GSH (#T10095; ThermoFisher Scientific, USA), MitoTracker RedCMXRos (#M7512; Thermo Fisher Scientific, USA) stosując się do zaleceń producenta [Traut i wsp. 2022, **I**]. Immunodetekcję białka TFAM określono z wykorzystaniem specyficznych przeciwciał (#sc-166965; RRID:AB_10610743; Santa Cruz Biotechnologies, USA) [Traut i wsp. 2022, **I**].

Detekcje komórek apoptotycznych w blastocystach przeprowadzono metodą TUNEL (Terminal-uridine nick-end labeling) przy użyciu zestawu In Situ Cell Death Detection Kit Fluorescein (#11684795910, Roche, Niemcy) [Traut i wsp. 2024, **II**].

Analizę statystyczną wyników przeprowadzono przy użyciu oprogramowania GraphPad PRISM 9.0 (GraphPad Software, Inc., USA). Zgodność rozkładów zmiennej z rozkładem normalnym oceniono z wykorzystaniem testów D'Agostino-Pearsona oraz Shapiro-Wilka [Traut i wsp. 2022, **I**]. Różnice statystyczne określono przy użyciu testów statystycznych: testu t-Studenta, dokładnego testu Fishera, których wybór był zgodny z projektem doświadczenia, liczbą prób oraz rozkładem Gauss'a [Traut i wsp. 2022, **I**].

Uzyskane dane przedstawiono jako średnią ± odchylenie standardowe (SD), a za poziom istotności przyjęto ($p < 0.05$) [Traut i wsp. 2022, **I**; Traut i wsp. 2024, **II**].

Doświadczenia przeprowadzone w niniejszej pracy doktorskiej przedstawiono wg schematu umieszczonego poniżej (Rycina 2).



Rycina 2. Schemat doświadczeń wykonanych w niniejszej rozprawie doktorskiej (Opracowanie własne wykonane z zastosowaniem programu Biorender).

Omówienie uzyskanych wyników i dyskusja

Zagadnienie skuteczności prowadzenia hodowli *in vitro* blastocyst bydlęcych jest od wielu lat przedmiotem zainteresowania różnych ośrodków badawczych na całym świecie [Gualtieri i wsp. 2024]. Najbardziej obiektywnym współczynnikiem świadczącym o skuteczności tej metody biotechnologicznej jest współczynnik uzyskania blastocyst, wyrażony liczbą morfologicznie prawidłowych zarodków uzyskanych w trakcie jednego cyklu produkcji zarodków, w stosunku do liczby komórek jajowych poddanych procedurze zapłodnienia pozaustrojowego [Ferré i wsp. 2020]. Z uwagi na fakt, że w praktyce hodowlanej produkcja zarodków *in vitro*, od zwierząt niedojrzałych płciowo, stale zyskuje na popularności, jednym z założeń niniejszej rozprawy doktorskiej było wyznaczenie współczynników rozwojowych oraz określenie jakości blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo. W wyniku badań prowadzonych w dysertacji, uzyskano porównywalną średnią liczbę pobranych komórek jajowych od jałówek dojrzałych i niedojrzałych płciowo [$p>0.05$; Tabela 2,Traut i wsp. 2022, **I;** $p>0.05$; Tabela 3, Traut i wsp. 2024, **II**]. Powyższe wyniki nie są spójne z danymi zaprezentowanymi przez Tervit [1996], w których autorzy wskazują na wyższą pulę komórek jajowych od cieląt w porównaniu do puli komórek u krów [Tervit, 1996]. W cytowanych badaniach brak jest jednak informacji o jakości i klasyfikacji pobieranych komórek, jak również liczba komórek wyznaczana była podczas badań laparoskopowych dokumentujących liczbę pęcherzyków antralnych, a nie liczby oocytów pobranych za pomocą procedury OPU. Co więcej, w badaniach przeprowadzonych w niniejszej pracy, przedstawiona do porównania liczba komórek jajowych dotyczyła jedynie komórek 1 i 2 klasy wg klasyfikacji (IETS). Komórki 3 i 4 klasy nie były przez nas poddawane dalszej procedurze hodowli zarodków *in vitro*, ani w grupie komórek pobieranych od zwierząt dojrzałych, ani niedojrzałych płciowo. W wyniku przeprowadzenia hodowli zarodków *in vitro* w obydwu tych grupach badawczych uzyskaliśmy wyższy współczynnik rozwoju blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych w porównaniu do niedojrzałych płciowo [$p<0.05$; Tabela 2, Traut i wsp. 2022, **I**; $p<0.05$; Tabela 3, Traut i wsp. 2024**, II**]. Przedstawione w niniejszej rozprawie wyniki są spójne z danymi zaprezentowanymi przez Rizos i wsp. [2005], którzy również udowodnili wyższą skuteczność produkcji *in vitro* zarodków od zwierząt dojrzałych płciowo. Jakkolwiek, w przeprowadzonych w ramach dysertacji badaniach, nie wykazano statystycznych różnic w morfologicznej ocenie jakości uzyskanych blastocyst w obydwu grupach badanych zwierząt [$p>0.05$; Tabela 2, Traut i wsp. 2022, **I**; $p>0.05$; Tabela 3, Traut i wsp. 2024**, II**], pomimo statystycznie mniejszej liczby wyhodowanych *in vitro* blastocyst w grupie jałówek niedojrzałych płciowo.

Kolejnym celem prowadzonych w ramach rozprawy doktorskiej badań, było porównanie profili transkryptomicznych blastocyst bydlęcych wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo. Już od pierwszej dekady XX wieku, wielu autorów, za pomocą analizy profili transkryptomicznych, przedstawiało różnice przebiegu kluczowych mechanizmów molekularnych zachodzących we wczesnym rozwoju embrionalnym bydła – takich jak np. aktywacja genomu zarodkowego (EGA, ang. embryonic genome activation) w zarodkach bydlęcych uzyskanych *in vivo* [Kues i wsp. 2008; Gad i wsp. 2012; Jiang i wsp. 2014] oraz zarodkach wyhodowanych *in vitro* [Gad i wsp. 2012; Graf i wsp. 2014; Kropp i wsp. 2017; Wei i wsp. 2017]. Pomimo wielu różnych analiz profili transkryptomicznych zarodków hodowanych w różnych warunkach *in vitro,* pozyskiwanych w różny sposób, nie istnieją dane literaturowe dotyczące porównania profili transkryptomicznych blastocyst bydlęcych wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo. Jedynie Morin- Doré i wsp. [2017] analizowali transkryptom blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo, jakkolwiek z zastosowaniem metody mikromacierzy. Autorzy wykazali, w przeciwieństwie do wyników przedstawionych w niniejszej rozprawie doktorskiej, występowanie szlaków związanych z regulacją proliferacji w komórkach - tj. mTOR (ang. mammalian target of rapamycin, szlak kinazy serynowo-treoninowej), PPAR (ang. peroxisome proliferator-activated receptors - receptory aktywowane przez proliferatory peroksysomów), i NRF2 (ang. nuclear factor erythroid 2-related factor 2, jądrowy czynnik transkrypcyjny pochodzenia erytroidalnego typu 2), jako głównych szlaków odpowiedzialnych za regulację żywotności i różnicowania komórek, jak również szlaków związanych z regulacją ekspresji genów metabolizmu lipidów oraz ochroną komórek przed stresem oksydacyjnym.

Przedstawiona w niniejszej rozprawie doktorskiej analiza transkryptomu pozwoliła na zidentyfikowanie 436 genów różniących się poziomem ekspresji. Zaobserwowano 247 genów o niższej i 189 genów o wyższej ekspresji w blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych płciowo w porównaniu do blastocyst od jałówek niedojrzałych. Przeprowadzona analiza ontologii genów (GO-ang. gene ontology, ontologia genów), z wykorzystaniem bazy DAVID (Database for Annotation, Visualization and Integrated Discovery, Baza danych do adnotacji, wizualizacji i zintegrowanego odkrywania) i bazy WebGestalt (WEB-based Gene SeT Analysis Toolkit) [Wang i wsp. 2017] umożliwiła wyodrębnić trzy główne kategorie z ontologii genów [Figura 3, Traut i wsp. 2024, **II**]:

1. proces biologiczny (BP-ang. biological process)
2. składnik komórkowy (CC-ang. cellular component)
3. funkcja molekularna (MF-ang. molecular function)

W badaniach własnych zidentyfikowano, że geny różniące się poziomem ekspresji w blastocystach uczestniczą w 38 procesach biologicznych, 23 procesach związanych ze składnikiem komórkowym i 26 odnoszących się do funkcji molekularnych. Analiza szlaków przy użyciu biblioteki KEGG (ang. Kyoto Encyclopedia of Genes and Genomes) wykazała, że geny różniące się poziomem ekspresji należą do następujących szlaków: szlaki metaboliczne, biosynteza kofaktorów, metabolizm glutationu, fosforylacja oksydacyjna, metabolizm węgla, kardiomiopatia przerostowa, metabolizm glicyny, seryny i treoniny, mikroRNA w nowotworach, ścieżki neurodegeneracji. Największa grupa genów różniących się poziomem ekspresji w dwóch badanych grupach została zakwalifikowana do szlaków metabolicznych - 52 geny [Tabela 4, Traut i wsp. 2024; **II**]. Ponadto, najmniej genów różniących się poziomem ekspresji należało do szlaku metabolizmu glicyny, seryny i treoniny - 4 geny. Warto nadmienić, iż przedstawione w niniejszej pracy doktorskiej analizy profili transkryptomicznych blastocyst bydlęcych, po raz pierwszy, pozwoliły na wskazanie różnic poziomu ekspresji genów biorących udział w szlaku OXPHOS, w zależności od wieku dawczyń komórek jajowych [Traut i wsp. 2024; **II**].

Przebiegający w mitochondriach komórek szlak OXPHOS jest głównym źródłem produkcji ATP. System OXPHOS tworzy pięć białkowych kompleksów wieloenzymatycznych, wchodzących w skład łańcucha oddechowego. Kompleks I łańcucha uważany jest za główne źródło i wejście dla elektronów [Zhang i wsp. 2019]. W przeprowadzonych badaniach w ramach rozprawy doktorskiej, w blastocystach bydlęcych wyhodowanych *in vitro* od jałówek dojrzałych płciowo, wykazano wyższy poziom ekspresji trzech ważnych enzymów lub podjednostek enzymów (*NDUFA3*,NADH: ubiquinone oxidoreductase subunit A3, NADH: podjednostka A3 oksydoreduktazy ubichinonu; *NDUFA13,* NADH: ubiquinone oxidoreductase subunit A13, podjednostka A13 oksydoreduktazy ubichinonowej oraz *NDUFS3,* ubiquinone oxidoreductase core subunit S3, podjednostka S3 rdzenia oksydoreduktazy ubichinonowej NADH); [$p<0.05$; Figura 5, Traut i wsp. 2024, **II**], biorących bezpośredni udział w funkcjonowaniu I kompleksu łańcucha, a więc zaangażowanych w prawidłowy transport elektronów z matriks mitochondrialnej do przestrzeni międzybłonowej [Zhang i wsp. 2019; Nolfi- Donegan i wsp. 2020]. Opisany wyżej mechanizm umożliwia powstanie gradientu elektrochemicznego na wewnętrznej błonie mitochondrialnej, pozwalając na przepływ protonów na końcu łańcucha oddechowego, prowadząc do właściwej syntezy ATP, poprzez aktywację kompleksu V- syntazy ATP. Niższy poziom ekspresji *NDUFA3, NDUFA13* i *NDUFS3* w blastocystach bydlęcych wyhodowanych od jałówek niedojrzałych płciowo może zatem świadczyć o mniej efektywnym transporcie elektronów i syntezie ATP w zarodkach wyhodowanych *in vitro* z komórek jajowych pobranych od jałówek niedojrzałych płciowo w porównaniu do blastocyst pochodzących od jałówek dojrzałych. Uzyskane w pracy doktorskiej wyniki są spójne z wynikami zaprezentowanymi w pracy Qin i wsp. [2022], w której wykazano, że ekspresja *NDUFA3* była niższa w oocytach mysich po dojrzewaniu *in vivo* w porównaniu do ekspresji po dojrzewaniu *in vitro*, które cechuje się suboptymalnymi warunkami hodowli. Podobnie, w badaniach Huang i wsp. [2004], Chao i wsp. [2015] oraz Cui i wsp. [2012], w których udowodniono, że obniżona ekspresja genu *NDUFA13* wpływała na obniżenie żywotności zarodków, wydajności dojrzewania oraz zdolności rozwojowych mysich oocytów. Warto zaznaczyć, iż przedstawione w niniejszej rozprawie wyniki są również zgodne z wynikami badań Zhang i wsp. [2020] w których stwierdzono, że wyższa ekspresja *NDUFS3* [$p<0.05$; Figura 5, Traut i wsp. 2024, **II**] była skorelowana z niższym poziomem reaktywnych form tlenu (ROS, ang. reactive oxygen species) [$p<0.05$, Figura 2A,C, Traut i wsp. 2022, **I**] w mysich oocytach, toksycznych dla ich funkcjonowania.

W niniejszej rozprawie doktorskiej odnotowano wyższą ekspresję białka oksydazy cytochromu c (*COX17,* Cytochrome c oxidase copper chaperone), cytochromu c somatyczny (*CYCS,* Cytochrome c somatic) oraz białka rdzeniowego reduktazy ubichinolu-cytochromu C I (*UQCRC1,* Ubiquinol-cytochrome C reductase core protein I), w blastocystach wyhodowanych *in vitro* od dojrzałych w porównaniu do niedojrzałych jałówek [$p<0.05$; Figura 5, Traut i wsp. 2024, **II**]. Białko oksydazy cytochromu c oraz *CYCS* zlokalizowane są w przestrzeni międzybłonowej mitochondriów, natomiast *UQCRC1* znajduje się w III kompleksie łańcucha oddechowego [Hoffman i wsp. 1993; Iwata i wsp. 1998; Garrido i wsp. 2006; Altmann i wsp. 2012; Unni i wsp. 2019; Li i wsp. 2022]. Pomimo odmiennej lokalizacji, wszystkie one pełnią istotną rolę podczas aktywnego transportu elektronów w czasie OXPHOS [Hoffman i wsp. 1993; Takahashi i wsp. 2002; Altmann i wsp. 2012]. Uzyskane w rozprawie wyniki mogą zatem świadczyć o mniej sprawnym transporcie elektronów w zarodkach wyhodowanych *in vitro* od zwierząt niedojrzałych płciowo. W badaniach przeprowadzonych przez Ntostis i wsp. [2021], również stwierdzono wyższą ekspresję genu *COX17* w ludzkich komórkach jajowych zdolnych do zapłodnienia – komórki dojrzałe po zakończeniu II podziału mejotycznego w porównaniu do komórek jajowych niedojrzałych - niezdolnych do zapłodnienia – w stadium GV (ang. germinal vesicle, stadium pęcherzyka zarodkowego), wskazując na istotną rolę *COX17* w produkcji energii oraz utrzymaniu prawidłowej fizjologii oocytów w stadium metafazy II niezbędnej dla utrzymania prawidłowej kompetencji rozwojowej. Ponadto, Li i wsp. [2000] zaobserwowali, że niższy poziom ekspresji genu *CYCS* skorelowany był z wyższą zamieralnością mysich zarodków. Dodatkowo, w badaniach Shan i wsp. [2019] udowodniono, że niższa ekspresja genu *UQCRC1* może prowadzić do zmniejszenia aktywności kompleksu III łańcucha oddechowego, czego konsekwencją była zmniejszona produkcja ATP związana ze wzrostem poziomu ROS. Przytoczone powyżej wyniki badań zgodne są z uzyskanymi w prezentowanej rozprawie, w której niższa ekspresja genu *UQCRC1* w blastocystach wyhodowanych *in vitro* od jałówek niedojrzałych również skorelowana była z wyższym poziomem ROS [$p<0.05$, Figura 2A, C, Traut i wsp. 2022, **I** ; $p<0.05$, Figura 5, Traut i wsp. 2024; **II**].

Ostatnim ogniwem łańcucha oddechowego w komórce jest zlokalizowana w wewnętrznej błonie mitochondrialnej syntaza ATP, definiowana w literaturze jako kompleks V łańcucha oddechowego. Syntaza ATP jest kompleksem złożonym z kilku podjednostek, kodowanych przez specyficzne geny [Jonckheere i wsp. 2012; Xu i wsp. 2015], między innymi:

ATP5MF (ATP synthase membrane subunit f, błonowa podjednostka f syntazy ATP), *ATP5PD* (ATP synthase peripheral stalk subunit, podjednostka d obwodowej syntazy ATP) oraz *ATP12A* (ATPase H^+/K^+ transporting non-gastric alpha2 subunit, ATPaza transportująca jony H^+, K^+). W przeprowadzonych badaniach zaobserwowano wyższą ekspresję *ATP5MF* i *ATP5PD* oraz niższą ekspresję *ATP12A* i *ATP5F1A* w zarodkach bydlęcych wyhodowanych *in vitro* z oocytów pobranych od zwierząt dojrzałych płciowo [p<0.05; Figura 5, Traut i wsp. 2024, **II;** p<0.05; Figura 7H, Traut i wsp. 2022, **I**]. Warto nadmienić, iż w badaniach Fu i wsp. [2014], autorzy zauważyli, że wyższa ekspresja genu *ATP5PD* skorelowana była z wyższą zdolnością implantacyjną zarodków mysich. Dodatkowo Salilew- Wondim i wsp. [2021] udowodnili bezpośredni związek skuteczności implantacji zarodków bydlęcych pozyskanych *in vivo* z wyższym poziomem ekspresji *ATP5PD*. Biorąc powyższe wyniki pod uwagę, wykazana w niniejszej pracy niższa ekspresja *ATP5MF* i *ATP5PD* w zarodkach bydlęcych wyhodowanych *in vitro* z oocytów od zwierząt niedojrzałych płciowo może również świadczyć o znacznie mniej efektywnej produkcji ATP, prowadzącej do niższej zdolności do implantacji tych zarodków w porównaniu do zarodków wyhodowanych *in vitro* od zwierząt dojrzałych płciowo. Z drugiej strony, w badaniach niniejszej rozprawy doktorskiej odnotowano wyższą ekspresję *ATP12A* oraz *ATP5F1A* w blastocystach wyhodowanych *in vitro* z oocytów pobranych od niedojrzałych jałówek w porównaniu do zarodków od jałówek dojrzałych [p<0.05; Figura 5, Traut i wsp. 2024, **II;** p<0.05; Figura 7H, Traut i wsp. 2022**, I**]. Ponadto, istotny wydaje się związek pomiędzy ekspresją *ATP12A* i poziomem ROS w blastocystach. W dysertacji wykazano również wyższy poziom ekspresji *ATP12A* i jednocześnie wyższe stężenie ROS w blastocystach wyhodowanych *in vitro* z oocytów pobranych od niedojrzałych jałówek [p<0.05, Figura 2A, C, Traut i wsp. 2022; **I;** p<0.05; Figura 5, Traut i wsp., 2024, **II**]. Powyższe dane są spójne z wynikami uzyskanymi przez Qin i wsp. [2022] w aspekcie zależności nadekspresji *ATP12A* oraz wyższego poziomu ROS w oocytach poddanych dojrzewaniu w warunkach optymalnych – *in vitro* w porównaniu do oocytów dojrzewanych w warunkach *in vivo*.

Podsumowując tę część badań, prawidłowe współdziałanie białkowych kompleksów wieloenzymatycznych łańcucha oddechowego, gwarantujące produkcję energii niezbędnej dla właściwego przebiegu większości procesów metabolicznych w komórce jest w pewnym stopniu upośledzone w zarodkach bydlęcych wyhodowanych *in vitro* od niedojrzałych płciowo zwierząt. W badaniach przeprowadzonych w ramach niniejszej pracy doktorskiej, udowodniono, że zarodki bydlęce pozyskane od zwierząt niedojrzałych płciowo posiadają mniejsze możliwości efektywnego transportu elektronów podczas OXPHOS oraz znacznie mniej efektywną produkcję ATP, prowadzącą do wyższego poziomu ROS.

Glukoza jako podstawowy i najprostszy monosacharyd stanowi główne źródło energii dla wszystkich komórek w organizmie, biorąc udział w przebiegu wielu istotnych przemian metabolicznych. Katabolizm glukozy podczas glikolizy stanowi wspólny etap oddychania tlenowego i beztlenowego prowadząc do powstania ATP oraz substratów energetycznych biorących udział w produkcji ATP podczas OXPHOS w mitochondriach. W niniejszej dysertacji zaplanowano zbadanie ekspresji wybranych genów związanych z regulacją procesów metabolicznych, odpowiedzialnych za syntezę substratów niezbędnych dla prawidłowego przebiegu OXPHOS w mitochondriach. Glikoliza przebiegająca w warunkach tlenowych prowadzi do powstania pirogronianu oraz dwóch cząsteczek ATP i NADH jako nośników transportu elektronów w łańcuchu oddechowym. W przeprowadzonych badaniach wykazano wyższy poziom ekspresji dehydrogenazy glukozo-6 fosforanowej (*G6PD*) katalizującej pierwszą reakcję szlaku pentozofosforanowego przekształcając glukozo-6-fosforan do NADPH i 6-fosfoglukonolaktonu w blastocystach wyhodowanych od zwierząt dojrzałych płciowo [$p<0.05$; Figura 7E, Traut i wsp. 2022, **I**], co może bezpośrednio świadczyć o sprawniejszym przebiegu glikolizy w zarodkach od tych zwierząt w porównaniu do blastocyst pozyskanych od jałówek niedojrzałych. Podobne wyniki uzyskali Fu i wsp. [2014], wykazując zależność poziomu ekspresji *G6PD* z wyższą zdolnością implantacyjną zarodków. Ponadto, przypuszczamy, że w zarodkach bydlęcych pozyskanych od jałówek niedojrzałych, w warunkach obniżonego poziomu glukozy, podczas glukoneogenezy, podwyższona ekspresja Sirtuiny 2 (*SIRT2*) poprzez deacetylację i aktywację karboksykinazy fosfoenolopirogronianowej (PEPCK-phosphoenolpyruvate carboxykinase) stymuluje syntezę glukozy w komórkach [Frydzińska i wsp. 2019]. Dodatkowo, Gomes i wsp. [2013] dowiedli, iż aktywność sirtuin obniża się wraz z wiekiem, co jest spójne z wynikami przedstawionymi w rozprawie.

W niniejszej rozprawie doktorskiej stwierdzono ponadto wyższą ekspresję genów aktywnego transportu glukozy przez błonę plazmatyczną, kodowanych przez *SLC2A1* i *SLC2A5,* w blastocystach wyhodowanych *in vitro* od jałówek niedojrzałych płciowo w porównaniu do ekspresji transporterów w zarodkach od zwierząt dojrzałych [$p<0.05$; Figura 7B, C, Traut i wsp. 2022, **I**]. Powyższe dane sugerują, iż nadekspresja *SLC2A1* i *SLC2A5* w blastocystach od jałówek niedojrzałych płciowo, wynika prawdopodobnie ze zwiększonego zapotrzebowania tych zarodków na glukozę, podobnie jak zaobserwowano w badaniach Arhin i wsp. [2019].

W badaniach Legault i wsp. [2000] oraz Goovaerts i wsp. [2011] wykazano, iż gen *GPX1* (ang. glutathione peroxidase 1, peroksydaza glutationowa), jako marker stresu oksydacyjnego, ulega ekspresji w zarodkach w okresie przedimplantacyjnym oraz że jego nadekspresja pełni ochronną rolę w niekorzystnych warunkach, poprzez zmniejszenie oksydacyjnego uszkodzenia mitochondrialnego DNA (mtDNA). W niniejszej rozprawie doktorskiej, w blastocystach od jałówek niedojrzałych płciowo, stwierdzono wyższą ekspresję *GPX1*, skorelowaną z podwyższonym poziomem ROS w tychże blastocystach [$p<0.05$; Figura 7I, Figura 2A, C Traut i wsp. 2022; **I**]. Na podstawie powyższych doniesień można zatem wnioskować, iż wyższa ekspresja *GPX1* może również odgrywać istotną rolę w ochronie mtDNA przed uszkodzeniami oksydacyjnymi w blastocystach wyhodowanych *in vitro* od jałówek niedojrzałych płciowo. Co więcej, w badaniach przeprowadzonych w dysertacji zaobserwowano wyższą liczbę kopii mtDNA w blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek niedojrzałych płciowo w porównaniu do liczby kopii mtDNA w zarodkach od zwierząt dojrzałych. Przedstawione wyniki są spójne z wynikami uzyskanymi przez Fragouli i wsp. [2015], w których zaprezentowano niższą liczbę kopii mtDNA w zarodkach ludzkich zaimplantowanych w porównaniu do niezaimplantowanych. Dowiedziono również, iż oocyty pobrane od starszych krów cechowały się niższą liczbą kopii mtDNA niż komórki pochodzące od osobników młodszych [Iwata i wsp. 2011]. Z drugiej strony, mitochondrialny czynnik transkrypcyjny A - *TFAM* jest głównym czynnikiem odpowiedzialnym za replikację i transkrypcję mtDNA w komórce [Ekstrand i wsp. 2004]. Również w zarodkach oraz komórkach jajowych udokumentowano bezpośrednią zależność między liczbą kopii mtDNA a całkowitym poziomem białka *TFAM* [Ekstrand i wsp. 2004, Thundathil i wsp. 2005, May-Panloup i wsp. 2005; Spikings i wsp. 2007]. Podobnie, w niniejszej rozprawie doktorskiej, zanotowano wyższą ekspresję *TFAM* w blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek niedojrzałych płciowo w porównaniu do ekspresji w zarodkach pozyskanych od zwierząt dojrzałych, co jest zgodne z wyższą liczbą kopii mtDNA w zarodkach od zwierząt niedojrzałych [$p<0.05$; Figura 3, Figura 7A, Traut i wsp. 2022; **I**]. Nasuwa się zatem wniosek, iż uzyskane wyniki wskazują na bezpośredni związek poziomu ekspresji *TFAM* oraz liczby kopii mtDNA z jakością wyhodowanych zarodków. Istnieją doniesienia literaturowe, że zaburzenia równomiernej dystrybucji mitochondriów w komórkach związane są z zaburzonym dojrzewaniem oocytów oraz nieprawidłowym rozwojem zarodków [Nagai i wsp. 2006; Selesniemi i wsp. 2011; Wang i wsp. 2020; Lee i wsp. 2022]. W niniejszej rozprawie doktorskiej, zaobserwowano wyższą intensywność fluorescencji aktywnych mitochondriów wyhodowanych *in vitro* z oocytów pobranych od jałówek niedojrzałych płciowo w porównaniu do dystrybucji w zarodkach od zwierząt dojrzałych [p<0.05; Figura 4, Traut i wsp. 2022; **I**]. Uzyskane w rozprawie wyniki nie są zgodne z wynikami Czernik i wsp. [2022], w których odnotowano wyższy poziom dystrybucji mitochondriów w mysich blastocystach pozyskanych *in vivo* w porównaniu do blastocyst wyhodowanych *in vitro*.

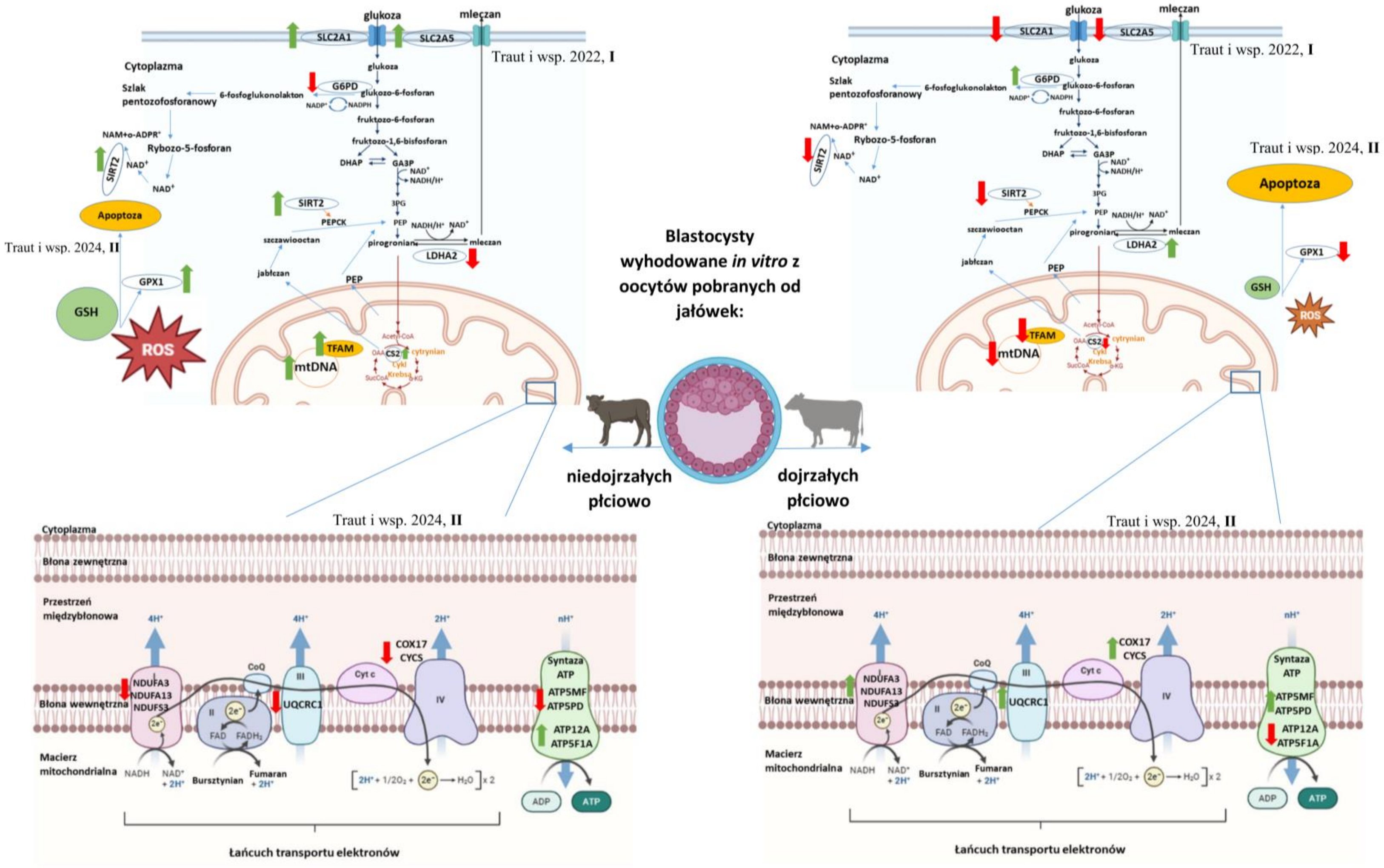
W przeprowadzonych doświadczeniach wchodzących w skład pracy doktorskiej analizowano również potencjał redoks wyhodowanych *in vitro* blastocyst. Stres oksydacyjny wywołany przez ROS może uszkadzać komórki, zaburzając homeostazę i prowadząc do apoptozy [Currin i wsp. 2021]. W dysertacji odnotowano istotnie statystycznie wyższy poziom ROS w zarodkach wyhodowanych od zwierząt niedojrzałych płciowo w porównaniu do poziomu w zarodkach od zwierząt dojrzałych [p<0.05; Figura 2A,C, Traut i wsp. 2022, **I**], co jak udowodniono wcześniej może być szczególnie niekorzystne we wczesnym stadium rozwoju embrionalnego [Ozawa i wsp. 2002; Sakatani i wsp. 2004]. Z drugiej strony, glutation poprzez zmniejszanie poziomu stresu oksydacyjnego w blastocystach wspomaga ich rozwój przedimplantacyjny [Yoshida i wsp. 1993, Gardiner & Reed, 1995]. W ramach niniejszych badań, stwierdzono wyższy wewnątrzkomórkowy poziom glutationu w blastocystach wyhodowanych *in vitro* od jałówek niedojrzałych w porównaniu do poziomu w blastocystach od jałówek dojrzałych [p<0.05; Figura 2B, D, Traut i wsp. 2022; **I**]. Powyższe dane wskazują, iż wyższy poziom glutationu w blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek niedojrzałych płciowo prawdopodobnie chroni te zarodki przed nadmierną akumulacją ROS w komórkach, kompensując występujące w tych zarodkach zaburzenia wewnątrzkomórkowej równowagi redoks.

W dysertacji wykazano niższą liczbę komórek apoptotycznych w blastocystach wyhodowanych *in vitro* z oocytów pobranych od niedojrzałych jałówek w porównaniu do blastocyst od zwierząt dojrzałych [p<0.05; Figura 6, Traut i wsp. 2024, **II**]. Apoptoza jest procesem fizjologicznym, występującym także podczas rozwoju embrionalnego [Gjørret i wsp. 2003]. Zależność między zachodzącym procesem apoptozy w oocytach i blastocystach, a ich kompetencją rozwojową jest niespójna w literaturze [Yang & Rajamahendran, 2002; Bilodeau-Goeseels & Panich, 2002; Lonergan i wsp. 2003; Yuan i wsp. 2005; Melka i wsp. 2010]. Badania przeprowadzone przez Bilodeau-Goeseels & Panich [2002], udowodniły, iż oocyty wykazujące wysoki potencjał rozwojowy wykazywały wyższy poziom apoptozy. Natomiast w doświadczeniach przeprowadzonych przez Zaraza i wsp. [2010] stwierdzono wyższą liczbę komórek apoptotycznych w blastocystach od jałówek niedojrzałych, w porównaniu do blastocyst pozyskanych od dojrzałych płciowo krów, co nie jest spójne z wynikami uzyskanymi w niniejszej pracy odnotowującymi wyższą liczbę komórek apoptotycznych w blastocystach wyhodowanych od dojrzałych jałówek [p<0.05; Figura 6, Traut i wsp. 2024, **II**].

Podsumowanie i wnioski

1. Uzyskano statystycznie niższy odsetek blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek niedojrzałych płciowo w porównaniu do odsetka blastocyst od zwierząt dojrzałych, jakkolwiek, w obydwu badanych grupach nie zanotowano różnic w jakości wyhodowanych zarodków [Traut i wsp. 2022, **I;** Traut i wsp. 2024, **II**],
2. Analiza profilu transkryptomicznego wyhodowanych *in vitro* blastocyst bydlęcych, po raz pierwszy, pozwoliła na wykazanie różnic poziomu ekspresji genów biorących udział w szlaku OXPHOS, w zależności od wieku dawczyń komórek jajowych [Traut i wsp. 2024, **II**],
3. W przeprowadzonych badaniach udowodniono, że zarodki bydlęce pozyskane od zwierząt niedojrzałych płciowo posiadają mniejsze możliwości efektywnego transportu elektronów podczas OXPHOS oraz znacznie mniej efektywną produkcję ATP, prowadząc do wyższego poziomu ROS [Traut i wsp. 2022, **I;** Traut i wsp. 2024, **II**].
4. Zmiany ekspresji *TFAM* oraz innych markerów molekularnych związanych z funkcją mitochondriów w blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek niedojrzałych płciowo, skutkują wyższą liczbą kopii mtDNA w tych zarodkach. [Traut i wsp. 2022, **I**].
5. Z drugiej strony, wyższy poziom glutationu w blastocystach wyhodowanych *in vitro* z oocytów pobranych od jałówek niedojrzałych płciowo może chronić te zarodki przed nadmierną akumulacją ROS w komórkach, kompensując występujące zaburzenia wewnątrzkomórkowej równowagi redoks, prowadząc do obniżenia liczby komórek apoptotycznych w tych zarodkach [Traut i wsp. 2022, **I;** Traut i wsp. 2024, **II**].

Weryfikacja postawionej w pracy doktorskiej hipotezy badawczej, pozwoliła na sformułowanie następującego wniosku: profil transkryptomiczny, ze szczególnym uwzględnieniem różnic przebiegu szlaku fosforylacji oksydacyjnej blastocyst wyhodowanych *in vitro*, ekspresja molekularnych markerów związanych z funkcją mitochondriów w blastocystach oraz liczba kopii mtDNA, odzwierciedlają niższe kompetencje rozwojowe zarodków pochodzących od zwierząt niedojrzałych płciowo. Ponadto, stworzona w niniejszej rozprawie doktorskiej baza danych profili transkryptomicznych blastocyst wyhodowanych *in vitro* z oocytów pobranych od jałówek dojrzałych i niedojrzałych płciowo, umożliwia dalsze poszukiwanie molekularnych markerów blastocyst, świadczących o ich kompetencjach rozwojowych po transferze. Jakkolwiek w celu jednoznacznego potwierdzenia znaczenia biologicznego uzyskanego w ramach rozprawy wniosku, potrzebne jest przeprowadzenie transferu wyhodowanych *in vitro* zarodków, analizę ich implantacji oraz określenie liczby żywo urodzonego potomstwa.



Rycina 3. Schemat podsumowujący uzyskane wyniki w rozprawie doktorskiej. Opracowanie własne wykonane z wykorzystaniem programu Biorender na podstawie **Wu & Sinclair [2014] oraz de Lima i wsp. [2020].** (3PG kwas 3-fosfoglicerynowy; Acetyl-Coa acetylokoenzym A; ADP adenozyno-5′-difosforan; ATP adenozyno-5′-trifosforan *ATP12A* ATPaza transportująca jony H^+, K^+; *ATP5F1A* mitochondrialna syntaza ATP; *ATP5MF* błonowa podjednostka f syntazy ATP; *ATP5PD* podjednostka d obwodowej syntazy ATP; *COX17* białka oksydazy cytochromu c ; *CS2* syntaza cytrynianowa ; *CYCS* cytochrom c somatyczny; DHAP Fosfodihydroksyaceton, e^- jony elektronów; FAD dinukleotyd flawinoadeninowy, forma utleniona; GA3P aldehyd-3-fosfoglicerynowy; *GPX1* peroksydaza glutationowa; GSH glutation; H^+ kationowa forma wodoru atomowego; H_2O tlenek wodoru; *LDHA2* dehydrogenaza mleczanowa,; mtDNA mitochondrialny DNA; NAD^+ dinukleotyd nikotynoamidoadeninowy**,** forma utleniona; NADH dinukleotyd nikotynoamidoadeninowy, forma zredukowana; NAM+o-ADPR nikotynamid adenozynodifosforanu rybozy; *NDUFA13* podjednostka A13 oksydoreduktazy ubichinonowej; *NDUFA3* NADH: podjednostka A3 oksydoreduktazy ubichinonu; *NDUFS3* podjednostka S3 rdzenia oksydoreduktazy ubichinonowej NADH; O_2 wzór dwuatomowej cząsteczki tlenu; OAA Kwas szczawiowooctowy; PEP kwas fosfoenolopirogronowy; PEPCK karboksykinaza fosfoenolopirogronianowa; ROS reaktywne formy tlenu; *SLC2A1* transporter glukozy typu 1; *SLC2A5* transporter glukozy typu 5; *SIRT2* mitochondrialna deacetylaza zależna od NAD*;* SucCoa bursztynylo-koenzym A; *TFAM* mitochondrialny czynnik transkrypcyjny A; *UQCRC1* białko rdzeniowe reduktazy ubichinolu-cytochromu C I; α -KG Kwas α-ketoglutarowy).

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Mitochondrial DNA content and developmental competence of blastocysts derived from pre-pubertal heifer oocytes

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ABSTRACT

In the cattle-breeding industry, there is an increasing demand for *in vitro* embryo production from pre-pubertal heifers. In this study, we evaluated the differences in mitochondrial DNA content, oxidative stress, and developmental competence in blastocysts derived from pre-pubertal and pubertal heifers.

We found higher mitochondrial DNA copy numbers in blastocysts produced from pre-pubertal heifers than from pubertal heifers. In the group of pre-pubertal animals, there was a significantly lower number of blastocysts produced *in vitro* from the same number of collected oocytes, and these blastocysts did not differ from those obtained from pubertal oocytes in terms of their morphological quality. The morphologically appropriate blastocysts derived from pre-pubertal heifers had higher concentrations of reactive oxygen species and glutathione. In blastocysts derived from pre-pubertal heifers, we found alterations in the expression of gene markers for developmental competence, which correlated with higher mitochondrial DNA content, suggesting a lower quality of blastocysts derived from pre-pubertal animals than from pubertal animals.

The inadequate redox balance in blastocysts obtained from pre-pubertal females, along with higher mitochondrial DNA copy number, as well as differential gene expression of markers of developmental competence, elucidate the low quality of blastocysts derived from pre-pubertal animals, despite their unaltered morphology.

1. Introduction

The dairy industry is one of the most crucial industries in global food production. An increase in production efficiency and the quality of animal products from livestock has been possible owing to the use of advanced reproductive biotechnologies, such as *in vitro* embryo production. This reproductive biotechnique is useful for the selection and breeding of genetically superior animals [1].

Currently, genomic selection has profoundly remodeled the bovine industry by reducing the breeding interval, augmenting selection efficiency, and decreasing the cost of offspring assessment [2], resulting in increased commercial interest in performing genomic analysis and collecting gametes from pre-pubertal animals that have a decent pedigree and presumably high genetic merit [3].

Oocyte quality is the main factor influencing blastocyst yield, which also determines the success rate of *in vitro* fertilization (IVF) [4]. It has been reported that oocyte competence, and consequently, development to the blastocyst stage, is positively associated with the size of the antral follicle, mainly because of the hormonal status of the donor female [5]. Moreover, a bovine model has been described in the literature to be appropriate for studies of oocyte developmental competence established on the basis of the puberty of oocytes, which are oocyte donors [6]. Despite documented evidence of biochemical defects in pre-pubertal oocytes, such as altered protein synthesis, aberrant energy metabolism, and reduced activity of maturation promoting factors [7,8], relatively little is known about the potential difference between blastocysts produced *in vitro* from pre-pubertal and pubertal heifer oocytes, manifested as the level of mitochondrial DNA (mtDNA) content as well as the mRNA transcript abundances of factors involved in

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mitochondrial function and markers of oocyte developmental competence and embryonic implantation ability. Therefore, in this study, we investigated mtDNA copy number as well as mitochondrial distribution in embryos derived from oocytes collected from pre-pubertal and pubertal heifers.

Mitochondria play an important role in oocyte maturation and subsequent embryonic development because they are responsible for the majority of energy production in the form of adenosine triphosphate (ATP), which is produced through oxidative phosphorylation [9] using glucose as a substrate. The mtDNA content is stable in metaphase II oocytes until the hatched blastocyst stage [10–12]. Therefore, mitochondria that initially exist in the oocyte are a major source of ATP during preimplantation embryonic development. This suggests that mtDNA content (mtDNA copy number) could be a useful indicator for assessing oocyte and blastocyst quality, although there is no consensus in the literature regarding this [13]. The threshold of mtDNA copy number in oocytes required for proper embryonic development is directly dependent on the appropriate expression of mitochondrial transcription factor (*TFAM*), an essential component of mitochondrial nucleoids [14]. It has been demonstrated that exogenous expression of *TFAM* increases mtDNA copy number in mice [15], and heterozygous deletion of mouse *TFAM* reduces mtDNA copy number in oocytes, impairing their post-implantation development [16].

On the other hand, glucose metabolism is an important process for energy production in the cells, as reflected by the level of expression of certain enzymes, which are also essential for proper mitochondrial function. The aim of this process is to facilitate effective glucose transport for the cells via the solute carrier family 2 facilitated glucose transporter member 1 and 5 (*SLC2A1* and *SLC2A5*) genes [17,18]. This process directs the energy substrates toward anaerobic glycolysis with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enzyme lactate dehydrogenase A (LDHA) playing the primary roles. Alternatively, the pentose phosphate pathway with mitochondrial glucose-6-phosphate dehydrogenase (G6PD) may be the governing enzyme, conclusively producing ribose 5-phosphate, a fundamental precursor for nucleotide synthesis, with mitochondrial NAD-dependent deacetylase (*SIRT2*) expression as regulators of G6PD [19,20]. In oocytes, mitochondria play a pivotal role in fertilization events and determine embryo developmental competence through different means, such as ATP production via mitochondrial ATP synthase (*ATP5A1*) expression and the regulation of reactive oxygen species (ROS) through expression of the oxidative stress marker, glutathione peroxidase 1 (*GPX1*), mediated by reactive oxygen species ROS [21–23].

Previous studies have described numerous candidate genes whose level of expression in early stage embryos is directly associated with developmental competence. In this group of genes, octamer-binding transcription factor 4 (*OCT4*) and sex determining region Y-box 2 (*SOX2*) can be considered the two most important transcription factors, as their levels of expression is involved in pluripotency, cell differentiation, and regulation of early embryonic development [24,25]. Transcription of *OCT4* is also increased in early-cleaved embryos when compared to late-cleaved embryos [26]. Another important transcription factor is the homeobox protein NANOG, which plays a key role in embryonic development and stem cell pluripotency [27]. This factor is synthesized by the embryo from the 8-cell stage onwards and has been proposed as a candidate factor for early inner cell mass specification and the maintenance of pluripotency during bovine preimplantation development [28]. There is also evidence that a higher mRNA abundance of insulin-like growth factors (*IGFs*) in bovine embryos correlates with improved embryonic development [29,30]. The expression pattern of bovine insulin-like growth factor receptors 1 and 2 (*IGF1R* and *IGF2R)* exhibited increased mRNA levels during oocyte maturation, then decreased gradually until the activation of the embryonic genome at the 8–16-cell stage, and finally increased again to reach the highest level in hatched blastocysts [31]. Another important molecular marker for developmental competence is the placenta-specific 8 gene (*PLAC8*), which is upregulated in hatched blastocysts compared to early blastocysts [32]. In the epithelial cells of the bovine endometrium, the transcription of *PLAC8* is induced by interferon tau (IFNτ), which is the main embryonic signal of pregnancy recognition in ruminants [33]. Desmocollin 2 (DSC2), a member of the cadherin superfamily, is a cell adhesion protein present in desmosomes [34] and is involved in the formation of desmosomal junctions during blastocyst expansion [35,36]. Higher expression of *DSC2* was also observed in high-quality blastocysts than in low-quality blastocysts [37]. DNA methyltransferase 3 alpha (*DNMT3A*) is an epigenetic embryo marker that belongs to the methyltransferase family and mediates the establishment and maintenance of dynamic patterns of DNA methylation in the genome. This is essential for embryo viability and the development of embryos [38]. Aquaporin 3 (AQP3) is very important in cryopreserved oocytes and blastocysts for cellular homeostasis and transport [39,40]. Downregulation of this gene is associated with low fertilization rates [41] and inhibition of embryonic development in embryos [42]. Rekik et al. [32] reported the overexpression of secreted vesicle seminal protein 1 (SSLP1) and plasminogen activator urokinase (*PLAU*) in hatched blastocysts. SSLP1 is involved in the immune response and pregnancy establishment [35], whereas *PLAU* is involved in the proteolysis of extracellular matrix proteins [43]. Rekik et al. [32] suggested that the overexpression of SSLP1 and *PLAU* during the hatching blastocyst stage could be used as a marker of blastocyst quality since they are highly involved in pregnancy establishment.

In view of the increased commercial demand for *in vitro* embryo production involving young heifers, in the present study, we hypothesized that there is a difference between the level of mtDNA content and the mRNA transcript abundance of factors involved in mitochondrial function and markers of embryonic developmental competence and implantation ability in blastocysts produced *in vitro* from oocytes of either pre-pubertal or pubertal heifers.

2. Materials and methods

2.1. Animals

For all experimental procedures, we obtained official consent from the Local Animal Care and Use Committee in Olsztyn, Poland (Agreement No. 76/2014/DTN). For *in vitro* embryo production, cumulus–oocyte complexes (COCs) were obtained via the ovum pick up (OPU) method from pre-pubertal animals, as presented in Fig. 1. The animals in the pubertal group were older than 15 months and served as the control group for all experiments. Animals in the pre-pubertal group were younger than 12 months, with the absence of a dominant follicle or corpus luteum in the ovary, as confirmed by ultrasound examination.

2.2. Chemicals and suppliers

For *in vitro* production of bovine embryos, all culture media were purchased from Minitube (Germany). Plastic dishes, 4-well plates, and tubes were obtained from Nunc (Thermo Scientific; Denmark). Unspecified reagents and supplements for *in vitro* culture were obtained from Merck (Germany). The chemicals for reverse transcription were purchased from Invitrogen (Carlsbad, CA, USA).

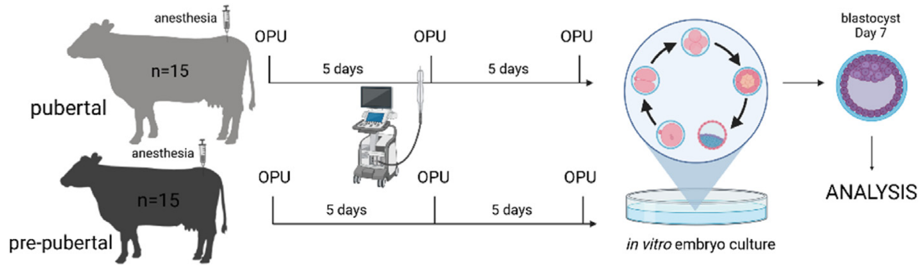

Fig. 1. Schematic illustration of the OPU procedure created with BioRender.com. OPU, ovum pick up.

2.3. Experimental design

The following three experiments were conducted on blastocysts produced from oocytes collected from pre-pubertal and pubertal (control) heifers in an *in vitro* manner:

Experiment 1. Determination of blastocyst development rates and quality of bovine embryos produced *in vitro.*

Experiment 2. Determination of oxidative stress and mtDNA content in bovine embryos produced *in vitro.*

2.3.1. Determination of ROS and glutathione (GSH) in bovine embryos produced *in vitro*
2.3.2. Determination of mtDNA copy number, mitochondrial distribution, and *TFAM* localization in bovine embryos produced *in vitro*
2.3.3. Determination of the mRNA expression of mitochondrial function genes in bovine embryos produced *in vitro*

Experiment 3. Determination of blastocyst quality marker expression in bovine embryos produced *in vitro.*

2.4. Ovum pick up

We collected COCs from two groups of animals, pre-pubertal (n = 15) and pubertal (n = 15) Polish Holstein Friesian heifers, via a transvaginal ultrasound-guided OPU method, according to Cavalieri et al. [44]. Each animal in both groups underwent three OPU sessions. The interval between each OPU procedure was five days, as shown in Fig. 1.

Before each procedure, the perineal region was cleaned using water and 70% ethanol. The animals received epidural anesthesia before each OPU session. After transvaginal insertion of the transducer, the ovary was positioned via transrectal manipulation together with the transducer to view and aspirate all visible follicles, except those with diameters larger than 5–6 mm. Follicular aspiration was performed using a real-time B-mode ultrasound scanner (Draminski 4Vet) with an 8 MHz microconvex transducer, follicular aspiration guide, and stainless-steel guide. Follicular puncture was performed using a disposable 18 G hypodermic needle connected to a 50 mL conical tube (Corning, Acton, MA, USA) via a suitable silicon tubing system (Minitube, Germany). Aspiration pressure was generated using a vacuum pump (Mofa Globals, USA) and adjusted between 90 and 100 mmHg. After the OPU of both ovaries, the aspiration system was washed with tissue culture medium (TCM; #M5017, Merck, Germany) containing 0.05% sodium heparin (Merck, #SRE0027) and 1% fetal bovine serum (Gibco®, Thermo Fisher Scientific, #11573397).

The COCs were viewed under an Olympus SZX10 stereomicroscope and washed twice in wash medium (TCM) supplemented with 20 mM HEPES (#H4034, Merck, Germany), 25 mM sodium bicarbonate (#S4019, Merck, Germany), 0.4% bovine serum albumin (#A9418 BSA; Merck, Germany), and 40 μg/mL gentamicin (#G1272, Merck, Germany). The COCs thus obtained were used for *in vitro* maturation.

2.5. In vitro maturation

Pooled groups of approximately 25 COCs (collected separately from pre-pubertal and pubertal animals) were placed into 4-well plates (#144444, Thermo Fisher Scientific, MA, USA) containing 500 μL of maturation medium (TCM 199 Maturation Medium [19990/0010, Minitube, Germany]) supplemented with 0.02 IU/mL of pregnant mare serum gonadotropin (PMSG, #G4527, Merck, Germany), 0.01 IU/mL human chorionic gonadotropin (hCG, #C0684, Merck, Germany), and 5% fetal bovine serum (FBS, #12106C, Merck, Germany) and incubated at 38.5 °C in a 5% CO_2 humidified atmosphere for 23 h for *in vitro* maturation (IVM).

2.6. Semen capacitation and IVF

For IVF, freeze-dried commercially available semen from the same bull (Sexing Technology, USA) was used in all experiments. Fertility parameters of semen were confirmed using the computer-assisted sperm analysis method. After thawing in a water bath at 38 °C for 60 s, semen was layered underneath capacitation medium (TL sperm capacitation medium (19990/0020, Minitube, Germany) supplemented with 1 mM sodium pyruvate, 0.6% BSA, and 0.1 mg/mL gentamicin) and incubated for 1 h at 38.5 °C in a humidified atmosphere with 5% CO_2 to allow the recovery of motile sperm using a swim-up procedure. After incubation, the upper two-thirds of the capacitation medium were recovered and centrifuged at 200 g for 10 min. The supernatant was discarded, and the sperm pellet was diluted in an appropriate volume of fertilization medium to produce a final concentration of 10^6 motile sperm/mL.

After *in vitro* maturation, groups of 25 COCs were placed in 500 μL of fertilization medium (TL fertilization medium; 19990/0030, Minitube, Germany) supplemented with 10 μg/mL of heparin (#H3393, Merck, Germany), 20 mM sodium pyruvate (#P3662, Merck, Germany), and 0.5% BSA) and co-incubated with spermatozoa in 4-well dishes containing 500 μL of fertilization medium for 24 h at 38.5 °C in a 5% CO_2 humidified atmosphere. The day of IVF was considered day 0.

2.7. In vitro embryo culture

At 24 h after IVF, embryos were separated from cumulus cells and washed twice in wash medium. Embryos (groups of 25) were then cultured in 4-well dishes containing 500 μL of culture medium (SOF; synthetic oviduct fluid medium (19990/0040) supplemented with basal medium Eagle-amino acids using 10 μL/mL (#B6766), 20 μL/mL MEM (#M7145), 3.3 mM sodium pyruvate, and 5% fetal bovine serum in 500 μL of mineral oil (NidOil, Nidacon). Culturing was carried out at 38.5 °C in an atmosphere of 5% CO_2, 5% O_2, and 95% N_2 with high humidity until the blastocyst stage was reached (day 7).

The developmental rates and embryo quality of embryos derived from pre-pubertal and pubertal animals were determined by morphological examination using an Olympus SZX10 stereomicroscope at approximately 100 × magnification according to the guidelines of the International Embryo Technology Society (IETS).

2.8. Intracellular determination of ROS and GSH levels

Intracellular ROS and GSH levels in blastocysts were measured using 2′,7′- dichlorodihydrofluorescein diacetate (DCHFDA) (#D6665; Merck, Germany) and ThiolTracker™ Violet GSH detection reagent (#T10095; ThermoFisher Scientific, USA), respectively, according to the manufacturer's protocol. For these assays, we used 12 blastocysts from pre-pubertal animals and 15 blastocysts from pubertal females. The blastocysts were incubated with 100 μmol/L DCHFDA or 20 μmol/L prewarmed ThiolTracker™ violet dye solution in the dark for 30 min at 37 °C; they were then washed three times in 0.1% polyvinyl alcohol and Dulbecco's phosphate-buffered saline (PVA/DPBS) and documented via a Fluorescence Axio observer (Carl Zeiss, Germany) using a 10 × /0.30 objective. The doses of the different dyes were selected according to data obtained by Pang et al. [45]. Subsequently, blastocysts were observed under an Axio Observer Microscope System (Carl Zeiss, Germany) using appropriate fluorescence filters with excitation/emission wavelengths of 495 nm/529 nm and 404/526 nm for DCHFDA and Thioltracker Violet™, respectively. The results are presented as the relative values of fluorescence intensity. Fluorescence intensity was analyzed using ZEN blue 2.5 pro software (Carl Zeiss, Germany).

2.9. Mitochondrial DNA copy number evaluation

Total DNA was extracted from embryos at the blastocyst stage (n = 6, one embryo per analysis) using the Sherlock AX Kit (#095–100, A&A Biotechnology, Poland), according to the manufacturer's instructions. Primers for assessing mtDNA copy number were designed based on a region of cytochrome B (*CYTB*) and NADH dehydrogenase subunit 2 (*ND2*). For the obtained embryos, diploid nuclear genome analysis was performed using primers designed from the POU class 5 homebox 1 (POU5F1) region. The primer sequences are listed in Table 1. Mitochondrial and nuclear DNA target sequences were quantified by quantitative real-time quantitative polymerase chain reaction (qPCR) using an ABI Prism 7900 SDS instrument (Thermo Fisher Scientific, USA). The quantification of mtDNA was accomplished in 10 μl volumes of PCR mixtures consisting of Maxima SYBR Green/ROX qPCR Master Mix (#K0223, Thermo Fisher Scientific, USA), 0.25 mM of each primer as listed in Tables 1 and 20 ng of embryonic DNA. The PCR was performed at 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s, 58 °C (for CYTB) or 55 °C (for ND2) or 59 °C (for POU5F1) for 15 s, and 72 °C for 15 s. The final elongation step was conducted at 72 °C for 15 s. Fluorescence signals were obtained using the FAM/SYBR channel during the elongation phase. Melting curve data were generated from the first fluorescence signals by heating PCR products from 47 to 98 °C, holding for 4 s at each step. To eliminate the negative effects of primer dimerization, the data described above were used to establish the second extension phase, which was set at the temperature just before the start of the melt curve phase. The second extension phase was set at 78 °C for CYTB, 74 °C for ND2, and 80 °C for POU5F1 for 15 s, and the second fluorescence signal was acquired. To generate standards for qPCR, the CYTB, ND2, and POU5F1 regions were amplified from the total DNA obtained from the blastocysts. The PCR products were purified using the GeneMATRIX Short DNA Clean-Up Purification Kit (#E3515, EURx, Poland) according to the manufacturer's instructions and used as standards. For each run, a standard curve was generated from 10-fold serial dilutions (10^{-1}–10^{-8}). The mtDNA copy number per cell was calculated from the number of CYTB and ND2 copies, with each copy divided by half the number of POU5F1 copies. Three analyses were performed for each sample to determine the mean number of mtDNA copies.

2.10. Mitochondrial distribution

Mitochondrial distribution was evaluated using MitoTracker Red CMXRos (#M7512; Thermo Fisher Scientific, USA). MitoTracker Red CMXRos is a red fluorescent dye that stains the mitochondria in live cells. For this study, we used seven blastocysts from pre-pubertal females and ten blastocysts from pubertal females. The blastocysts were treated with 100 nmol/L MitoTracker for 20 min at 37 °C in the dark [45]. The embryos were then washed three times in 0.1% polyvinyl alcohol/phosphate-buffered saline (0.1% PVA/PBS) and placed in 4% paraformaldehyde (PFA) in PBS for 15 min at 37 °C. After fixation, the blastocysts were observed under an LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany) using a 40 × /1.2NA immersion objective. Appropriate fluorescence filters with excitation and emission wavelengths of 579 nm and 599 nm were used. For image analysis, the intensity of red fluorescence was measured and analyzed using the ZEN blue 2.5 pro software (Carl Zeiss, Germany).

2.11. Immunocytochemistry

Embryos derived from pre-pubertal and pubertal animals were stained using specific antibodies for the expression of the mitochondrial-encoded subunit of TFAM (#sc-166965; RRID: AB_10610743; 1:100; Santa Cruz Biotechnologies, USA), a factor involved in mtDNA replication and transcription. For this study, we used seven blastocysts from pre-pubertal females and 12 blastocysts from pubertal females. Blastocysts were fixed in 2% formaldehyde for 1 h, permeabilized with 1% (v/v) Triton X-100 for 30 min, and blocked with PBS containing 200 mM glycine and 2 mg/mL BSA for 30 min. For embryos, primary antibodies were added and incubated for 1 h at 37 °C. The specificity of primary antibodies in bovine oocytes has been previously confirmed [46]. To confirm the lack of nonspecific binding of the secondary antibodies, embryos were stained without the primary antibody. Embryos were then washed in 0.1% Triton X-100 for 30 min before being labeled with the CruzFluor™ 594 secondary antibody (#516250, Santa Cruz Biotechnologies, USA). Embryos were washed and counterstained with 4′,6-diamidyno-2-fenyloindol (DAPI; Vectashield, Vector Laboratories, USA). Blastocysts were observed under an LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany) using a 40 × /1.2NA immersion objective. Fluorescence intensity was analyzed using ZEN blue 2.5 pro software (Carl Zeiss, Germany).

2.12. RNA isolation, reverse transcription, and real-time qPCR analysis

For mRNA expression analysis, total RNA was extracted from blastocysts from pre-pubertal (n = 4 × 5 blastocysts) and pubertal (n = 4 × 5 blastocysts) animals. Total RNA was extracted using an Arcturus PicoPure RNA Isolation Kit (#KIT0204, Applied Biosystems, USA) according to the manufacturer's instructions. The RNA samples were stored at −80 °C until the real-time qPCR was

Table 1
Primers used for real-time PCR.

Gene symbol	Gene name	Primer sequence 5′–3′	Fragment size, bp	Gen Bank accession no.
AQP3	Aquaporin 3	F:TCTTCGACCAGTTCATTGGCACA R:GGGGACGGGGTTGTTGTAGG	136	NM_001079794
ATP5F1A	ATP synthase	F:GCTGCGAAGATGCTGTCCGT R:TTTTTGGAGACCAGCCCCGC	80	NM_174684.2
CS	Citrate synthase	F:CATGGCTTTACTCACTGCGGC R:TCAAATTCGTGGAAGAAGCACTGGC	101	NM_001044721.1
DNMT3A	DNA methyltransferase 3 alpha	F:CGTCCGCAGCGTTACACAGA R:CAGTGCCCTCGTAGAGTCCCT	125	NM_001206502.2
DSC2	Desmocollin 2	F:CCAGTAGCAACCGACAACTGC R:TGGACCTTTTCACCAAGACGGG	80	NM_001166526.1
G6PD	Glucose-6-phosphate dehydrogenase	F:GAGCAGCGAAGCACAGAGAGC R:CTGGTACAGCTCTTCTCGCAGG	90	NM_001244135.2
GPX1	Glutathione peroxidase 1	F:TGGGCATCAGGAAAACGCCAA R:TCTCGCCATTCACCTCGCAC	119	NM_174076.3
IGF1R	Insulin-like growth factor 1 receptor	F:GAGTGGAGAAATCTGCGG R:AAATGAGCAGGATGTGGAGG	110	NM_001244612
IGF2R	Insulin-like growth factor 2 receptor	F:ACCTCCGATCCTCAATCCC R:TGTAGTTGAAGTGCCGGTC	82	NM_174352.2
LDHA2	Lactate dehydrogenase 2	F:CCCAGGTGCATGGAGGAAGTG R:ACCCTAAAGGAACCTGTCCTACCT	77	NM_174099.2
NANOG	Homeobox transcription factor	F:GCCTCAGGGGCCAGCAATGG R:GAGAGTTCACCAAACACCCCTGG	71	NM_001025344.1
OCT4	Octamer-binding protein	F:GAGAAAGACGTGGTCCGAGTG R:GACCCAGCAGCCTCAAAATC	101	NM_174580.2
PLAC8	Placenta-specific gene	F:TTTACCGCTCTGTGCCCTTT R:CCATGTGAACTTGACCAAGCAT	95	NM_001025325.2
PLAU	Urokinase-type plasminogen activator	F:CATGAGGGTCCTGCTGGCAT R:ACAGCCACAGTTCGATTCACCA	106	NM_001034016.2
SIRT2	Sirtuin 2	F:GTGCAGGAGGCTCAGGACTCA R:AGTCCATCTCTGCTTCTCCGCC	70	NM_001113531.1
SLC2A1	Solute carrier family 2 member 1	F:GTCCGGCATCAACGCTGTTTT R:GTTGACGATGCCAGAGCCGA	100	NM_174602.2
SLC2A5	Solute carrier family 2 member 5	F:GGCGGGTACTGTTCACTGGC R:TCCGAGGCCCACTTGGATGA	73	NM_001101042.2
SOX2	SRY-box transcription factor 2	F:TGGATCGGCCAGAAGAGGAG R:CAGGCGAAGAATAATTTGGGGG	89	NM_001105463.2
SSLP1	Secreted seminal vesicle Ly-6 protein 1	F:GGGACTTGAGCCAGATCCAGAG R:GGAAACATTCTAGAGCTTGCGGGA	101	NM_001105478.1
TFAM	Mitochondrial transcription factor, A	F:GCGTATGGGGCGTGCTGAAT R:TGGAACATACGCAAAACTAAAGGGA	101	NM_001034016.2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	F:CACCCTCAAGATTGTCAGCA R:GGTCATAAGTCCCTCCACGA	103	NM_001034034.2
CYTB	Cytochrome B	F:CCTCTGTTACCCATATCTGCCG R:GTGCCGATGTATGGGATTGC	282	MZ901759.1
ND2	NADH dehydrogenase subunit 2	F:TATACGACTCACGTATTCTACC R:CTTTGAAGGCTCTTGGTCTG	199	MZ901759.1
POU5F1	POU class 5 homebox 1	F:CCCAGTGGAAGAGGGGGTGA R:ACCCCACACCGGCTACTCTT	370	AH007191.2

performed. Before use, the RNA content and quality were evaluated using spectrophotometric measurements. The average RNA level from each sample (pool of five blastocysts) was 20 ng, and the purity was confirmed by measuring the 260:280 ratio in the range of 1.8–2.0. The SuperScript™ III First-Strand Synthesis SuperMix for qPCR (#11752250, Thermo Fisher, USA) was used for RT. RT products were diluted and stored at −20 °C until qPCR amplification.

Quantification of mRNA for the studied genes was conducted via qPCR using specific primers for the selected genes (Table 1). The ABI Prism 7900 sequence detection system (Applied Biosystems, Life Technologies, USA) and Maxima SYBR Green/ROX qPCR Master Mix (#K0222, Thermo Fisher Scientific, USA) were used to perform qPCR. qPCR was performed using 384-well plates. For each reaction, a quantity of cDNA equivalent to 0.15 blastocysts was used. The mRNA transcript levels were normalized to GAPDH (internal control) and then expressed as arbitrary units. The internal control was selected using NormFinder software by comparing the expression of the following candidate genes: GAPDH, beta-actin (β-actin), and histone macro-H2A.1 (H2A.1). Primers were designed using an online software package (https://primer3.ut.ee/). The primer sequences and sizes of the corresponding amplified fragments for all transcripts are listed in Table 1. The Miner software (version 4.0; http://ewindup. info/miner/) was used for the relative quantification of mRNA levels.

2.13. Statistical analysis

Statistical analyses were conducted using the statistical analysis system software (GraphPad PRISM v. 9.0 Software, Inc., USA). The distribution of data was tested using the D'Agostino Pearson and Shapiro–Wilk tests, and the Student's t-test was subsequently used for statistical analysis in the case of independent pairs. Categorical data was analyzed using Fisher's exact test. Before statistical analysis, percentage data were transformed using an arcsine function. All experimental data is shown as mean ± standard error of the mean (SEM), and the differences were considered significant at the 95% confidence level ($p < 0.05$).

3. Results

3.1. Determination of the developmental rates and quality of the in vitro-produced bovine embryos

As shown in Table 2, the average number of oocytes per animal received from pubertal versus pre-pubertal animals did not differ ($p > 0.05$). A higher average blastocyst rate was obtained in the pubertal group than in the pre-pubertal group ($p < 0.05$). We did not find any differences in the rates of selected quality blastocysts between the pre-pubertal and pubertal groups ($p > 0.05$).

3.2. Determination of the oxidative stress in the in vitro-produced bovine embryos

Fig. 2 presents fluorescent images of bovine blastocysts labeled for the determination of intracellular ROS and GSH levels, with GSH depicted by blue fluorescence and ROS by green fluorescence.

The ROS level was significantly increased in blastocysts derived from pre-pubertal oocytes compared to that in pubertal heifers ($p < 0.05$; Fig. 2A and C). Moreover, blastocysts derived from pre-pubertal animals displayed higher intracellular levels of GSH compared to blastocysts derived from pubertal animals ($p < 0.05$; Fig. 2B and D).

3.3. Determination of the mtDNA copy number, mitochondrial distribution, and TFAM localization in the in vitro-produced bovine embryos

Fig. 3 shows the mtDNA copy number calculated per blastocyst and cell. The mtDNA copy number in blastocysts derived from pre-pubertal animals was significantly higher (453667 ± 17243, mean ± SEM) than that in blastocysts derived from pubertal heifers (403667 ± 7371, mean ± SEM) (Fig. 3A; $p < 0.05$). The mtDNA copy number re-calculated per cell in blastocysts derived from pre-pubertal animals was also significantly higher (2741 ± 78,21, mean ± SEM) than in blastocysts derived from pubertal heifers (2541 ± 81,98, mean ± SEM) (Fig. 3B; $p < 0.05$).

Fig. 4 shows photographs of the mitochondrial distribution in the *in vitro* obtained bovine blastocysts derived from pre-pubertal and pubertal animal oocytes. Mitochondrial fluorescence intensity in bovine blastocysts derived from pre-pubertal heifers was significantly higher than that in blastocysts from pubertal animals (Fig. 4C; $p < 0.05$), as determined by Student's t-test.

Fig. 5 depicts representative fluorescent images of TFAM abundance in bovine blastocysts derived from pre-pubertal and pubertal animals. Blastocysts derived from pre-pubertal animals displayed higher TFAM expression than blastocysts derived from pubertal heifers ($p < 0.05$; Fig. 5A and C).

3.4. Determination of the mRNA expression of genes involved in mitochondrial function in in vitro-produced bovine embryos

We analyzed the mRNA expression levels of different genes involved in mitochondrial function, including *TFAM*, *SCL2A1*, *SLC2A5*, *LDHA2*, *G6PD*, *SIRT2*, *CS2*, *ATP5F1A*, and *GPX1*, in blastocysts derived from oocytes collected from pubertal and pre-pubertal animals.

We found higher relative mRNA expression of *TFAM*, *SLC2A1*, *SLC2A5*, *SIRT2*, *CS2*, *ATP5F1A*, and *GPX1* in blastocysts derived from oocytes collected from pre-pubertal as opposed to pubertal animals ($p < 0.05$; Fig. 6A, B, 6C, 6F, 6G, 6H and 6I). In contrast, the expression of *G6PD* and *LDHA2* was higher in blastocysts derived from pubertal animals than in blastocysts derived from pre-pubertal heifers ($p < 0.05$; Fig. 6D and E).

3.5. Determination of the expression of blastocyst quality markers in the in vitro-produced bovine embryos

As shown in Fig. 7, we measured the relative mRNA expression of the blastocyst quality marker genes in the *in vitro*-produced bovine embryos.

The mRNA expression levels of the genes involved in embryonic implantation and developmental competence were determined. We analyzed the expression levels of *OCT4*, *SOX2*, NANOG, *PLAC8*, *IGF1R*, *IGF2R*, *PLAU*, *SSLP1*, *DSC2*, *DNMT3A*, and *AQP3* in blastocysts derived from pre-pubertal and pubertal oocytes.

We found higher mRNA levels of *NANOG*, *PLAC8*, *IGF2R*, *PLAU*, *SSLP1*, *DSC2*, and *DNMT3A* in blastocysts derived from pubertal animals than in those derived from pre-pubertal animals ($p < 0.05$; Fig. 7C, D, F–J). The relative mRNA abundances of *OCT4*, *SOX2*, *IGF1R*, and *AQP3* were significantly higher in blastocysts derived from pre-pubertal animals than in those derived from pubertal animals ($p < 0.05$; Fig. 7A, B, 7E, and 7K).

4. Discussion

In the first part of the study, we revealed that the activity of the main intracellular energy-generating pathways in blastocysts differed depending on the oocyte donor age, in which blastocysts derived from pre-pubertal animals demonstrated significantly lower quality despite their unaltered morphology.

Mitochondria play a key role in ATP generation during oocyte and embryonic development [47]. In the present study, we found that blastocysts derived from pre-pubertal animals exhibited

Table 2
Developmental rates of embryos illustrated by adequate photographs.

Experimental group	Total number of oocytes (n)	Average number of oocytes (n) per one animal	Total number of blastocysts (n)	Blastocyst rate (%)	Total number of selected quality blastocysts (%)
Pre-pubertal (n = 15)	364	7.69	52	14.4 [a]	Grade 1/20 (40) [a] Grade 2/24 (45) [a] Grade 3/4 (8.5) [a] Grade 4/4 (5.7) [a]
Pubertal (n = 15)	346	7.07	101	29.2 [b]	Grade 1/37 (36.5) [a] Grade 2/49 (48.5) [a] Grade 3/9 (8.9) [a] Grade 4/6 (5.9) [a]

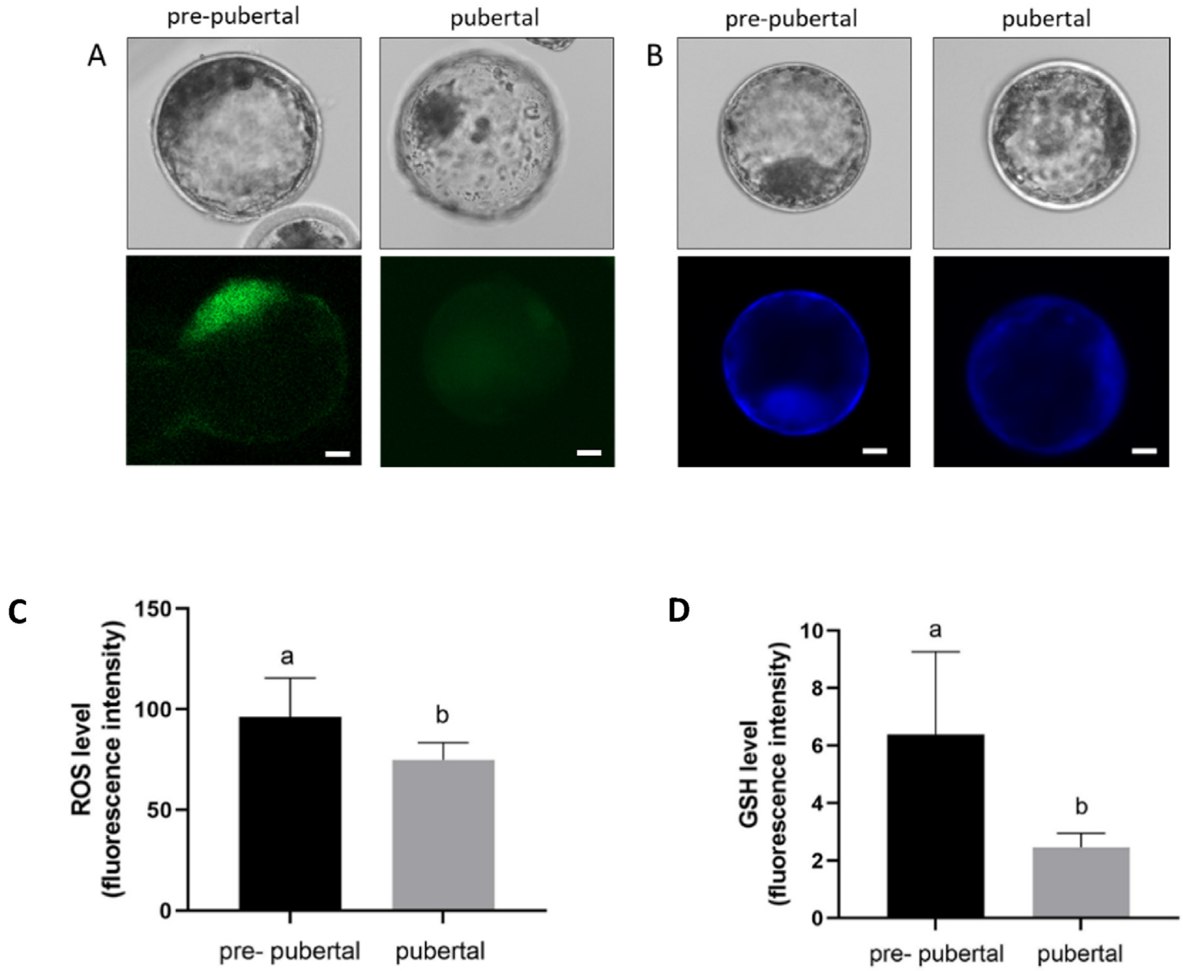

Fig. 2. ROS and GSH levels in *in vitro* obtained blastocysts (n = 6 for ROS and n = 6 for GSH in the pre-pubertal group; n = 7 for ROS and n = 8 for GSH in the pubertal group). Representative images of (A) ROS and (B) GSH levels of the embryo from pre-pubertal and pubertal animals. The quantitative results from the fluorescence intensities of ROS (C) and GSH (D) in blastocysts. Fluorescence intensity was quantified using ZEN blue 2.5 pro. Values with different superscripts in the same column are significantly different ($p < 0.05$), as determined by Student's t-tests. The scale bar in A and B is 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
ROS, reactive oxygen species; GSH, glutathione.

higher mtDNA copy numbers, more intense TFAM localization, and different mRNA expression compared to blastocysts derived from pubertal heifers. The increased mtDNA copy number might result from elevated levels of *TFAM* transcripts that increase concurrently with mtDNA replication during bovine embryogenesis, as documented by May-Panloup et al. [48]. However, there is no consensus in the literature regarding whether higher mtDNA copy number unequivocally accounts for better or worse embryo quality [13,49].

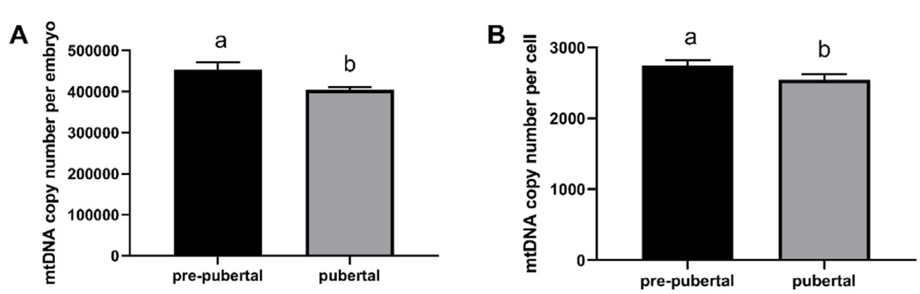

Fig. 3. mtDNA copy number (A) per embryo and (B) per cell in the *in vitro* obtained bovine blastocysts (n = 6 for the pre-pubertal group; n = 6 for the pubertal group) derived from pre-pubertal and pubertal animal oocytes. The number of mtDNA copies was assessed by qPCR. To determine the quantity of mtDNA products per embryo, *CYTB* and *ND2*, the genes specific to the mitochondrial genome, were used. To determine the relative quantity of mtDNA products per cell, *CYTB* and *ND2*, the genes specific to the mitochondrial genome, and the POU5F1 domain, a housekeeping gene acting as a nuclear control gene with a known copy number of two per cell, were used. On the graphs, mean values ± SEM are presented. Different letters above the columns indicate a significant difference between groups ($p < 0.05$), as determined by Student's t-tests.
mtDNA, mitochondrial DNA; SEM, standard error of the mean; qPCR, quantitative PCR.

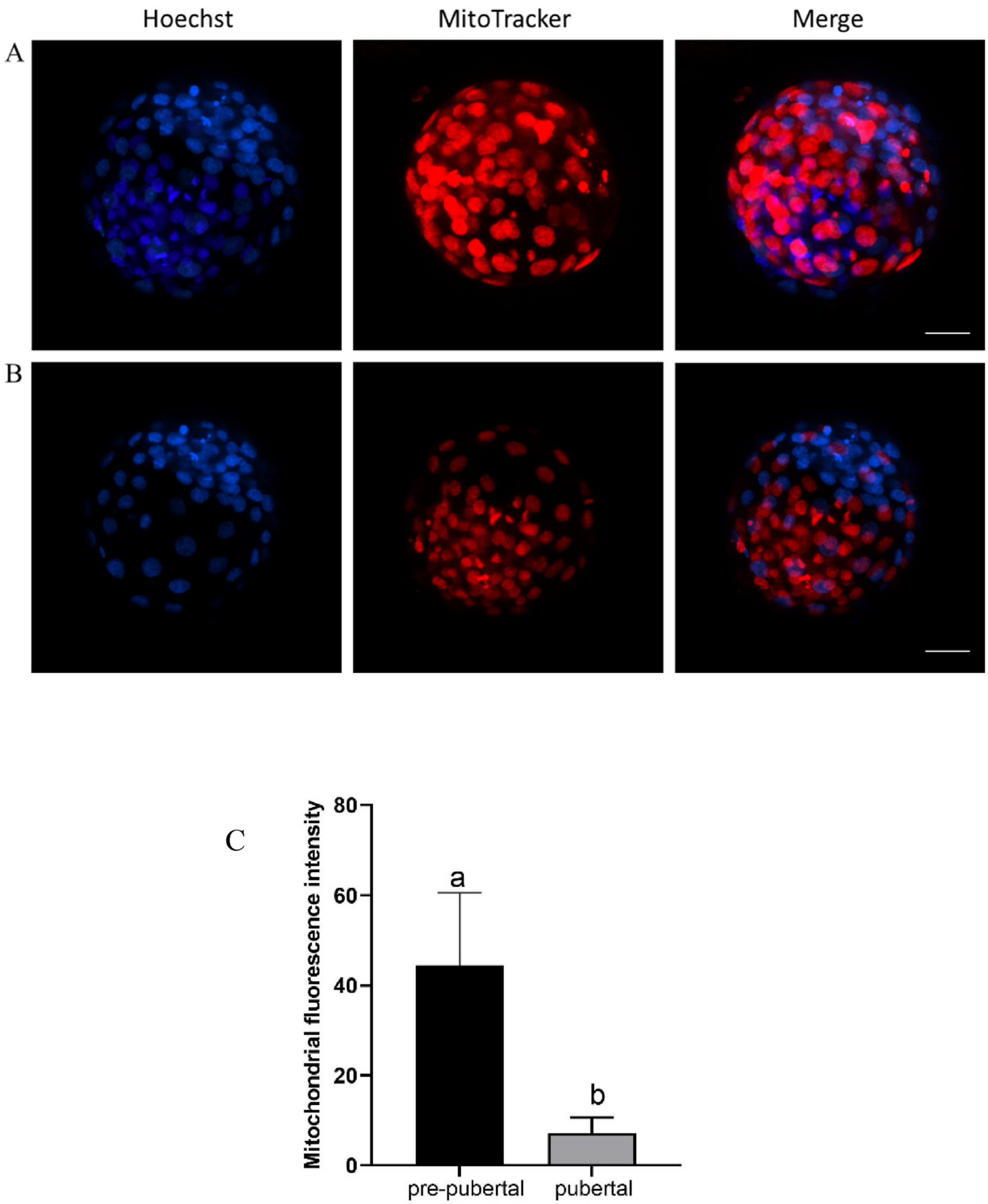

Fig. 4. Representative photographs showing mitochondrial distribution in the *in vitro* obtained bovine blastocysts (n = 7 for the pre-pubertal group; n = 10 for the pubertal group) derived from (A) pre-pubertal and (B) pubertal animal oocytes. Mitochondria were stained with MitoTracker Red and nuclei were stained with Hoechst 33342. (C) Mitochondrial fluorescence intensity in *in vitro* bovine blastocysts derived from pre-pubertal and pubertal animal oocytes. Different letters above the columns indicate a significant difference between groups ($p < 0.05$), as determined by Student's t-test. The scale bar in A and B is 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Most scientists claim that the higher the mtDNA copy number, the lower the blastocyst quality. Fragouli et al. [50] support this hypothesis. In the cited experiments, the authors quantified mtDNA levels in trophectoderm (TE) biopsies of euploid blastocysts depicted for transfer and proved that, on average, the blastocysts that led to a clinical pregnancy had a lower mtDNA copy number than unsuccessful blastocysts [50]. Therefore, the blastocyst stage quantitation of mtDNA appears to be a new predictor of the implantation potential of the embryo. The biological rationale for this phenomenon was postulated by Leese et al. [51] in connection with the “Quiet Embryo Hypothesis,” which states that an embryo with normal, stable development is characterized by low (or “quieter”)

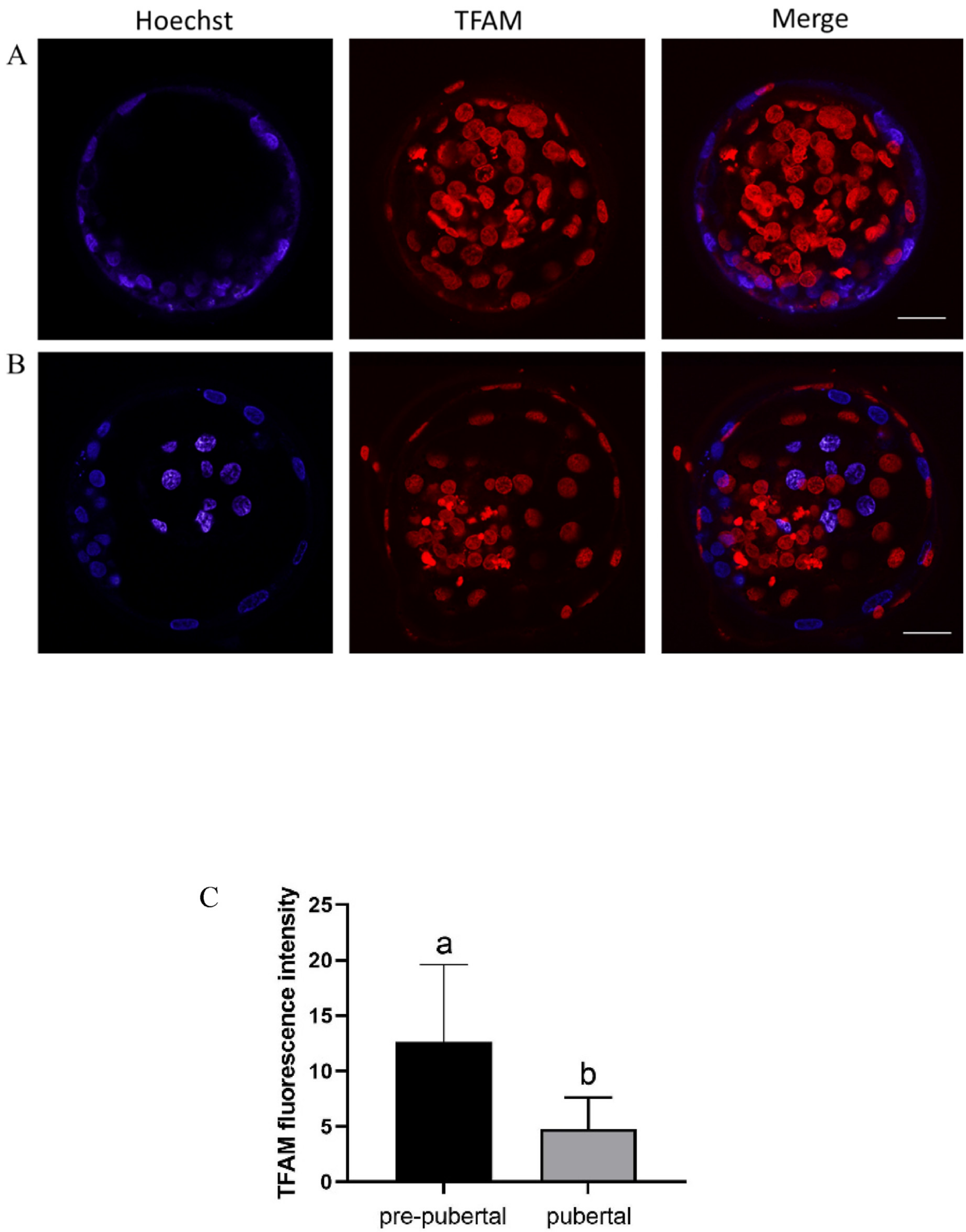

Fig. 5. Representative photographs showing transcription factor A (TFAM) protein of *in vitro* obtained bovine blastocysts (n = 7 for the pre-pubertal group; n = 12 for the pubertal group) derived from (A) pre-pubertal and (B) pubertal animal oocytes. Fluorescence intensity was quantified using ZEN blue 2.5 pro. (C) TFAM protein fluorescence intensity in *in vitro* bovine blastocysts derived from pre-pubertal and pubertal animal oocytes. Different letters above the columns indicate a significant difference between groups ($p < 0.05$), as determined by Student's t-test. The scale bar in A and B is 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

metabolic activity [51]. Conversely, a stressed embryo engages in compensatory mechanisms that increase metabolic output. Therefore, as suggested by Leese et al. [51], elevated mtDNA numbers could be considered a biomarker of a stressed embryo that is unlikely to be implanted [51]. On the other hand, Victor et al. [52] documented that the difference in mtDNA copy number between euploid blastocysts that did implant upon transfer and those that failed to do so was statistically insignificant, and the predictive

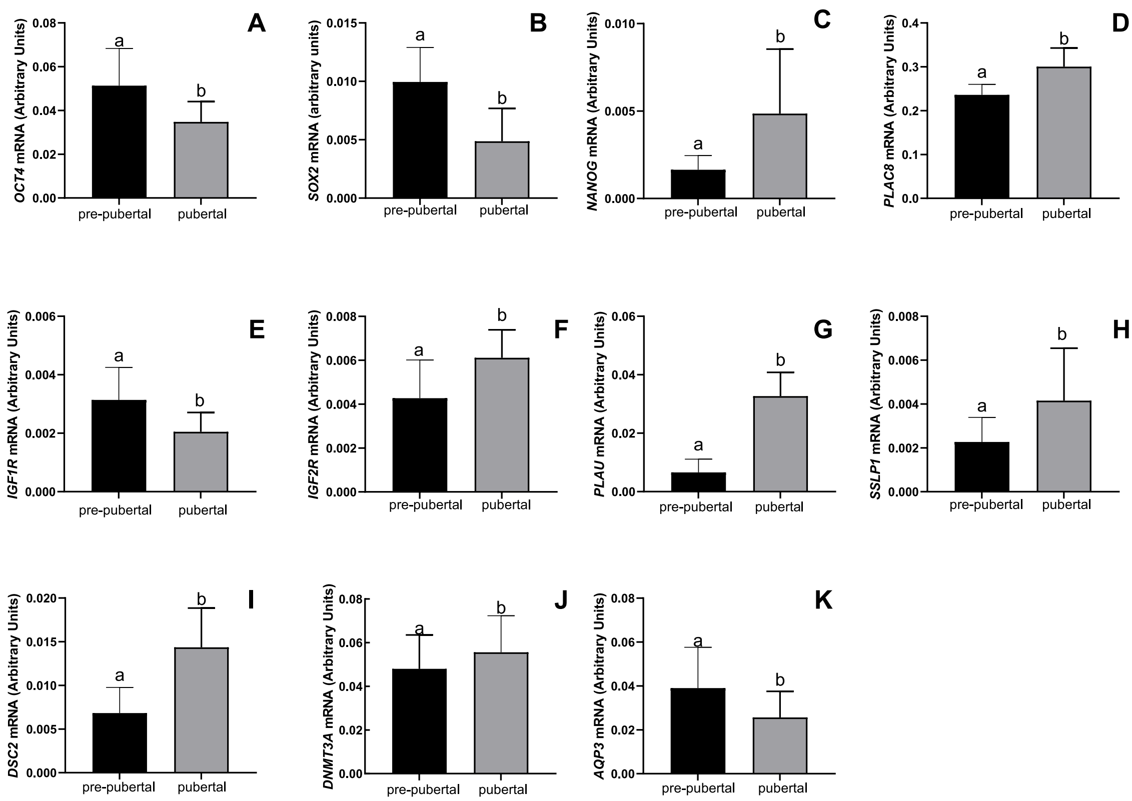

Fig. 6. The transcription profiles of *OCT4* (A), *SOX2* (B), *NANOG* (C), *AQP3* (D), *IGF1R* (E), *IGF2R* (F), *DSC2* (G), *DNMT3A* (H), *PLAC8* (I), *SSLP1* (J), and *PLAU* (K) in *in vitro* obtained bovine blastocysts (n = 4 × 5 for the pre-pubertal group; n = 4 × 5 for the pubertal group) derived from pre-pubertal and pubertal animal oocytes. Different letters above columns indicate a significant difference between groups ($p < 0.05$), as determined by Student's t-test. The values are presented as arbitrary units and are expressed as the mean ± SEM.

value of mtDNA quantitation in terms of viability was negligible.

In our study, relative ROS levels were significantly higher in blastocysts derived from pre-pubertal oocytes than in pubertal heifers. Moreover, blastocysts derived from pre-pubertal animals exhibited higher intracellular levels of GSH than blastocysts derived from pubertal animals. Ozawa et al. [53] and Sakatani et al. [54] revealed that high levels of intracellular ROS are responsible for the deleterious effects on the development of early stage embryos. In contrast, GSH, a low molecular weight thiol, is well known for protecting cells against ROS and maintaining the intracellular redox balance. GSH reduced oxidative stress while promoting embryonic development [55,56]. Therefore, the higher intracellular levels of GSH in blastocysts derived from pre-pubertal heifers compared to those from pubertal animals probably accounts for the fact that GSH in blastocysts cultured from pre-pubertal heifer oocytes probably protects cells against excessive accumulation of ROS in an attempt to maintain the appropriate intracellular redox balance.

During the OPU sessions conducted in the present study, we collected comparable average numbers of oocytes per animal, whether pubertal or pre-pubertal. However, a significantly higher average blastocyst rate was obtained in the group of pubertal animals than in the group of pre-pubertal animals, which agrees with the data of Rizos et al. [57], who highlighted the advantage of oocytes from cows over those from heifers in relation to embryo production efficiency under both *in vivo* and *in vitro* conditions. However, despite the higher blastocyst rates in the group of pubertal heifers, we did not find any differences in the rates of selected quality blastocysts between the groups of pre-pubertal and pubertal animals. This means that in the group of pre-pubertal animals, there may be a significantly lower number of blastocysts produced *in vitro* from the same number of collected oocytes, but these blastocysts did not differ from those obtained from pubertal oocytes relative to their morphological quality. However, morphologically appropriate blastocysts derived from pre-pubertal heifers had higher concentrations of deleterious ROS and also higher concentrations of protective GSH. This inadequate redox balance, associated with the increased mtDNA copy number, suggests a lower quality of blastocysts derived from pre-pubertal heifers.

Moreover, in blastocysts derived from pre-pubertal heifer oocytes, energy production through glucose metabolism, which is essential for proper mitochondrial function, also appears to be altered. We found higher gene expression of active glucose and fructose transport across the plasma membrane, encoded by *SLC2A1* and *SLC2A5* genes, in blastocysts derived from oocytes from pre-pubertal heifers, directing the energy substrates toward anaerobic glycolysis. However, the rate-limiting enzymes *LDHA* and *G6PD*, which ultimately produce ribose 5-phosphate, an essential precursor for nucleotide synthesis, were not overexpressed in blastocysts from pre-pubertal animals. The overall results presented in this manuscript imply that blastocysts derived from pre-pubertal heifer oocytes have disturbed anaerobic glycolysis, which can be beneficial for mtDNA synthesis, as it avoids overloading immature mitochondria in Krebs cycle metabolism, which was previously documented to be directly associated with altered developmental competence [58]. Additionally, in pre-pubertal heifer blastocysts, we found that the tricarboxylic acid cycle enzyme citrate synthase (*CS*), a transcriptionally regulated rate-

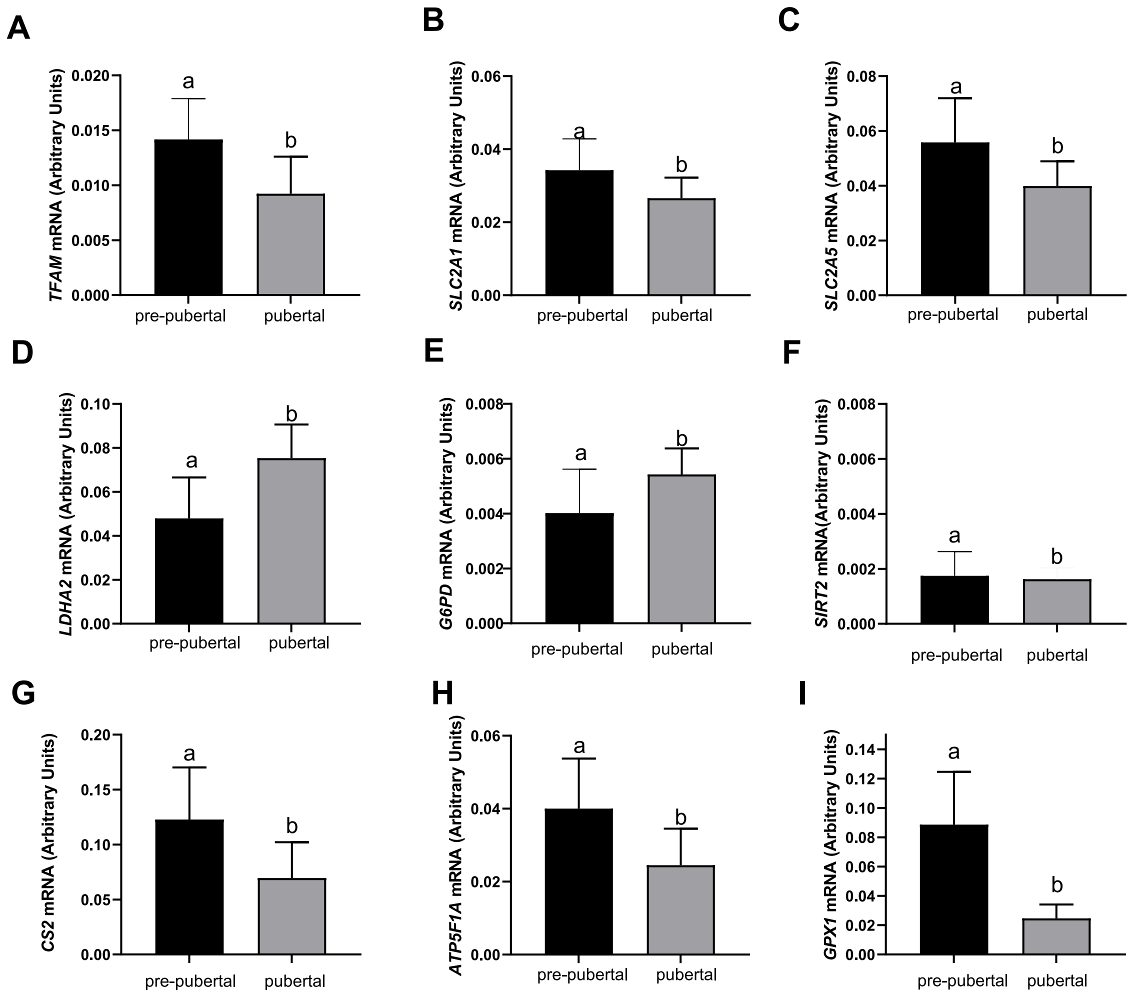

Fig. 7. The transcription profiles of *TFAM* (A), *SLC2A1* (B), *SLC2A5* (C), *LDHA2* (D), *G6PD* (E), *SIRT2* (F), *CS2* (G), *ATP5F1A* (H), and *GPX1* (I) in *in vitro* obtained bovine blastocysts (n = 4 × 5 for the pre-pubertal group; n = 4 × 5 for the pubertal group) derived from pre-pubertal and pubertal animal oocytes. Different letters above columns indicate a significant difference between groups ($p < 0.05$), as determined by Student's t-test. The values are presented as arbitrary units and are expressed as the mean ± SEM.
SEM, standard error of the mean.

limiting enzyme [59], and mitochondrial ATP synthase (*ATP5A1*) were overexpressed, which resulted in an increased mtDNA copy number in those blastocysts [21–23]. In blastocysts derived from pre-pubertal heifer oocytes, we found overexpression of the oxidative stress marker *GPX1*, most likely resulting in higher levels of ROS, compared to blastocysts derived from pubertal heifer oocytes.

To confirm the quality of the obtained blastocysts at the molecular level, we examined the mRNA expression levels of genes involved in embryonic developmental competence and implantation ability. The relative mRNA levels of *OCT4* and *SOX2* were significantly higher in blastocysts derived from pre-pubertal animals than in those derived from pubertal animals. *SOX2* is part of the pluripotency network in which it plays a central role, along with *OCT4* and *NANOG* [27]. These genes self-regulate and regulate the expression of each other, and their products often bind to the same target genes, maintaining the cell pluripotency program [60–63]. According to Khan et al. [28], low-quality bovine preimplantation embryos displayed overexpression of *OCT4* and *SOX2,* which was not reflected in their morphology, but they suggested that it could have consequences in the subsequent embryonic developmental stages. These data are consistent with our results that elevated *OCT4* and *SOX2* mRNA expression in blastocysts derived from pre-pubertal animals accounts for the lower quality of these blastocysts. However, Velásquez et al. [64] highlighted the importance of a proper balance between OCT4 and SOX2 during bovine embryonic development, which is required for the proper regulation of pluripotency. We also found lower mRNA expression of *NANOG* in blastocysts derived from pre-pubertal as opposed to pubertal animals. In cattle, NANOG was detected in bovine embryos beginning at the 8-cell stage, prior to the establishment of polarization and blastomere compaction [65], and became restricted to the inner cell mass (ICM) subpopulation at the blastocyst stage [66]. The decreased mRNA expression of *NANOG* in blastocysts derived from pre-pubertal animals might account for the fact that those blastocysts might have a lower ICM cell number than blastocysts derived from pubertal heifer oocytes, which is further evidence of their lower quality.

It has been previously suggested that IGF and its receptors, IGF1R and IGF2R, are also interdependent on the growth potential of embryos [67–69]. In our study, blastocysts derived from pre-pubertal animals exhibited higher mRNA expression of *IGF1R* but lower expression of *IGF2R* than those derived from pubertal animals. Yaseen et al. [70] and Wrenzycki et al. [71] found higher mRNA expression of both *IGF1R* and *IGF2R* in preimplantation bovine embryos produced in a more optimal *in vitro* culture system. The literature suggests that the elevation of both *IGF1R* and *IGF2R* mRNA expression accounts for better embryo quality. However, in our study, blastocysts from pre-pubertal animals exhibited higher mRNA expression of *IGF1R* but lower expression of *IGF2R* compared to those derived from pubertal animals. We presume that the obtained data supports the hypothesis that the impaired redox balance in blastocysts derived from pre-pubertal heifers, as well as higher mtDNA copy number, might be linked to some compensation effect and poor adaptation of blastocysts derived from pre-pubertal heifers to *in vitro* conditions, leading to altered *IGFR* expression.

The same hypothesis regarding the compensation effect in blastocysts derived from pre-pubertal heifers was demonstrated for *AQP3* and *DSC2* expression. Aquaporin 3 is a transmembrane channel protein that allows the rapid and passive movement of water and other tiny neutral solutes across the membrane to improve plasma membrane permeability and blastocyst cavity formation [72]. In the present study, the mRNA expression of *AQP3* was higher in blastocysts derived from pre-pubertal animals than in those derived from pubertal animals. In contrast, the mRNA expression of *DSC2* was higher in blastocysts derived from pubertal than in those derived from pre-pubertal animals. The expression of the *DSC2* transcript was detected at the highest level at the blastocyst stage [73], and the DSC2 protein was derived predominantly from the trophectoderm cells of bovine embryos [74]. DSC2 is involved in the formation of desmosomal junctions rather than TE stabilization during blastocyst expansion [35]. As the expression of the *DSC2* gene was significantly higher in high-quality blastocysts than in low-quality blastocysts [37], we might presume that blastocysts derived from pubertal animals have enhanced blastocyst expansion.

In this study, the mRNA expression of *SSLP1*, *PLAU*, and *PLAC8* was higher in embryos obtained from pubertal than in pre-pubertal heifers. SSLP1 is involved in immune response and pregnancy establishment [32], whereas PLAU is involved in the proteolysis of extracellular matrix proteins during implantation [43]. Moreover, *PLAC8* was found to be upregulated in hatched blastocysts compared to early blastocysts [32] and was also reported to be induced by IFNτ, which is the main embryonic signal of pregnancy recognition in ruminants [33]. Considering the above data, we postulate that the overexpression of *SSLP1*, *PLAU*, and *PLAC8* in blastocysts derived from pubertal heifer oocytes could account for their higher quality, as these genes are known to be involved in pregnancy establishment in ruminants [32].

The last gene used to confirm the quality of the blastocysts obtained at the molecular level was *DNMT3A*. It is an epigenetic embryo marker belonging to the methyltransferase family that mediates the establishment and maintenance of dynamic patterns of DNA methylation in the embryonic genome [38]. In the present study, the mRNA expression of *DNMT3A* was higher in blastocysts derived from pubertal animals than in those derived from pre-pubertal animals. The findings suggest that higher *DNMT3A* mRNA expression in blastocysts derived from pubertal heifer oocytes can facilitate a window of DNA methylation reprogramming in bovine embryos post-blastocyst development during implantation.

5. Conclusions

In conclusion, the results obtained in this study revealed that the oxidative stress, mtDNA content, and developmental competence of *in vitro*-produced blastocysts derived from pre-pubertal heifer oocytes differed from those of blastocysts obtained from pubertal heifers. In the group of blastocysts derived from pre-pubertal heifers, we found a higher mtDNA copy number and a significantly lower number of blastocysts produced *in vitro* from approximately the same number of collected oocytes, although those blastocysts did not differ from those obtained from pubertal oocytes in terms of their morphological quality. Morphologically appropriate blastocysts derived from pre-pubertal heifers had higher concentrations of deleterious ROS and higher concentrations of GSH. The impaired redox balance in blastocysts derived from pre-pubertal heifers, in addition to higher mtDNA copy number as well as altered gene expression of markers of developmental competence, suggest significantly lower quality of blastocysts derived from pre-pubertal animals, despite their unaltered morphology. However, to conclusively assess the quality of blastocysts obtained *in vitro*, an *in vivo* study concerning the embryo transfer of those blastocysts is required to calculate the embryonic implantation rate and, subsequently, the number of live births of the calves.

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Author contributions

M.T. took part in *in vitro* embryo production, was the main person performing the molecular biology analyses and immunostaining of embryos, and contributed to writing the paper; I. K-Z. took part in the design of the study, ovum pick up, and *in vitro* embryo production, was the main supervisor of the molecular biology analyses and immunostaining of embryos, and contributed to writing the paper; D.B. took part in the study design and *in vitro* embryo production; J.J. took part in the ovum pick up and contributed to writing the paper; K.Ł. contributed to writing the paper, I·W.-P. was the main designer of the study, the performer of ovum pick up, took part in *in vitro* embryo production, and contributed to writing the paper.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

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RESEARCH

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Deregulation of oxidative phosphorylation pathways in embryos derived in vitro from prepubertal and pubertal heifers based on whole-transcriptome sequencing

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Abstract

Background Although, oocytes from prepubertal donors are known to be less developmentally competent than those from adult donors it does not restrain their ability to produce full-term pregnancies. The transcriptomic profile of embryos could be used as a predictor for embryo's individual developmental competence. The aim of the study was to compare transcriptomic profile of blastocysts derived from prepubertal and pubertal heifers oocytes. Bovine cumulus-oocyte complexes (COCs) were obtained by ovum pick- up method from prepubertal and pubertal heifers. After in vitro maturation COCs were fertilized and cultured to the blastocyst stage. Total RNA was isolated from both groups of blastocysts and RNA-seq was performed. Gene ontology analysis was performed by DAVID (Database for Annotation, Visualization and Integrated Discovery).

Results A higher average blastocyst rate was obtained in the pubertal than in the pre-pubertal group. There were no differences in the quality of blastocysts between the examined groups. We identified 436 differentially expressed genes (DEGs) between blastocysts derived from researched groups, of which 247 DEGs were downregulated in blastocysts derived from pubertal compared to prepubertal heifers oocytes, and 189 DEGs were upregulated. The genes involved in mitochondrial function, including oxidative phosphorylation (OXPHOS) were found to be different in studied groups using Kyoto Encyclopedia of Genes (KEGG) pathway analysis and 8 of those DEGs were upregulated and 1 was downregulated in blastocysts derived from pubertal compared to prepubertal heifers oocytes. DEGs associated with mitochondrial function were found: ATP synthases (ATP5MF-ATP synthase membrane subunit f, ATP5PD- ATP synthase peripheral stalk subunit d, ATP12A- ATPase H+/K+transporting non-gastric alpha2 subunit), NADH dehydrogenases (NDUFS3- NADH: ubiquinone oxidoreductase subunit core subunit S3, NDUFA13- NADH: ubiquinone oxidoreductase subunit A13, NDUFA3- NADH: ubiquinone oxidoreductase subunit A3), cytochrome c oxidase (COX17), cytochrome c somatic (CYCS) and ubiquinol cytochrome c reductase core protein 1 (UQCRC1). We

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found lower number of apoptotic cells in blastocysts derived from oocytes collected from prepubertal than those obtained from pubertal donors.

Conclusions Despite decreased expression of genes associated with OXPHOS pathway in blastocysts from prepubertal heifers oocytes, the increased level of *ATP12A* together with the lower number of apoptotic cells in these blastocysts might support their survival after transfer.

Keywords Next-generation sequencing, Mitochondria, Blastocyst, Prepubertal heifer, Cow

Background

In vitro embryo production (IVP) in cattle demands obtaining embryos of decent developmental competence, which is a great challenge for assisted reproductive technology [1]. Although the IVP of embryos from prepubertal animals is even more challenging, this procedure provides many more possibilities for the cattle breeding industry due to the greater chance for faster genetic progress. However, during IVP procedure of embryos from prepubertal animals, the developmental potential of the collected oocytes is still the major limiting factor for embryonic developmental potential, resulting in the procurement of various amounts of transferable blastocysts and subsequent pregnancies [2, 3].

In every single cell of the body, including the oocyte, the major mechanism involved in energy metabolism is ATP production, which depends on the normal function of mitochondria and is significant for proper embryo developmental competence [4, 5]. Mitochondria, the powerhouses of the cells, are not only involved in cell death and homeostasis by supporting redox homeostasis and producing intermediate metabolites for the pathways of cell signaling and gene expression; their crucial role is ATP production throughout the process of oxidative phosphorylation (OXPHOS) [6]. Moreover, in cows, the enzymes involved in the Krebs cycle are also responsible for epigenetic remodeling, which occurs during embryonic genome activation (EGA) [7].

Bovine embryonic development occurs in accordance with specific metabolic patterns, among which the production of energy is mainly associated with pyruvate oxidation through oxidative phosphorylation. During this process, one molecule of glucose is transformed into two molecules of pyruvate to produce 2 molecules of ATP [8]. During OXPHOS, 33 molecules of ATP are produced by the electron transport chain in the mitochondrial membrane [9, 10]. In the cow, it has been demonstrated that during early embryonic development, an increasing function of energy acquired from glycolysis was correlated with compaction and blastulation [11, 12]. The remarkable increase in glucose metabolism during early embryo development was also reported by Gardner [13] to significantly increase embryo viability. Moreover, it is worth noting that the deficient ATP content was associated with fertilization failure and inability to achieve the blastocyst stage in cattle [5].

To the best of our knowledge, there are no data in the literature on global gene expression in embryos derived from oocytes from prepubertal and pubertal heifers or whether the impaired early embryonic development from oocytes collected from prepubertal heifers depends on different ATP production capacities in the mitochondria of these embryos. We also planned to gain the knowledge if the oxidative phosphorylation pathway of embryos derived from oocytes collected from prepubertal and pubertal heifers may be a good predictor for embryo's developmental competence. In the current study we presume that there is a potential for selecting transferable embryos not only on the basis of morphological criteria, but also on the basis of candidate transcripts. The studies of Chitwood et al. [14] presented the first application of RNA-seq technology in single bovine embryos, Graf et al. [15] provided detailed insight into the timing of gene activation during early bovine embryo development and Jiang et al. [16] demonstrated the comprehensive examinations of gene expression in bovine oocytes and preimplantation embryos and taking above the studies presented the transcriptome analysis of whole bovine embryos. Moreover, the analysis of transcriptome profiles of bovine embryos derived in vivo Kues et al. [17], Gad et al. [18] Jiang et al. [16] and Kropp et al. [19] or in vitro Gad et al. [18], Graf et al. [15], Kropp et al. [19] and Wei et al. [20] allowed us to understand the regulatory mechanisms concerning EGA and metabolic demands before preimplantation development of bovine embryos. Previous studies also proved that the different environmental conditions of embryo culture in vitro influenced the transcriptomic profiles of the obtained embryos [1, 18]. Moreover, Gad et al. (2012) reported that the culture conditions during maturation and early embryo development also influenced the metabolic activity and gene expression patterns of bovine embryos [18]. Finally, Morin-Doré et al. (2017) demonstrated the transcriptome profile of blastocysts derived from prepubertal and pubertal heifers oocytes by microarray method, however we decided to use NGS sequencing due to its better potential to gain deeper insight into the transcriptome profile.

The purpose of this study was to examine whether different developmental competence of the embryos derived from prepubertal and pubertal animals is affected by the heterogeneity of their transcriptomic profiles. Moreover, the expression of molecular markers associated with mitochondrial function, expressed by the genes involved in OXPHOS, might be applied as a predictable marker for embryonic developmental capability. Thus, in this study, the leading objective was to identify and compare the transcriptional status of bovine blastocysts obtained in vitro from prepubertal and pubertal heifers. The presented study is the continuation of the previous research published in Theriogenology [21] which aimed to demonstrate the differences between the level of the mitochondrial DNA content and the expression of genes involved in mitochondrial function and embryo's developmental competence in blastocysts derived from prepubertal and pubertal heifers. On the basis of the literature and our previous study [21], we presume that the regulatory gene pathways involved in the OXPHOS pathway in blastocysts derived from prepubertal versus pubertal heifers can reflect different developmental competence of the embryos.

Methods

Animals

All experiments were carried out in accordance with the Local Animal Care and Use Committee in Olsztyn, Poland (Agreements No. 76/2014/DTN and 55/2023). All experiments were conducted in accordance with relevant guidelines and regulations and adheres to the ARRIVE guidelines. The experiment took place in the owned by the University of Warmia and Mazuria in Olsztyn, Scientific and Educational Station in Bałdy. The animals used in the study resided at the Scientific and Educational Station in Bałdy, which belongs to the Warmia and Mazuria University in Olsztyn. During the experiment, the heifers stayed in their habitat and their environmental conditions did not change. All cattle were healthy and disease-free and were maintained under the same standard management conditions with free access to feed and water. Two groups of Polish Holstein-Friesian heifers were examined. The animals in the prepubertal group were younger than 10-months-old with the absence of a dominant follicle or corpus luteum in the ovary, which was confirmed by ultrasound examination. In the pubertal group, animals were older than 15-months-old and were confirmed by ultrasound examination to have a corpus luteum or dominant follicle on the ovary and were used as a control group.

Chemicals and suppliers

All culture media for the in vitro production of bovine embryos were purchased from Minitube (Germany). From Nunc (Thermo Scientific Denmark), we received plastic dishes, 4-well plates, and tubes. Unspecific reagents and supplements for in vitro culture were obtained from Merck (Germany). The chemicals for reverse transcription were purchased from Invitrogen (Carlsbad, CA, USA). The SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian Components (cat. no. 634,489; Takara Bio Inc) was purchased to perform next-generation sequencing.

Experimental design

The transcriptomic profiles of genes involved in oxidative phosphorylation pathway from blastocysts derived from oocytes collected from prepubertal and pubertal heifers were compared. TUNEL assay was performed to detect apoptotic cells in situ in blastocysts derived from oocytes collected from prepubertal and pubertal heifers.

Ovum pick -up

Bovine cumulus oocyte complexes (COCs) were collected from the follicles from two studied groups of animals, prepubertal and pubertal via a transvaginal ultrasound quided OPU method, according to Cavalieri et al. [22]. Before each procedure of the ovum pick up, the perineal region was cleaned using water and 70% ethanol. The animals received standard epidural anesthesia before each OPU session with the use of polocainum hydrochloricum 2% and adrenalinum 0.005%. The dose of epidural anesthesia was determined based on the weight of the animal and ranged between 1,5 and 3 ml. Directly, after the experiment the animals came back to the herd and underwent normal reproductive management, as the rest of the animals leading to pregnancy at the age of approximately 13–16 months. None of the animals used for the study was sacrificed. The COCs were obtained in the same way as described in our previous study [21]. During the experiment fifty-one OPU sessions were performed. The oocytes collected by the OPU procedure were used for in vitro embryo production. COCs from researched groups were observed under an Olympus SZX10 stereomicroscope and washed twice in wash medium (TCM) supplemented with 20 mM HEPES, 25 mM sodium bicarbonate, 0.4% bovine serum albumin, and 40 μg/mL gentamicin. In vitro maturation was performed using obtained COCs.

In vitro maturation (IVM)

The obtained COCs were pooled and placed (grouped separately from prepubertal and pubertal heifers) into 4-well plates (#144,444) containing 500 μL of maturation medium (TCM 199 Maturation Medium (19,990/0010), supplemented with 0.02 IU/mL of pregnant mare serum gonadotropin (PMSG, #G4527), 0.01 IU/mL human chorionic gonadotropin (hCG, #C0684,), and 5% fetal bovine serum (FBS, #12,106 C) and incubated at 38.5 °C in a 5% CO_2 humidified atmosphere for 23 h for in vitro maturation (IVM).

In vitro fertilization (IVF)

In all experiments frozen commercially available semen from the same bull (Sexing Technology, USA) was used for the IVF procedure. Computer- assisted sperm analysis method was used to assess and confirm the fertility parameters of the semen. The sperm were placed under capacitation medium [(TL sperm capacitation medium (19,990/0020) supplemented with 1 mM sodium pyruvate sodium pyruvate, 0.6% BSA and 0.1 mg/ml gentamicin)] after thawing in a water bath at 38 °C for 60 s, then incubated for one hour at 38.5 °C in a humidified atmosphere with 5% CO_2 to allow recovery of motile spermatozoa using a swim-up procedure. Following incubation, the upper of two thirds of the capacitation medium were recovered and then centrifuged at 200 g for 10 min. Finally, the supernatant was removed and the sperm pellet was diluted in a appropriate volume of fertilisation medium to obtain the final concentration of 10^6 motile sperm/mL. Groups of COCs, obtained from prepubertal and pubertal heifers were placed in 500 µl of fertilisation medium [(TL fertilization medium; 19,990/0030) supplemented with 10 µg/mL of heparin (#H3393), 20 mM sodium pyruvate (#P3662), and 0.5% BSA)] and co-incubated with spermatozoa in 4-well dishes containing 500 µL of fertilization medium for 24 h at 38.5 °C in an atmosphere humified with 5% CO_2. The day of in vitro fertilization was regarded as day 0.

In vitro culture (IVC)

Embryos (from oocytes collected from prepubertal and pubertal heifers) were separated from the cumulus cells and attached sperm, then were washed twice in wash medium at 24 h after IVF. Then, blastocysts (groups of 25) were cultured in 4-well dishes cointaining 500 µL of culture medium [(SOF; 198 synthetic oviduct fluid medium (19,990/0040)] supplemented with basal medium Eagle-amino acids using 10 µL/mL (#B6766), 20 µL/mL MEM (#M7145), 3.3 mM sodium pyruvate, and 5% fetal bovine serum in 500 µL of mineral oil (NidOil, Nidacon). The culture conditions were carried at 38.5 °C in an atmosphere of 5% CO_2, 5% O_2, and 95% N_2 with high humidity until the blastocyst stage was achieved (day 7). An Olympus SZX10 stereomicroscope was used to evaluate the development rate and quality of embryos obtained from oocytes collected from prepubertal and pubertal animals by morphological examination at approximately 100× magnification according to the guidelines of the International Embryo Technology Society (IETS). Embryo grading was used to select the appropiate blastocysts for the current study. For further analysis we used only blastocysts assesed as grade 1 and 2 (according to IETS). For RNAseq analysis blastocysts from both groups were stored separately in Lysis Mix (Takara Bio Inc) at −80 °C. For quantitative polymerase chain reaction (qPCR) blastocysts from the two examined groups were stored at −80 °C in Extraction Buffer (KIT0204, Arcturus PicoPure RNA Isolation Kit Applied Biosystems, CA, USA).

Isolation of RNA from blastocysts for NGS sequencing

Total RNA was isolated from blastocysts derived from oocytes collected from prepubertal and pubertal heifers, as described previously [21]. For each group (blastocysts derived from prepubertal and pubertal heifers oocytes), total RNA was isolated from four replicates at an average of 5 pooled blastocysts ($n=4\times5$), and 4 vs. 4 samples were finally obtained for RNA-seq. The quality and quantity of mRNA were detected with a NanoDrop spectrophotometer (ND200C; Fisher Scientific, Hampton, PA, USA). The concentration of analyzed samples ranged between 0.6 and 2 ng/µl, and the RIN coefficient was 5.5 to 7.

Moreover, the RNA quality was controlled with TapeStation2200 (Agilent, Santa Clara, CA, USA) and High Sensitivity RNA ScreenTape (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Only RNA samples with RNA integrity values between 5.5 and 7 were used for further analysis.

Library preparation and whole transcriptome sequencing

Whole transcriptome sequencing was carried out for blastocysts derived from oocytes collected from prepubertal and pubertal heifers. For the analyzed blastocysts, the cDNA libraries were prepared according to the SMARTer Stranded Total RNA Seq Kit v3- Pico Input Mammalian (Takara Bio, USA, Inc). The libraries were prepared from 250 pg of total RNA according to the protocol. The samples were ligated with different adaptors and amplified in 5 cycles due to manufacturer's recommendations depend on RNA input (5 cycles for 0.25 ng-10ng regular RNA samples). The final RNA-seq library amplification was performed in 12 cycles according to manufacturer protocol and preceded by personal validation of optimal number of cycles dedicated to the input amount of material. The quality and quantity of the obtained libraries were measured with Qubit (Invitrogen, ThermoFisher Scientific), TapeStation2200 (Agilent, Santa Clara, CA, USA) and High Sensitivity D1000 ScreenTape RNA ScreenTape (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. The concentrations of the libraries obtained were normalized to 10 nm, and the next samples were pooled. NGS sequencing was performed on a NextSeq500 Illumina platform in 75 single-end cycles according to the manufacturer's instructions.

Data analysis, DEGs identification and functional enrichment analysis

First, the raw reads were checked for quality using FastQC software, followed by a trimming procedure, which led to removing reads that did not meet the following criteria: <20 phred quality, reads shorter than 35 bp, and presence of adapters - adapters trimming (Flexbar) [23]. After the trimming procedure, the filtered reads were mapped to the Bos taurus UCD1.2 reference genome using STAR software [24].

In the next step, HTSeq-count software was used to count the mapped reads into the respective Ensembl GTF annotation file version 105. After assessing the read counts, DESeq2 software was used to calculate differentially expressed genes. The resulting genes with an adjusted p value < 0.05 and a fold change above > 1.2 were treated as differentially expressed for further analysis.

Differentially expressed genes in blastocysts obtained from oocytes collected from prepubertal and pubertal heifers were reported as gene lists in the DAVID v. 6.8 software [25] and KEGG databases (according to the *Bos Taurus* reference) and WebGestalt (WEB-based Gene SeT Analysis Toolkit [26] to confirm and perceive appreciably enriched Gene Ontology terms (GO) and pathways. KEGG enrichment analysis for differentially expressed genes was performed by http://www.bioinformatics.com.cn/srplot (Supplemental Figure S1).

RNA isolation and qRT–PCR for validation

Total RNA was isolated as described previously [21] from blastocysts obtained from prepubertal and pubertal heifers oocytes. RNA was extracted from (n=4×5) blastocysts from prepubertal and (n=4×5) blastocysts from pubertal heifers with an Arcturus PicoPure RNA Isolation Kit (#KIT0204, Applied Biosystems, USA) in accordance with the manufacturer's instructions. First, SuperScript™ III First-Strand Synthesis SuperMix for qPCR (#11,752,250, Thermo Fisher, 278 USA) was used, and the products were stored at -20 °C.

Validation of NGS data using real-time PCR (n = 4 × 5)

Complementary cDNA and qPCR analysis were performed using the same RNA from the prepubertal (n=4×5) and pubertal (n=4×5) blastocysts. The validation of RNA-seq results was designed using nine differentially expressed genes (DEGs). Genes were selected with an FC (>1.2) and an adjusted p value < 0.05. Real-time PCR was used to check the expression of differentially expressed genes between the two examined groups (DEGs). The primers for real-time PCR were designed using the NCBI database (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are presented in Table 1. Relative mRNA levels of genes used for validation were normalized to the *GAPDH* internal control. The obtained results were examined to be statistically significant for a p value < 0.05 using statistical analysis system software (GraphPad PRISM v. 9.0 Software, Inc., USA).

Table 1 Primers used for real-time PCR

Gene symbol	Gene name	Primer sequence 5′–3′	Amplicon size, bp	Gen Bank accession no.
ATP5MF	Bos taurus ATP synthase membrane subunit f	**F**: ACTGATGCGGGATTTCACCC **R**: TGCTCCCTTTCTTCACGTTCA	98	NM_001113719
ATP5PD	ATP synthase peripheral stalk subunit d	**F**: ACTTCCGTTCTCTGCTGCTGT **R**: CCCCAAAAGCTACCCAGTCAA	110	NM_174724.4
ATP12A	ATPase H+/K+transporting non-gastric alpha2 subunit	**F**: AAGCCTCGCCACAAGAAGAA **R**: CATGAGGCCGATATGCAGGT	78	XM_002691870.5
NDUFA13	NADH: ubiquinone oxidoreductase subunit A13	**F**: GCCCTATCGACTACAAGCGG **R**: TCCAGTACCCGAACAGCAAG	96	NM_176672
CYCS	cytochrome c, somatic	**F**: GCGTGTCCTTGGGCTTAGAA **R**: TTCTTTCTCTGTGCGCGACC	74	NM_001046061
COX17	cytochrome c oxidase copper chaperone COX17	**F**: GGACACCTAATTGAAGCCCAC **R**: ACCATGCTCACCATTTCATATCTT	72	NM_001207032.1
UQCRC1	ubiquinol cytochrome c reductase core protein 1	**F**: AGGACCTGCCAAAAGCTGTA **R**: CCCGCTCCTTCTCAATCTGG	84	NM_174629.2
NDUFS3	NADH: ubiquinone oxidoreductase core subunit S3	**F**: GGGAGGCTTTCCCTGCCTAT **R**: CCACATGCCTTCCCTGAAAC	99	NM_174819.3
NDUFA3	NADH: ubiquinone oxidoreductase subunit A3	**F**: GACCAAGATGGCTGAGAGAGT **R**: AATGGCGAAGGATGCCACTA	82	NM_176659
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	**F**: CACCCTCAAGATTGTCAGCA **R**: GGTCATAAGTCCCTCCACGA	103	NM_001034034.2

Detection of apoptosis in blastocysts

For the detection of apoptotic cells in blastocysts derived from oocytes collected from prepubertal and pubertal heifers the terminal-uridine nick-end labeling (TUNEL) was conducted using the In Situ Cell Death Detection Kit, Fluorescein (#11,684,795,910, Roche, Germany). Blastocysts after culture from two examined groups were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature (RT). Then, the blastocysts were permeabilized in 0.3% Triton X-100 (#T9284) in 0.1% sodium citrate for 2 min on ice and washed twice in PBS. To induce DNA strand breaks before TUNEL labelled positive control of blastocysts were treated with 3000 U/ml DNase (#79,254; Qiagen, Hilden, Germany) in the reaction buffer and incubated at RT for 10 min. Positive control and sample blastocysts were transferred to 50 μL of TUNEL enzyme (terminal deoxynucleotidyl transferase) reaction mixture and then incubated at 37 °C for 1 h in the dark. At the same time, negative control blastocysts were incubated in TUNEL label solution without the enzyme. After the incubation blastocysts were washed three times in PBS, and were stained with 10 μg/ml DAPI (#D9564) for 30 min at 30 °C in the dark. The blastocysts were observed under an LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany) with a 40×/1.2NA immersion objective. The DAPI filter was used to estimate the total number of nuclei and the FITC filter to assess TUNEL positive cells.

Assessment of apoptotic cells in blastocysts

The number of TUNEL positive cells per embryo was measured in two examined groups. To calculate the percentage of apoptotic cells a number of apoptotic cells divided by the total number of blastomeres per embryo. The experiment was conducted with 5 blastocysts per group. The percentage of apoptotic cells was calculated for each group. The obtained data were determined to be statistically significant for a p value <0.05 using statistical analysis system software (GraphPad PRISM v. 9.0 Software, Inc., USA).

Results

Gene ontology analyses

RNA-Seq analysis was performed to compare the transcriptomic profiles of blastocysts derived from oocytes collected from prepubertal and pubertal heifers and to demonstrate the molecular mechanisms underlying the differences in the transcriptomic profiles.

The NGS statistic showed that the average raw reads obtained per sample were 53.9 mln, and 99% of them passed the quality filter (Table 2, Supplemental Figure S2). Moreover, an average of 79.4% of reads were uniquely mapped.

We performed KEGG pathway enrichment analysis and found that mitochondrial OXPHOS was significantly overrepresented (FDR < 5.3E-1). We also presented significant interactions between analyzed differentially expressed genes (DEGs). The Principal Component Analysis (PCA) clustering of samples representing two groups pre-pubertal (red) and pubertal (blue) was presented in the Fig. 1. The volcano plot depicted the up and down-regulated DEGs in the experiment and was presented in Fig. 2. The DEGs belonged to oxidative phosphorylation pathway were red marked.

Developmental competence of blastocyst stage and quality of the in vitro-produced blastocysts

The average number of oocytes per animal obtained from the pubertal group compared to the prepubertal group did not differ ($p > 0.05$). As shown in Table 3, the blastocyst rate was higher in the pubertal group than in the prepubertal group ($p < 0.05$). All collected blastocysts were graded (Grades 1–4) morphologically based on IETS criteria. Number and percentage of blastocysts in the different grades (Grades 1–4) are showed in Table 3. There were no differences in the quality of the obtained blastocysts between the two examined groups ($p > 0.05$). The total of 40 blastocysts Grade 1 were derived from prepubertal heifers oocytes and the total of 77 blastocysts were obtained from pubertal heifers oocytes. The percentage of Grade 1 embryos derived from oocytes collected from prepubertal heifers was higher than from the

Table 2 The basic NGS statistic of all analyzed libraries

Sample	Raw reads	Filtered reads	Uniquely mapped	Mapped to multiple loci	Percent of uniquely mapped reads	Assigned to annotation database
1	52,571,938	52,051,424	41,581,031	6,363,643	79.88	24,828,302
2	54,708,852	54,167,180	41,703,329	6,705,325	76.99	24,164,077
3	56,123,912	55,568,230	44,273,299	6,886,066	79.67	20,112,044
4	57,650,297	57,079,502	46,430,720	7,192,302	81.34	29,549,143
5	53,208,628	52,681,810	43,023,223	6,788,176	81.66	27,266,940
6	57,591,774	57,021,558	45,434,202	8,041,295	79.67	28,098,361
7	43,469,751	43,039,357	35,545,624	4,607,069	82.58	18,510,164
8	56,320,387	55,762,759	41,149,301	8,518,737	73.79	24,831,086

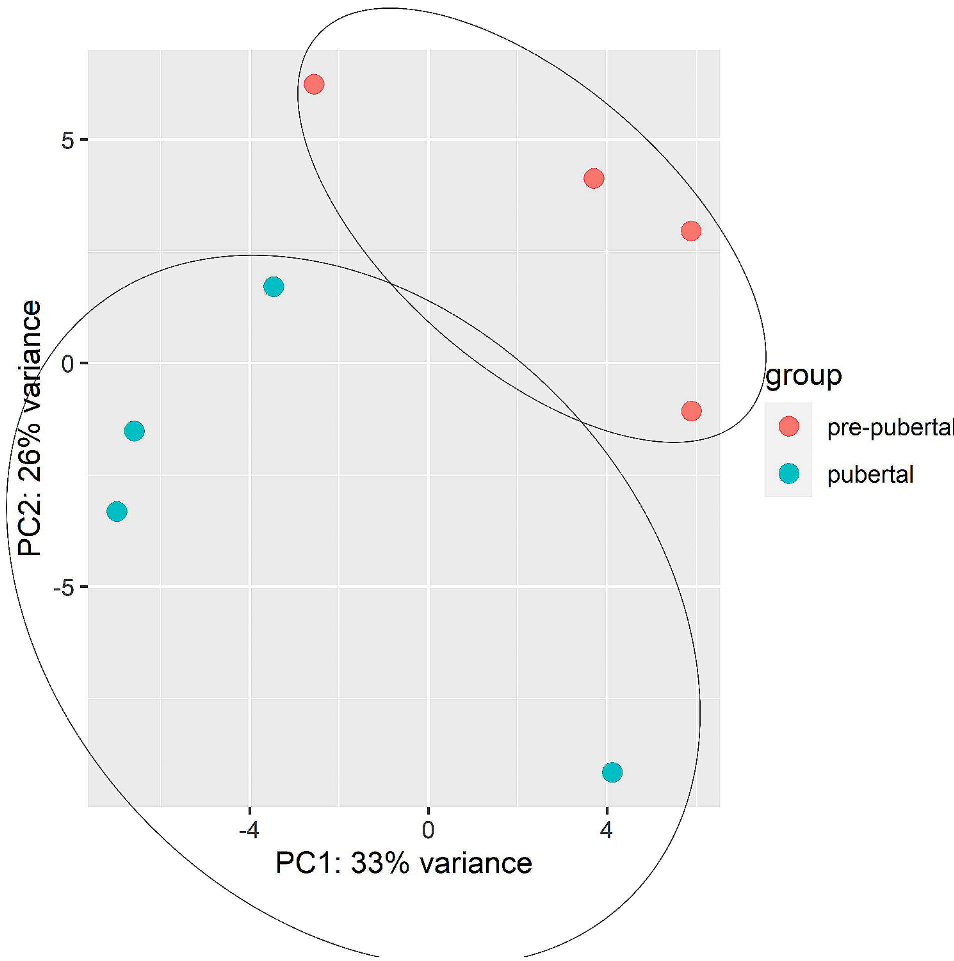

Fig. 1 The Principal Component Analysis (PCA) clustering of samples representing two groups pre-pubertal (red) and pubertal (blue)

pubertal heifers, however this difference was not statistically significant. The percentage of Grade 2 blastocysts derived from oocytes collected from pubertal heifers was higher than that from pubertal, but not significantly different. The percentage of Grade 3 and 4 blastocysts were found to be similar in both groups and were no statistically different.

Sequencing data Summary

Identification of differentially expressed genes (DEGs)

RNA-Seq of bovine blastocysts derived from oocytes collected from prepubertal and pubertal heifers was performed to identify, characterize and compare their transcriptomic profiles. Comparison of transcriptomic profiles showed that there were 436 differentially expressed genes between the two examined groups.

We noticed that 247 genes were downregulated in blastocysts derived from pubertal heifers compared to prepubertal heifers oocytes, and 189 differentially expressed genes were upregulated in blastocysts derived from pubertal heifers compared to prepubertal heifers oocytes. In the metabolic pathway 29 genes were up-regulated and 23 were down-regulated in embryos derived from pubertal compared to pre- pubertal heifers oocytes (Supplemental Figure S3, Supplemental Figure S4, Supplemental Table S1). In the oxidative phosphorylation pathway 8 genes were up-regulated and 1 was down-regulated in blastocysts derived from pubertal compared to prepubertal heifers oocytes (Supplemental Figure S5, Supplemental Figure S6, Supplemental Table S2).

Gene ontology analysis

Gene Ontology (GO) analysis was performed by DAVID (Database for Annotation, Visualization and Integrated Discovery) and WebGestalt [26]. Three categories from gene ontology were determined: biological process (BP), cellular component (CC) and molecular function (MF). Thirty-eight biological processes were found to be different in blastocysts derived from oocytes collected from prepubertal and pubertal heifers. A higher number of differentially expressed genes between the two examined groups was found in cell differentiation (GO: 0030154, 16 DEGs). The least number of differentially expressed genes were specified in negative regulation of primary

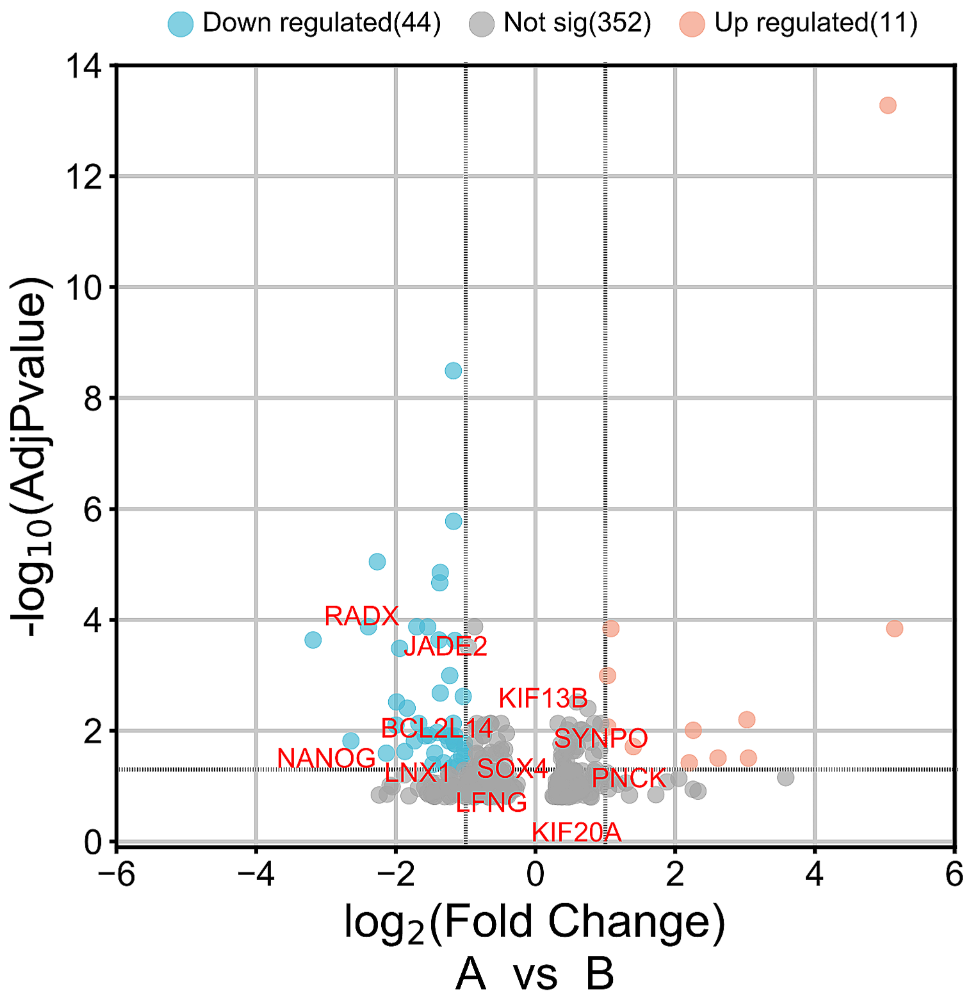

Fig. 2 Volcano plot showing the significant up and down regulated genes across experiment designed. The DEGs belonged to oxidative phosphorylation pathway were red marked

Table 3 Developmental rates of embryos

Experimental group	Total number of oocytes (*n*)	Average number of oocytes (*n*) per one animal	Total number of blastocyst (*n*)	Blastocyst rate (%)	Total number of selected quality blastocysts (%)
pre- pubertal	365	7.16	50	13.6[a]	Grade 1/40 [80][a] Grade 2/5 [10][a] Grade 3/4 [8][a] Grade 4/1 [2][a]
pubertal	347	6.8	115	33.1[b]	Grade 1/77 [67][a] Grade 2/30 [26][a] Grade 3/5 [4][a] Grade 4/3 [3][a]

miRNA processing (GO: 2,000,635, 2 DEGs), positive regulation of sodium ion transmembrane transporter activity (GO: 2,000,651, 2 DEGs), positive regulation of calcium- transporting ATPase activity (GO: 1,901,896, 2 DEGs), negative regulation of sodium ion transport (GO: 0010766, 2 DEGs), retina vasculature morphogenesis in camera- type eye (GO: 0061299, 2 DEGs) and mitral valvae morphogenesis (GO: 0003183, 2 DEGs).

Differentially expressed genes were involved in twenty-three processes in cellular component and twenty-six involved in molecular function.

We observed that several genes involved in oxidative phosphorylation were also related to other biological

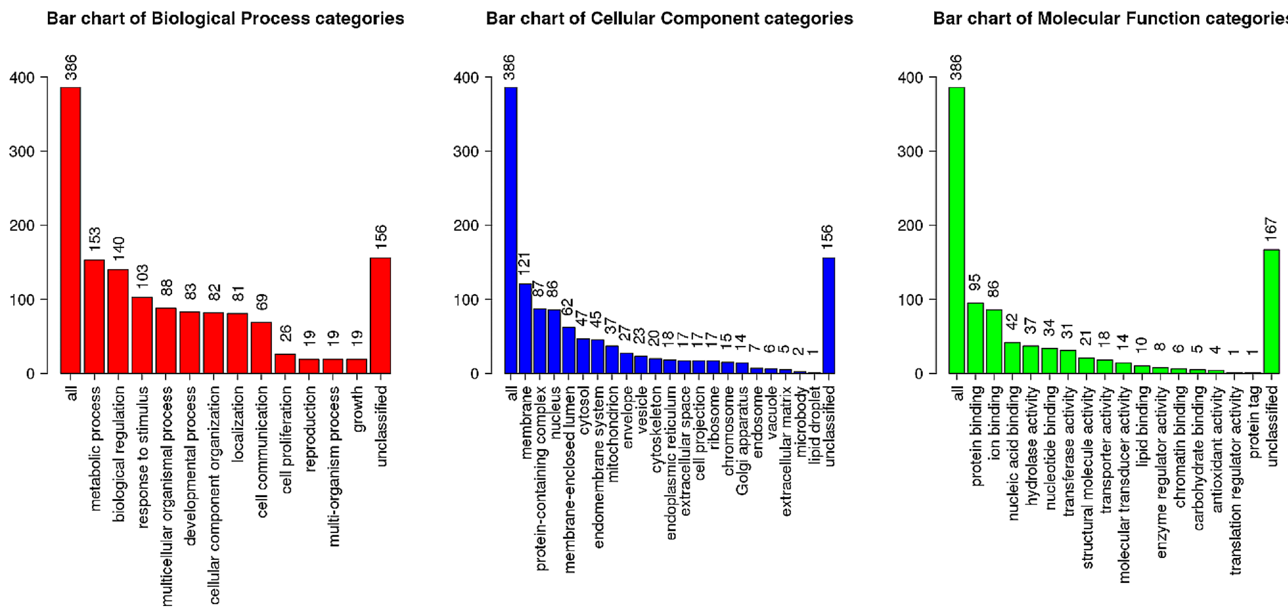

Fig. 3 WebGestalt Analysis of the different Gene Ontology terms. Bar chart showing the number of genes from the nex-generation sequencing that are involved in the different Gene Ontology terms as predicted by the Gene Set Enrichment Analysis (GSEA) via WebGestalt. The graph is showing the number of genes involved in the different biological processes (Red), Cellular components (Blue) and Molecular functions (Green)

processes. Thus, the *CYCS* gene is also involved in the apoptotic process (GO: 0006915, 12 DEGs), *COX17* in mitochondrial respiratory chain complex IV (GO: 0033617, 3 DEGs) and *NDUFA13* in positive regulation in cystein-type endopeptidase activity involved in the apoptotic process (GO:0043280, 4 DEGs).

The analysis of differentially expressed genes distinguished from the three categories is shown in Fig. 3. designed by WebGestalt [26].

Pathway enrichment analysis

The identified DEGs were enriched in thirteen biological pathways, which was established by the KEGG (Kyoto Encyclopedia of Genes) tool (Table 4, Supplemental Figure S7, Supplemental Table S3). The most differentially expressed genes were observed in metabolic pathways (13 DEGs). The other biological pathways significant by DAVID were as follows: ribosome (13 DEGs), biosynthesis of cofactors (10 DEGs), glutathione metabolism (6 DEGs), oxidative phosphorylation (9 DEGs), carbon metabolism (7 DEGs), hypertrophic cardiomyopathy (6 DEGs), glycine, serine and threonine metabolism (4 DEGs), and microRNAs in cancer (11 DEGs).

Oxidative phosphorylation pathway

To demonstrate the importance of mitochondrial function in the oxidative phosphorylation pathway in blastocysts derived from prepubertal and pubertal heifers oocytes, we chose the oxidative phosphorylation pathway.

The expression of eight genes involved in the oxidative phosphorylation pathway was upregulated in blastocysts derived from pubertal heifers compared to prepubertal heifers (Fig. 3, Supplemental Figure S6, Supplemental Figure S7, Supplemental Table S2). One of the genes associated with this pathway was downregulated in blastocysts derived from pubertal heifers compared to prepubertal heifers (Fig. 4).

Network between differentially expressed genes

The interaction network was constructed by STRING and Cytoscape (Supplemental Figure S8).

qPCR validation of DEGs that were enriched in KEGG pathways

We used real-time PCR to confirm the expression of nine DEGs involved in oxidative phosphorylation that were distinguished in blastocysts derived from prepubertal and pubertal heifers. Genes were selected with the FC (>1.2) and adjusted p value < 0.05. The expression of *ATP5MF, ATP5PD, CYCS, COX17, UQCRC1, NDUFA3, NDUFA13* and *NDUFS3* was upregulated in blastocysts derived from pubertal compared to prepubertal heifers. The expression of *ATP12A* was downregulated in blastocysts derived from prepubertal heifers compared to pubertal heifers. The qPCR method showed that the expression of the *ATP5MF, ATP5PD, ATP12A, CYCS, COX17, UQCRC1, NDUFA3, NDUFA13* and *NDUFS3* genes was in agreement with the NGS analysis (Fig. 5). The obtained results confirmed the precision of highly developed RNA-Seq analysis.

Table 4 Analysis of pathways included DEGs[1]

Term	Accession number	Gene number	*p*-Value	Genes involved in the process
Ribosome	bta03010:Ribosome	13 (13- DEGs up-regulated)	2,2E-4	*RPL5, MRPL18, RPL13A, RPS3A, MRPL34, MRPL10, MRPL4, RPS25, MRPL2, RPS17, RPL14, RPL36, RPL27*
Metabolic pathways	bta01100:Metabolic pathways	52 (29- DEGs up-regulated, 23-DEGs down-regulated)	1,3E-3	*NDUFA13, ALAS2, ST6GALNAC2, ENO1, ATP12A, GPHN, GCSH, STS, ALDH2, ANPEP, CA4, ACP5, CD38, KMT5A, PSPH, PCYT1B, GPX3, MARS1, ITPK1, AMT, PGD, NME1, ALDH1A3, UQCRC1, NDUFS3, ALDOC, AGPAT5, DNMT1, ISYNA1, MGST3, COX17, MGST2, PTGS2, SELENBP1, GULO, DGUOK, UGP2, GMDS, RDH12, INPP5F, PNPO, ARSB, ATP5MF, PCK2, ATP5PD, NDUFA3, IDH2, ETNPPL, DHODH, MGAT4B, ALPL, CYCS*
Biosynthesis of cofactors	bta01240:Biosynthesis of cofactors	10 (5- DEGs up-regulated, 5-DEGs down-regulated)	4,9E-3	*GULO, ALAS2, UGP2, ALDH2, RDH12, PNPO, ALPL, GPHN, DHODH, NME1*
Glutathione metabolism	bta00480:Glutathione metabolism	6 (1- DEGs up-regulated, 5-DEGs down-regulated)	9,8E-3	*GPX3, MGST3, ANPEP, IDH2, MGST2, PGD*
Oxidative phosphorylation	bta00190:Oxidative phosphorylation	9 (8-DEGs up-regulated, 1-DEGs down-regulated)	1,1E-2	*NDUFA13, ATP5PD, NDUFA3, COX17, NDUFS3, UQCRC1, CYCS, ATP12A, ATP5MF*
Carbon metabolism	bta01200:Carbon metabolism	7(4-DEGs up-regulated, 3-DEGs down-regulated)	3,1E-2	*GCSH, IDH2, AMT, ALDOC, ENO1, PGD, PSPH*
Hypertrophic cardiomyopathy	bta05410:Hypertrophic cardiomyopathy	6(4-DEGs up-regulated, 2-DEGs down-regulated)	4,5E-2	*MYBPC3, RYR2, IL6, TPM3, TPM1, ITGB8*
Glycine, serine and threonine metabolism	bta00260:Glycine, serine and threonine metabolism	4(3-DEGs up-regulated, 1-DEGs down-regulated)	6,9E-2	*GCSH, ALAS2, AMT, PSPH*
MicroRNAs in cancer	bta05206:MicroRNAs in cancer	11(6-DEGs up-regulated, 5-DEGs down-regulated)	9,2E-2	*NOTCH3, ZEB2, DNMT1, TNN, STMN1, STAT3, TPM1, PTGS2, SLC7A1, SOX4, CDC25A*
Pathways of neurode-generation- multiple diseases	bta05022: Pathways of neurodegeneration-multiple diseases	16(9-DEGs up-regulated, 7-DEGs down-regulated)	9,5E-2	*ATP5PD, NDUFS3, NDUFA13, NDUFA3, BDNF, CYCS, GPX1, IL6, NEFH. PTGS2, RYR2, TUBA4A, TUBB4A, TUBA1A, UQCRC1, UBE2G2*

[1] The analysis was performed in two examined groups (embryos from prepubertal and pubertal heifers) according to DAVID database and demonstrated up and down regulated genes

TUNEL assay

Blastocysts derived from prepubertal and pubertal heifers oocytes were assessed for apoptosis. Figure 6 depicts representative fluorescent images of bovine blastocysts labeled by TUNEL assay. The number of apoptotic cells was significantly higher in blastocysts derived from oocytes collected from pubertal heifers than those from prepubertal ($p<0.05$) (Fig. 6).

Discussion

The presented study showed that oocytes derived from prepubertal heifers had lower blastocyst rate than that obtained from pubertal heifers. Accurate knowledge about the transcriptomic profiles will enable us to gain insight into the molecular mechanisms involved in early embryonic development as well as early-fading fertility in cattle [27–29]. Morin-Dore et al. [30] demonstrated the analysis of transcriptome profile of blastocysts from prepubertal and pubertal heifers using microarray data analysis and presented the differences between examined groups and on the contrary with our study identified pathways such as mTOR (mammalian target of rapamycin), the peroxisome proliferator-activated receptor (PPAR) and NRF2-mediated oxidase stress response pathway. In the present study, we performed transcriptomic profile analysis of blastocysts derived from oocytes collected from prepubertal and pubertal heifers, but to the best of our knowledge for the first time demonstrated that the analysis of enriched KEGG pathways showed that DEGs were involved in the oxidative phosphorylation (OXPHOS) pathway, suggesting that this pathway was the most relevant in the conducted study and differed depending on the oocyte donor age. We have demonstrated that the expression of genes involved in OXPHOS had a crucial impact on embryo developmental competence, both in blastocysts derived from oocytes collected from prepubertal and pubertal heifers. Moreover, here, we also aimed to unravel the specific molecular markers of the embryos derived from prepubertal and pubertal heifers and their relationship with the developmental competence of examined embryos. In addition, identification of transcriptome profiles that favour early embryo development seems to be crucial for elucidating the genetic markers used to select developmentally competent embryos for embryo transfer.

In the conducted study, we identified 436 significantly differentially expressed genes, among which 247 genes were downregulated and 189 were upregulated when comparing the group of blastocysts derived from pubertal to the group of blastocysts derived from prepubertal

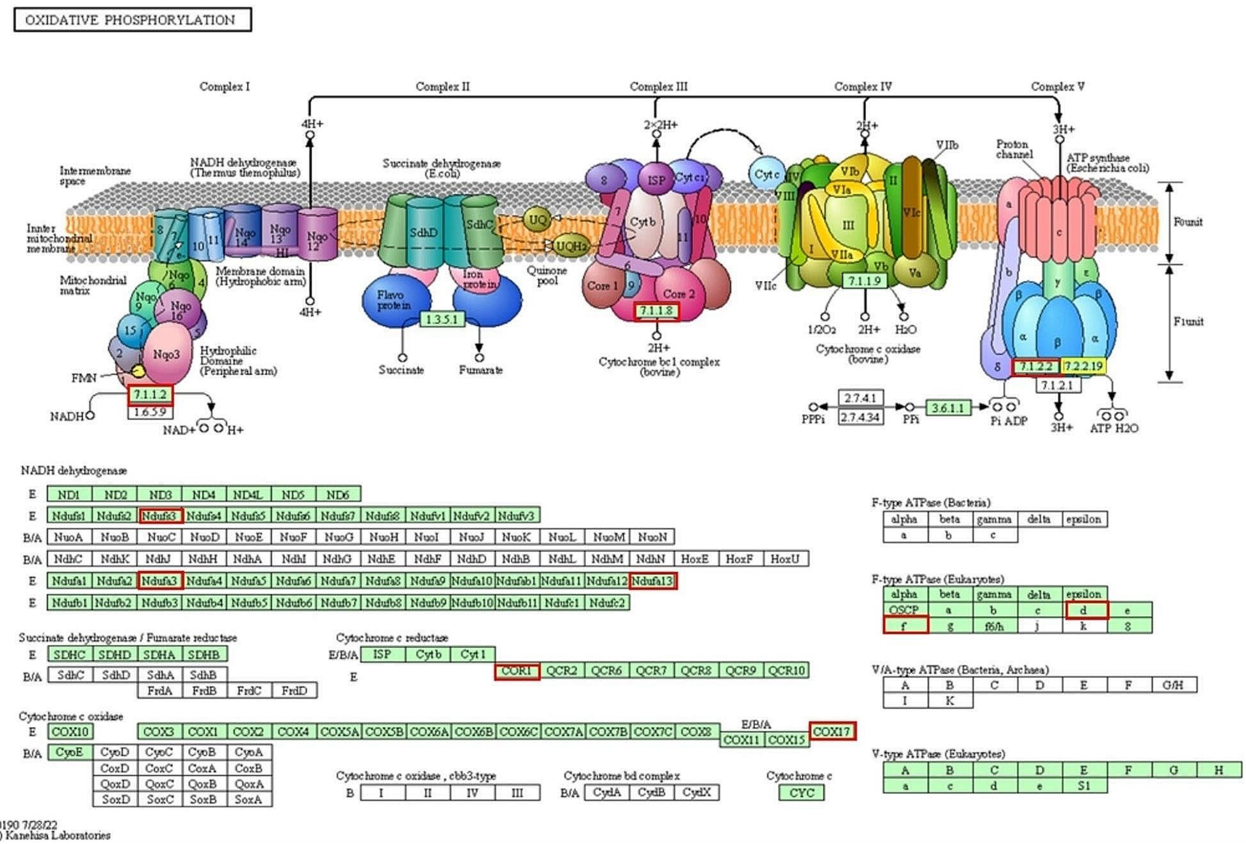

Fig. 4 KEGG pathway diagram for oxidative phosphorylation. The functional pattern of the gene expression between pre- pubertal and pubertal groups. Boxes with red borders represent up- regulated genes and boxes with the yellow border represent down-regulated genes when the pre- pubertal group was compared with the pubertal group

heifers. Using the DAVID database, we identified that most of the differentially expressed genes were related to cellular components, biological processes and molecular function. Our study demonstrated that mitochondrial function exemplified by OXPHOS differed in blastocysts derived from oocytes obtained from prepubertal vs. pubertal heifers. Whereas mitochondria are significant organelles, described as powerhouses of cells, that are responsible for energy metabolism and ATP production [4], which in turn is associated with the growth, homeostasis or death of the cells [6, 27] the deregulation of their function might result in lower ATP production. It was previously demonstrated that the higher the ATP content in human and mouse MII oocytes, the better the embryo developmental and implantation ability [5]. Moreover, oocyte quality and embryo developmental ability depended directly on the better function of mitochondria expressed by significantly higher mtDNA copy number [31–33].

The OXPHOS pathway is a significant source of ATP production in mitochondria and depends on five multisubunit respiratory chain protein complexes that are directly involved in oocyte maturation [34], fertilization [35] and preimplantation development of embryos [36, 37]. In the current study, we found that the first and fifth complexes of the respiratory chain had the highest number of DEGs. The expression of nine genes (*NDUFA3, NDUFA13, NDUFS3, UQCRC1, ATP5MF, ATP5PD, ATP12A, CYCS, COX17*) involved in the OXPHOS pathway differed in blastocysts derived from oocytes collected from the pubertal group compared to the prepubertal group. The expression of *NDUFA3, NDUFA13, NDUFS3, ATP5MF, ATP5PD, CYCS* and *COX17* was higher in blastocysts derived from oocytes collected from pubertal heifers than in those derived from prepubertal heifers. Only the expression of *ATP12A* was higher in blastocysts derived from oocytes collected from prepubertal than pubertal heifers. Moreover, differences in oocyte competence derived from prepubertal heifers yield lower developmental competence compared to oocytes derived from pubertal heifers [38]. Our previous study mentioned that the expression of genes associated with developmental competence was lower in blastocysts

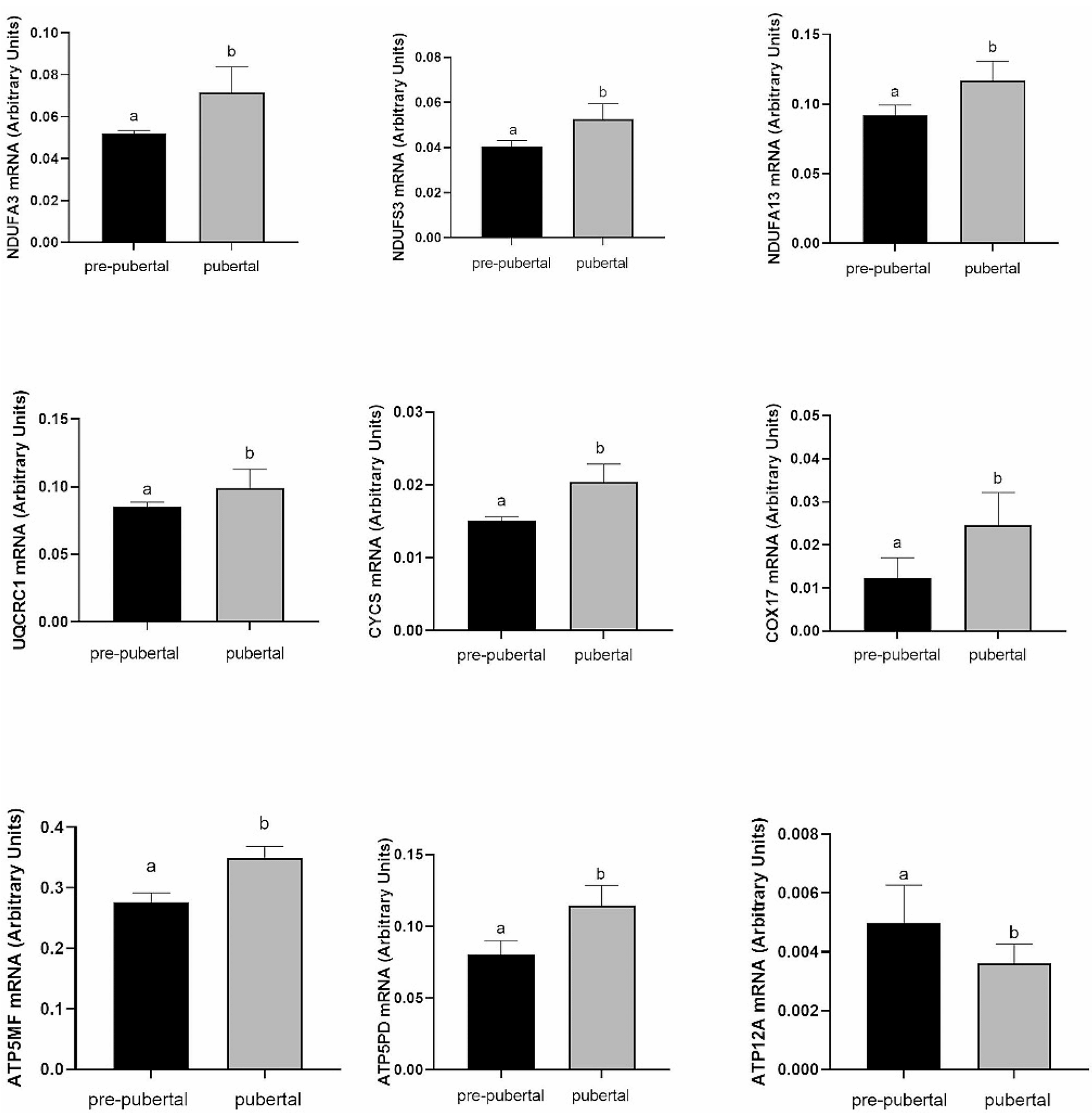

Fig. 5 Real-time PCR validation in in vitro obtained bovine blastocysts ($n = 4 \times 5$ for pre- pubertal and $n = 4 \times 5$ for pubertal group) obtained from pre-pubertal and pubertal oocytes). Different letters above the column mean a significant differences between groups ($p < 0.05$), as determined by Student's test. The data as presented as arbitrary units and are expressed as the mean ± SEM

derived from oocytes collected from prepubertal heifers than in those derived from pubertal heifers [21].

The largest complex in the respiratory chain of OXPHOS is complex I, called nicodine amide adenine dinucleotide [39–41]. This complex is considered a main source and entrance for electrons to the respiratory chain [42]. NADH, which is oxidized by NADH dehydrogenase, is produced in several processes, such as glycolysis, pyruvate dehydrogenase, the tricarboxylic acid (TCA) cycle, and beta-oxidation of fatty acids [43]. Furthermore, dysfunction of respiratory chain complex I is associated with many genetic diseases, including lactic acidosis, cardiomyopathy, leukoencephalopathy and muscle atrophy [44]. Therefore, the disorder in NADH dehydrogenase genes could cause dysfunction of the mitochondrial respiratory chain and a decrease in ATP production, as was demonstrated in blastocysts derived from prepubertal heifers. We found three differentially expressed

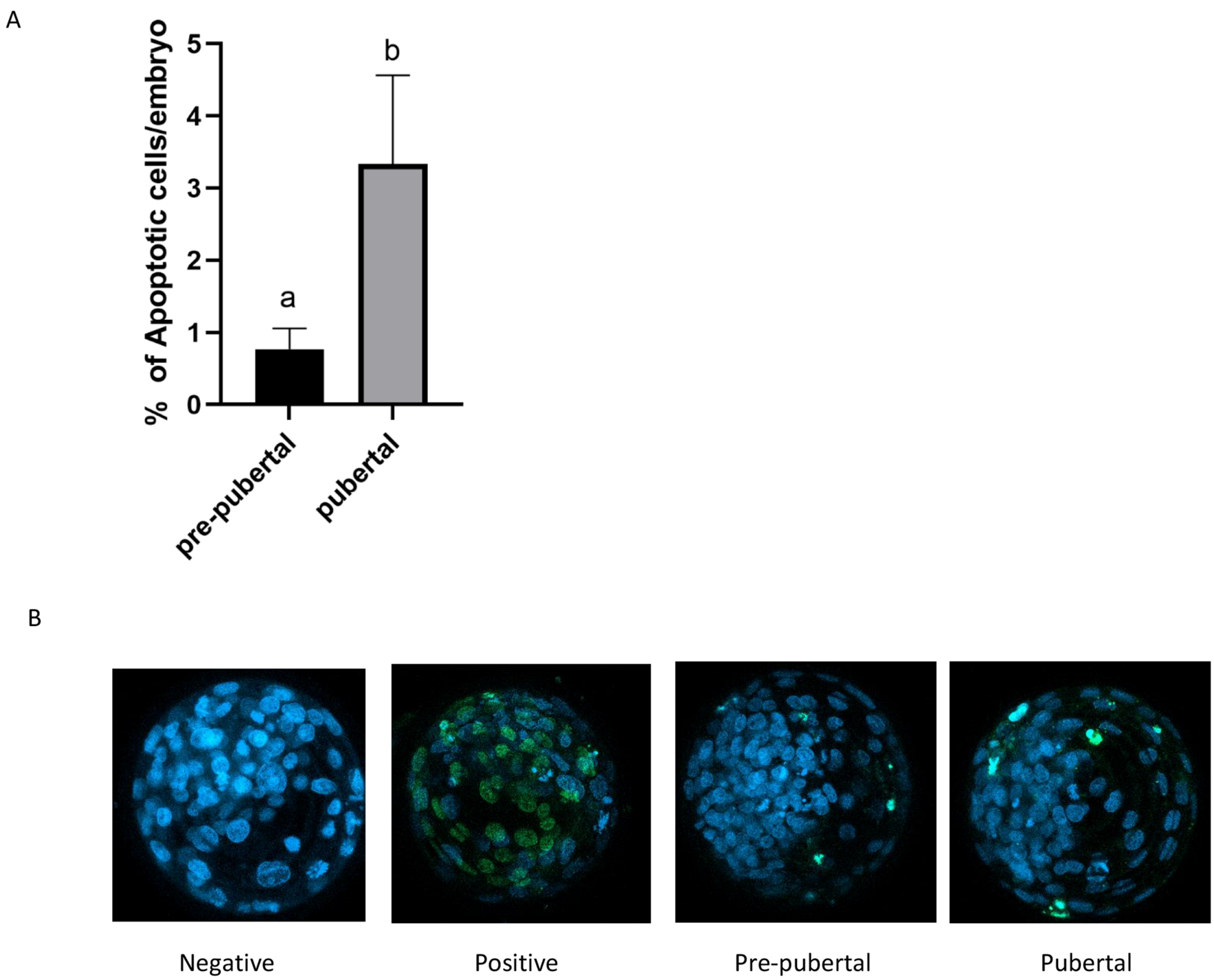

Fig. 6 (**A**) TUNEL was used to asses the level of apoptosis in blastocysts derived from oocytes obtained from pre-pubertal and pubertal heifers. The number of individual cells that were TUNEL positive was counted in each blastocyst and is represented as the average number of cells that are TUNEL positive per blastocyst. Embryos derived from oocytes collected from pre-pubertal heifers displayed significant decrease the average number of TUNEL positive cells compared with blastocysts derived from oocytes obtained from pubertal heifers; mean ± SEM, $P < 0.05$ (**B**) Representative images of embryos from TUNEL assay. Negative: No fluorescence control; Positive: Embryos pretreated with DNAse to ensure TUNEL staining was successful; 5 embryos in each group

genes, NADH: ubiquinone oxidoreductase subunit A3 (NDUFA3), NADH: ubiquinone oxidoreductase subunit A13 (NDUFA13), and NADH ubiquinone oxidoreductase core subunit S3 (NDUFS3), to be downregulated in blastocysts derived from oocytes collected from pre-pubertal compared to pubertal heifers, which might account for some dysfunctions of the mitochondrial respiratory chain action in those embryos. Moreover, NDUFA3, NDUFA13, and NDUFS3 have been previously considered to be predictive molecular markers of embryo developmental competence [1]. NADH: ubiquinone oxidoreductase subunit A3 and NADH: ubiquinone oxidoreductase subunit A13 are part of the ND1- module assembly, and NDUFA3 interacts with the transmembrane domain of NDUFA13. Moreover, NDUFA3 is located in the inner mitochondrial membrane and communicates with the previously mentioned core subunits ND1, ND3 and NDUFS8 and with the supernumerary subunits NDUFA8 and NDUFA13. Qin et al. [37] reported that the expression of NDUFA3 was downregulated in in vivo matured oocytes compared to in vitro matured oocytes, which are generally considered to have lower developmental competence [37]. In contrast, in our study, we demonstrated higher expression of NDUFA3 in blastocysts derived from oocytes collected from pubertal heifers than in those derived from prepubertal heifers, which in turn accounts for their better developmental competence.

Taking into consideration the higher expression of *NDUFA3, NDUFA13* and *NDUFS3* in pubertal heifers found in our study, we might presume that the blastocysts derived from heifers after they reach puberty have considerably higher developmental competence, caused by more efficient respiratory chain action. To support the hypothesis that lower expression of *NDUFA13* found in the blastocysts derived from oocytes collected from prepubertal heifers accounts for the probable higher lethality of those embryos, expressed by lower blastocyst rate in that group of blastocysts compared to the group from pubertal heifers, we would like to cite the studies of Huang et al. [45], Chao et al. [46] and Cui et al. [47] in mice. In those experiments, it was shown previously that the downregulation of the *NDUFA13* gene caused a reduction in mouse oocyte viability, maturation performance, and the developmental and implantation ability of embryos. Taking the above studies into consideration, we presume that the importance of NDUFA13 in bovine blastocysts obtained from oocytes collected from prepubertal and pubertal heifers is associated with the developmental competence of the embryos and suggest that the embryos derived from pubertal heifers had better developmental ability.

The other DEG we found in complex I of the respiratory chain is *NDUFS3*, which is the current precursor of mitochondrial assembly. Suhane et al. [48] indicated that the silencing of *NDUFS3* expression can systematically induce mitochondrial dysfunction. We found lower expression of *NDUFS3* in blastocysts derived from prepubertal heifers oocytes than in those derived from pubertal heifers oocytes. Zhang et al. [49] demonstrated significantly decreased expression of *NDUFS3* in Ca^{2+}-overloaded oocytes and suggested its correlation with mitochondrial dysfunction in those oocytes [49]. The authors also mentioned that higher expression of *NDUFS3* could act as an ROS scavenger in those oocytes [49]. In our previous study [21], we also demonstrated higher ROS levels in blastocysts derived from oocytes collected from prepubertal heifers than in blastocysts derived from oocytes collected from pubertal animals. Furthermore [50], also suggested that higher Ca^{2+} levels in oocytes could induce mitochondrial oxidative stress and lead to apoptosis [50]. In our study, we might presume that the higher level of ROS, additionally associated with the lower expression of *NDUFS3* in blastocysts derived from oocytes collected from prepubertal heifers, might subsequently impair ROS scavenger efficiency in the blastocysts. Similarly, Sasaki et al. [51] showed that the higher expression of *NDUFS3* played a key role in reducing ROS levels and protected cells from cellular damage [51]. Zhang et al. [49] also demonstrated a negative correlation between ROS levels and the expression of the *NDUFS3* gene in murine oocytes [49]. Taking the obtained data into account, we presume that higher expression of the *NDUFS3* gene can act as an ROS scavenger in blastocysts derived from pubertal heifers as well as be a potential hypoxia marker associated with the status of the developmental competence of the embryo.

Another characteristic of embryos capable of surviving after transfer was the downregulation of genes associated with energy metabolism. Zolini et al. [52] indicated that 11 downregulated genes involved in oxidative phosphorylation were less expressed in blastocysts capable of surviving after transfer [52]. Similarly, other experiments conducted on embryos produced in vitro [53] and in vivo [54] confirmed this statement. In the studies carried out by El-Sayed et al. [53] and Ghanem et al. [54], embryos that resulted in calf delivery also had lower expression of two mitochondrial transcripts [53, 54]. Zolini et al. [52] also suggested that the downregulation of the *ND1* gene, which encodes one of the NADH dehydrogenase genes, could be one of the genes involved in oxidative phosphorylation, and its downregulation of expression facilitates a metabolically quiet phenotype [52]. Although we did not assess the expression of the *ND1* gene, we speculate that there is a predictable correlation in our study with lower expression of genes also associated with oxidative phosphorylation in blastocysts derived from prepubertal heifers and facilitating the metabolically quiet phenotype by those embryos.

The “quiet embryo” hypothesis was proposed by Leese and colleagues, according to which “thrifty” embryos that do not require high rates of metabolism have a better chance of sustaining successful development than embryos that are characterized by a high rate of metabolism [55, 56]. Additionally, we propose that the lower expression of genes involved in OXPHOS in blastocysts derived from oocytes collected from prepubertal heifers could be associated with the statement mentioned above. Such embryos would likely benefit from reduced oxygen consumption, energy and nutrient requirements [52]. However, some studies on embryo transfer have not confirmed this statement [52]. Considering the research on human embryos produced in vitro and their pregnancy establishment ability after transfer, the authors noted significantly higher use of glucose by those embryos [13]. Moreover, in the cow, significant differences were demonstrated in oxygen consumption by the blastocysts developed in vivo; nevertheless, there was a nonsignificant tendency for a higher pregnancy rate for blastocysts using higher oxygen [57].

Additionally, many gene clusters associated with OXPHOS were also found in the present study in blastocysts derived from oocytes collected from prepubertal vs. pubertal heifers. In complex V, we identified three differentially expressed genes, namely, *ATP5MF, ATP5PD* and *ATP12A*. *ATP5MF* (ATP synthase membrane subunit f) and *ATP5PD* (ATP synthase peripheral stalk subunit), that, as demonstrated by Fu et al. [58], could be considered molecular markers for embryo implantation [58]. The upregulation of *ATP5PD* in reactivated blastocysts compared to dormant blastocysts was demonstrated by Fu et al. [58], who subsequently suggested that ATP production was reinforced in the mitochondria during the reactivation of blastocysts [58]. Moreover, Salilew et al. [1] found higher expression of *ATP5PD* in competent compared to noncompetent in vivo-derived embryos, which is consistent with data obtained in our study. We noticed higher expression of the *ATP5MF* and *ATP5PD* genes in blastocysts derived from oocytes collected from pubertal heifers compared to prepubertal heifers, which in turn accounts for the better developmental competence and implantation ability of those embryos.

In our previous study, we documented that *ATP5F1A* (ATP synthase subunit F1 alpha, also known as ATP5A1) was also overexpressed in blastocysts derived from prepubertal compared to pubertal heifers [21]. Liu et al. [59] demonstrated that the decreasing expression of *ATP5A1* was correlated with the aging process and that this gene had an essential role in the disorder of the function of mitochondria in human granulosa cells of aging women [59].

Ubiquinol-cytochrome C reductase core protein I (UQCRC1), a mitochondrial protein, is known as a biomarker of Alzheimer's disease and is located in complex III in the respiratory chain [60, 61]. UQCRC1 acts only as a core protein to simplify the interaction between cytochrome c (complex IV component) and cytochrome c1 (complex III component) [62]. The downregulation of *UQCRC1* expression may lead to decreased complex III activity, the consequence of which is the disruption of mitochondrial membrane potential as well as the reduced production of ATP with the increase in the level of ROS [63]. UQCRC1 plays an essential role in embryo survival and development [64]. Previous studies demonstrated that the knockout of *UQCRH* reinforced the Warburg effect [65]. This phenomenon has a crucial role in embryogenesis in the process of supporting rapid cell proliferation [66]. Qin et al. [37] demonstrated the downregulation of *UQCRC1* in in vitro matured oocytes from mice, which had higher levels of ROS than in vivo matured oocytes [37]. We presume that the lower expression of the *UQCRC1* gene in blastocysts derived from prepubertal heifers oocytes compared to pubertal heifers oocytes could also lead to a higher level of ROS, which we demonstrated in a previous study in those blastocysts [21], and at the same time reinforce the Warburg effect. We therefore assume that a higher level of that gene in blastocysts derived from pubertal heifers can be associated with better embryo developmental competence.

The inner- intermembrane electrochemical potential generated by OXPHOS in addition to ATP production is a significant feature of the organelle, which is useful for other important mitochondrial functions, such as dysfunction of mitochondria in response to protein import [67]. We investigated the expression of genes, such as *COX17* and *CYCS*, which are located in the mitochondrial intermembrane space and play a role in embryo developmental competence. Cytochrome c oxidase copper chaperone (COX17), also involved in OXPHOS, is located in the mitochondrial intermembrane space and is essential for the transport of copper to the mitochondria as well as cytochrome c oxidase activity [68], which is required for mitochondrial intermembrane space assembly and Cu homeostasis in cells [69]. In blastocysts, cytochrome c oxidase activity subunits were documented to be involved in embryo developmental competence and implantation ability [58]. Fu et al. [58] who investigated the global protein profiles in blastocysts at dormancy versus at reactivation, showed the downregulation of the *COX17* gene in reactivated blastocysts [58]. Similarly, in our study, we documented lower *COX17* expression in blastocysts derived from oocytes collected from prepubertal heifers compared to pubertal animals, which in turn accounts for the lower embryo developmental competence of those embryos. Moreover, Ntostis et al. [70] compared the transcriptomal profile of GV and MII oocytes from advanced maternal age (AMA) and young maternal age (YMA) groups [70]. Notably, those authors indicated that 31 genes involved in OXPHOS were highly expressed in young maternal age (YMA), such as cytochrome c oxidases (COX gene family), including the *COX17* gene, highlighting the significance of genes from that family in energy production and potential results in MII oocyte physiology and developmental capability [71]. In contrast, we propose that higher expression of *COX17* in blastocysts derived from pubertal heifers oocytes in our study could also be associated with energy production and have an impact on developmental competence in those blastocysts.

Cytochrome c somatic (CYCS), also located in the mitochondrial intermembrane space, is required for the electron transfer protein during OXPHOS, acting by transferring one electron from the cytochrome bc1 complex to cytochrome c oxidase [72], and its activity is essential for life [73]. Moreover, cytochrome c is also directly involved in apoptosis while being released into cytosol as a coactivator for caspase-3- activation as it binds the protease activating factor 1, leading to downstream activation of effector caspases [74–77]. It was previously demonstrated that the disruption of the unique *CYCS* gene caused embryonic lethality in mice [78]. We also demonstrated lower expression of the *CYCS* gene in blastocysts derived from prepubertal compared to pubertal heifers oocytes, suggesting that the lower expression of *CYCS* in blastocysts derived from prepubertal heifers oocytes was associated with lower developmental competence via modulation of the apoptotic process in the cells.

However, in our study, only one of the DEGs involved in OXPHOS, ATPase H+/K+transporting non gastric alpha2 subunit (*ATP12A*), which belongs to the cation transport ATPase (P-type), was demonstrated to have higher expression in blastocysts derived from oocytes collected from prepubertal heifers than in those derived from pubertal heifers. Qin et al. [37] demonstrated higher expression of *ATP12A* and ROS levels in in vitro matured (IVM) oocytes compared to in vivo matured (IVO) oocytes derived from mice [37]. We also indicated higher expression of *ATP12A* in blastocysts derived from oocytes collected from prepubertal heifers than in blastocysts derived from oocytes collected from pubertal animals, which correlated with previously demonstrated higher expression of ROS in those blastocysts, as confirmed by immunofluorescence staining [21]. Zhang et al. [42] also demonstrated that genes involved in OXPHOS were downregulated in GV-stage oocytes from 32-week-old mice in comparison with 5-week-old mice, which suggests that aging could have a detrimental impact on the mitochondrial respiratory chain in those oocytes [42]. Jakab et al. [79] suggested that in monocytes, higher expression of *ATP12A* could play a role in counteracting apoptosis via apoptotic volume decrease (AVD), intracellular acidification and a decrease in intracellular K^+ [79]. Programmed cell death depends on changes in the intracellular ion composition and subsequently the volume of cells, associated directly with the impaired function of Na^+, K^+ ATPase, which in turn leads to the loss of K^+ and gain of Na^+ ions [80]. AVD and cell shrinkage are triggered by the activation of cell volume regulation connected with potassium levels and anion channels. These processes lead to the cellular exit of K^+, Cl^- and HCO_3^- ions and osmotically obligated water [81–83]. Intracellular acidosis associated with the intracellular loss of these ions is necessary for the execution of apoptosis. Therefore, the expression of *ATP12A* could be considered the gene for blunting intracellular acidosis and counteracting the loss of K+ions and AVD. As we demonstrated higher expression of *ATP12A* in blastocysts derived from oocytes collected from prepubertal compared to pubertal heifers, we presume that ATP12A is involved in counteracting the early stages of apoptosis and maintaining the growth of the prepubertal blastocyst. Therefore, higher expression of *ATP12A* could be considered a potential candidate to counteract the process of early apoptosis.

Apoptosis is a physiological process which occurs during embryonic development [84]. Several studies demonstrated that the association between the presence of apoptosis in oocytes and blastocysts associated with their developmental competence seen to be inconsistent [85–89] Study of Bilodeau-Goeseels [86] demonstrated that oocytes which are developmentally competent have early signs for atresia. However, increased apoptosis is often in association with the worse embryo development and may lead to arrest the embryonic development before compaction. The study of Zaraza [90] aimed to compare the apoptosis level in blastocysts derived from oocytes collected from prepubertal calves, postpubertal calves and adult cows and demonstrated the lower level of apoptotic cells in blastocysts from adult cows compared to both prepubertal and postpubertal animals, which is in contrast to our results.

However, in the conducted study we demonstrated lower level of apoptotic cells in embryos derived from oocytes collected from prepubertal heifers which in turn can be beneficial to reach the blastocyst stage leading to their better chance for survival after transfer as well as. Zaraza [90] compared the apoptotic level in blastocysts derived from prepubertal, postpubertal and adult cows and found that the most significant factor determining the apoptosis was the age of oocytes donor. Moreover, the excess of glucose or glucose deprivation could cause the apoptosis. Several studies [90–92] mentioned that the relationship between decreased level of GLUTs transporter is related to programmed cell death by triggerring the apoptotic cascade in blastocysts. With the agreement on the above statement, in our previous study [21] we demonstrated the higher level of glucose transporters such as SLC2A1 and SLC2A5 in blastocysts derived from oocytes collected from prepubertal heifers and in the current study the higher level of ATP12A cause the decreased level of apoptosis in those blastocysts. The lower expression of ATP12A could act as counteracting the early stages of apoptosis in blastocysts derived from oocytes collected from prepubertal heifers and facilitates a metabolically quiet phenotype. As Singh et al. [93] documented that ATP12A was a transcriptional marker of committed lineages in the human blastocysts, therefore we presume that higher expression of ATP12A in blastocysts derived from oocytes collected from prepubertal heifers in our experiments can account for better early embryo preimplantation development. Moreover, Bogliotti et al. [94] demonstrated transcripts associated with the maternal-to-embryo transition and using GO analysis of transcripts documented enrichment of ATP12A in MII eggs, not in 8–16 cell embryo in terms associated with ion transport and nucleotide biosynthetic process. Furthermore, in the study conducted by Sood et al. [95] demonstrated the downregulation of ATP12A in blastocysts derived from cloned than IVF group and mentioned that the genes involved in embryo development are uniqely expressed in IVF group. Considering above, we pressume that the higher expression of ATP12A in blastocysts derived from oocytes collected from prepubertal heifers counteract the early stages of apoptosis, but is associated with poor developmental competence in those embryos.

Moreover, in our study, to broaden our knowledge about the function of all the DEGs in the examined blastocysts, we analyzed the molecular pathways in which these clusters were involved. ATP production in blastocysts could occur via aerobic and anaerobic energy-providing pathways. Salilew-Wondim et al. [1] compared *in vivo*- and in vitro-derived embryos using NGS sequencing and previously documented that a higher ATP accumulation ability in more competent in vivo-derived embryos was directly associated with the ability to utilize both aerobic and anaerobic energy-providing pathways [1]. Thompson et al. [12] also documented long ago that 86% of ATP production in produced bovine blastocysts in vitro was obtained via OXPHOS, which in turn explains that aerobic pathways are the main pathways of ATP production at the blastocyst stage [12]. Similarly, we compared DEGs involved in OXPHOS in blastocysts derived from prepubertal and pubertal heifers oocytes and confirmed the findings of Salilew-Wondim et al. [1] and Thompson et al. [12] that ATP production is obtained through aerobic pathways. Zolini et al. [52] mentioned that the decreased expression of genes involved in OXPHOS was associated with the survival of in vivo-produced female blastocysts to Day 60 of pregnancy [52]. Similarly the lower expression of genes involved in OXPHOS in blastocysts derived from prepubertal heifers oocytes found in our study does not have to account for their lower implantation ability, which is reflected by lower number of apoptotic cells in those blastocysts. However to explicitly confirm our hypothesis further experiments concerning embryo transfer are needed. Moreover, gaining new knowledge about the transcriptome analysis in embryos derived from prepubertal and pubertal heifers oocytes will enable us to search for more comprehensive overview about the possibility for selecting the embryos for transfer. Moreover, created for this study new data base of the whole transcriptome profiles of blastocysts derived from prepubertal and pubertal heifers oocytes gives unlimited potential for searching new molecular candidates for selecting embryos for embryo transfer.

Conclusions

In conclusion, the findings of our study provide new insight into the transcriptomic profile of blastocysts derived from prepubertal and pubertal heifers. We demonstrated differences in the expression of genes involved in mitochondrial function and OXPHOS in two groups of examined blastocysts, can be used for choosing the embryos with higher developmental competence for embryo transfer. So far, the selection of transferable embryos is mainly based on morphological criteria. Therefore, created for this study data base of the whole transcriptome profiles of blastocysts derived from prepubertal and pubertal heifers oocytes gives new possibilities for searching molecular markers of implantation ability. Moreover, the increased expression of ATP12A, together with the lower number of apoptotic cells in blastocysts derived from prepubertal heifers oocytes does not restrain their ability to produce full-term pregnancies. The possibility of using blastocysts derived from prepubertal heifers in embryo transfer programs will directly provide possibilities to enhance genetic gain in domestic livestock via the reduction of generation interval.

Abbreviations

ATP	adenosine triphosphate
COCs	cumulus-oocyte complexes
DAVID	Database for Annotation, Visualization and Integrated Discovery
KEGG	Kyoto Encyclopedia of Genes
DEGs	differentially expressed genes
ATP5MF	ATP synthase membrane subunit f
ATP5PD	ATP synthase peripheral stalk subunit d
ATP12A	ATPase H+/K + transporting non-gastric alpha2 subunit
NDUFS3	NADH: ubiquinone oxidoreductase subunit core subunit S3
NDUFA13	NADH: ubiquinone oxidoreductase subunit A13
NDUFA3	NADH: ubiquinone oxidoreductase subunit A3
COX17	cytochrome c oxidase
CYCS	cytochrome c somatic
UQCRC1	ubiquinol cytochrome c reductase core protein 1
OXPHOS	oxidative phosphorylation
IVP	in vitro production
EGA	embryonic genome activation
OPU	ovum pick up
WebGestalt	WEB-based Gene SeT Analysis Toolkit
Rna-seq	RNA sequencing
GO	Gene Ontology
TUNEL	The terminal-uridine nick-end labeling
IVM	In vitro maturation
IVC	In vitro culture
IETS	International Embryo Technology Society
qPCR	quantitative polymerase chain reaction

Supplementary Information

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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

Supplementary Material 10

Supplementary Material 11

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Author contributions
M.T. took part in in vitro embryo production, was involved in OPU, was the main person performing the molecular biology analyses and contributed to writing the paper, conceptualization, investigation, methodology, data curation, writing-original draft preparation, writing-review & editing,; I. K-Z. took part in the design of the study, ovum pick up, and in vitro embryo production, was the main supervisor of the molecular biology analyses and contributed to writing the paper, conceptualization, investigation, methodology, writing-original draft preparation, writing-review & editing, supervision; D.B. took part in the study design and in vitro embryo production, conceptualization, writing-original draft preparation, writing-review & editing; J.J. conceptualization, took part in the ovum pick up and contributed to writing the paper, writing-original draft preparation, writing-review & editing; S. G. was involved in OPU, conceptualization, K. Ł. contributed to writing the paper, writing-original draft preparation, writing-review & editing, IW.-P. was the main designer of the study, the performer of ovum pick up, took part in in vitro embryo production, and contributed to writing the paper, conceptualization, investigation, methodology, writing –original draft preparation, writing-review & editing, supervision, project administration, funding acquisition. K.R.-M and K.P.: performed RNA-seq analysis, conceptualization, software, validation, formal analysis, data curation, was contributed to writing the paper and original draft preparation, T.S: software, validation, data curation, formal analysis, was contributed to writing the paper and original draft preparation. All authors read and approved the final manuscript.

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Data Availability
The data have been submitted to the Gene Expression Omnibus (GEO) database and are available under GSE242449 accession number.

Declarations

Ethics approval and consent to participate
All experiments were carried out in accordance with the Local Animal Care and Use Committee in Olsztyn, Poland (Agreements No. 76/2014/DTN and 55/2023). All experiments were conducted in accordance with relevant guidelines, regulations and adheres to the ARRIVE guidelines (https://arriveguidelines.org/). We obtained the formal consent of the Director of the Scientific and Educational Station in Bałdy, which belongs to the Warmia and Mazuria University in Olsztyn.

Consent for publication
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Competing interests
The authors declare no competing interests.

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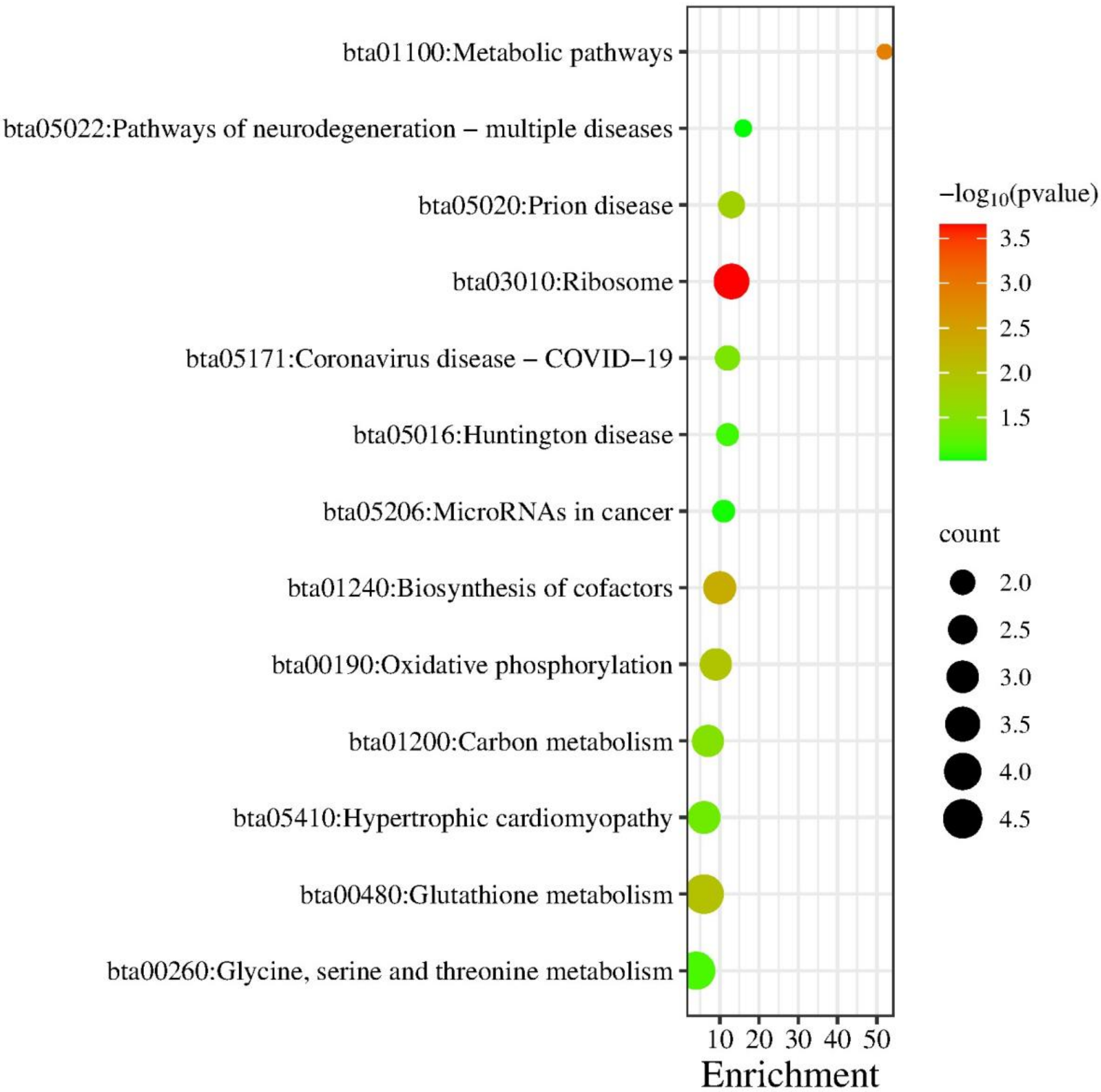
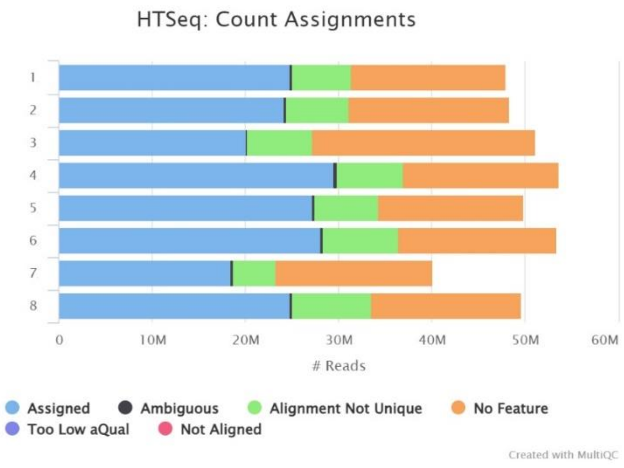
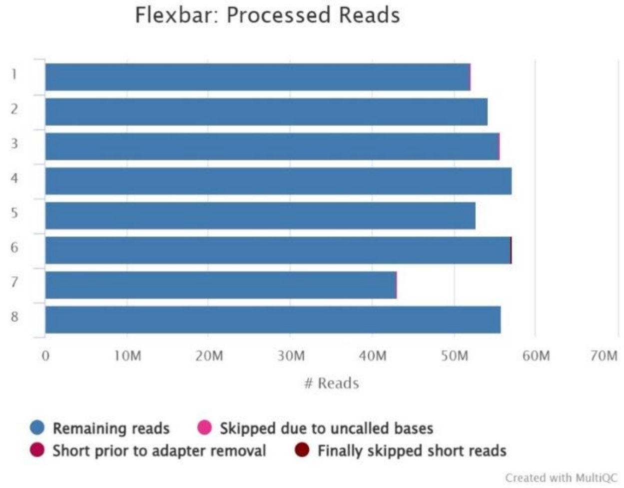


Supplemental Figure S1. KEGG pathway enrichment analysis. Y-axis indicate the pathway name, x-axis indicates the enriched factor in each of pathways. The bubble sizes are associated with the numer of DEGs. KEGG pathway enrichment analysis was perofrmed by http://www.bioinformatics.com.cn/srplot.

A



B



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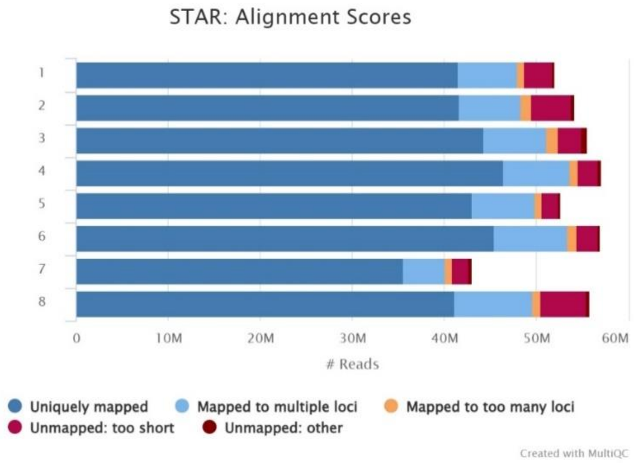
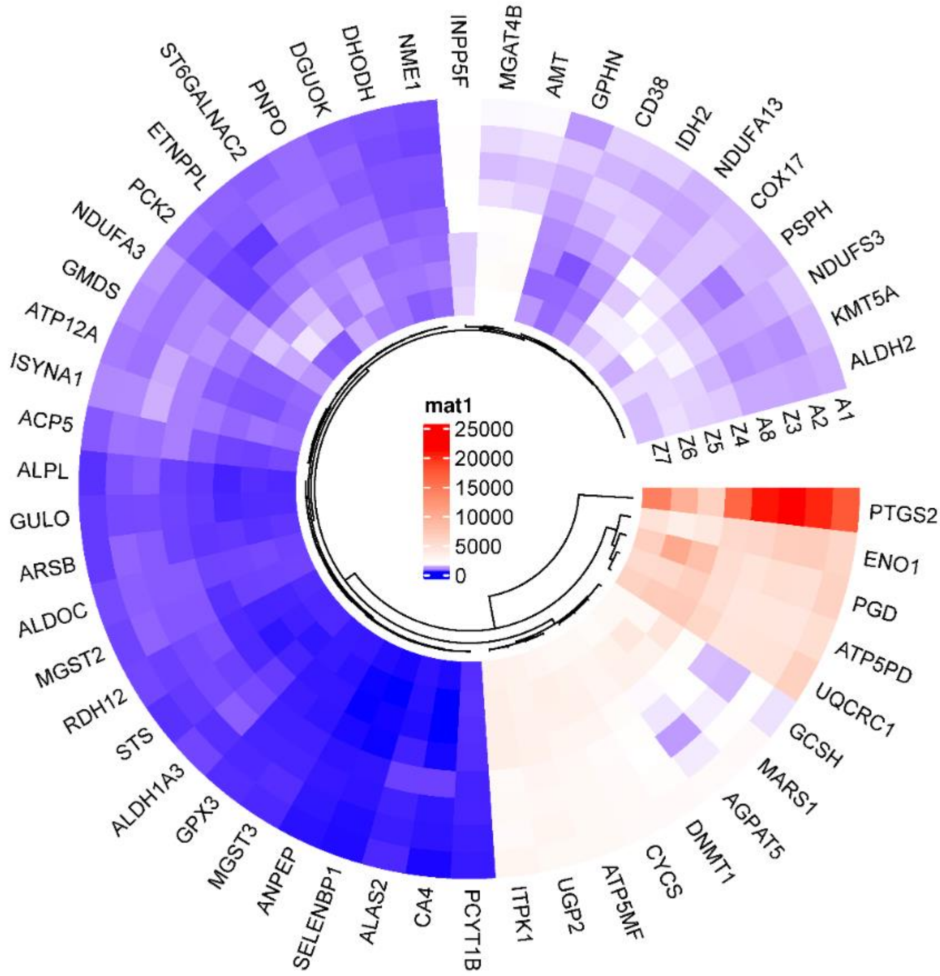
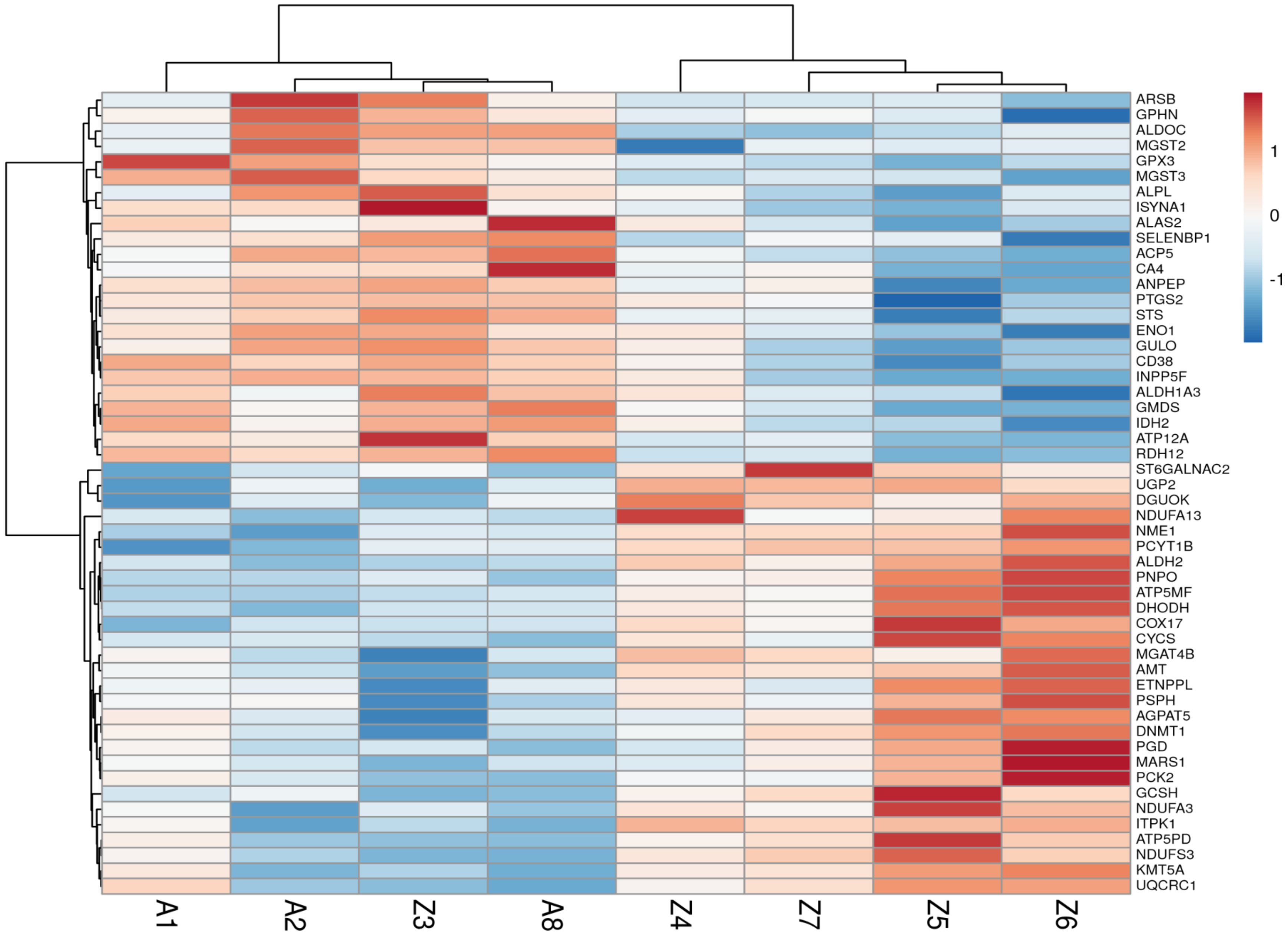


Supplemental Figure S2. (A). HTSeq Counts Assignments, (B) Flexbar: Processed reads, (C) STAR: Alignment Score.

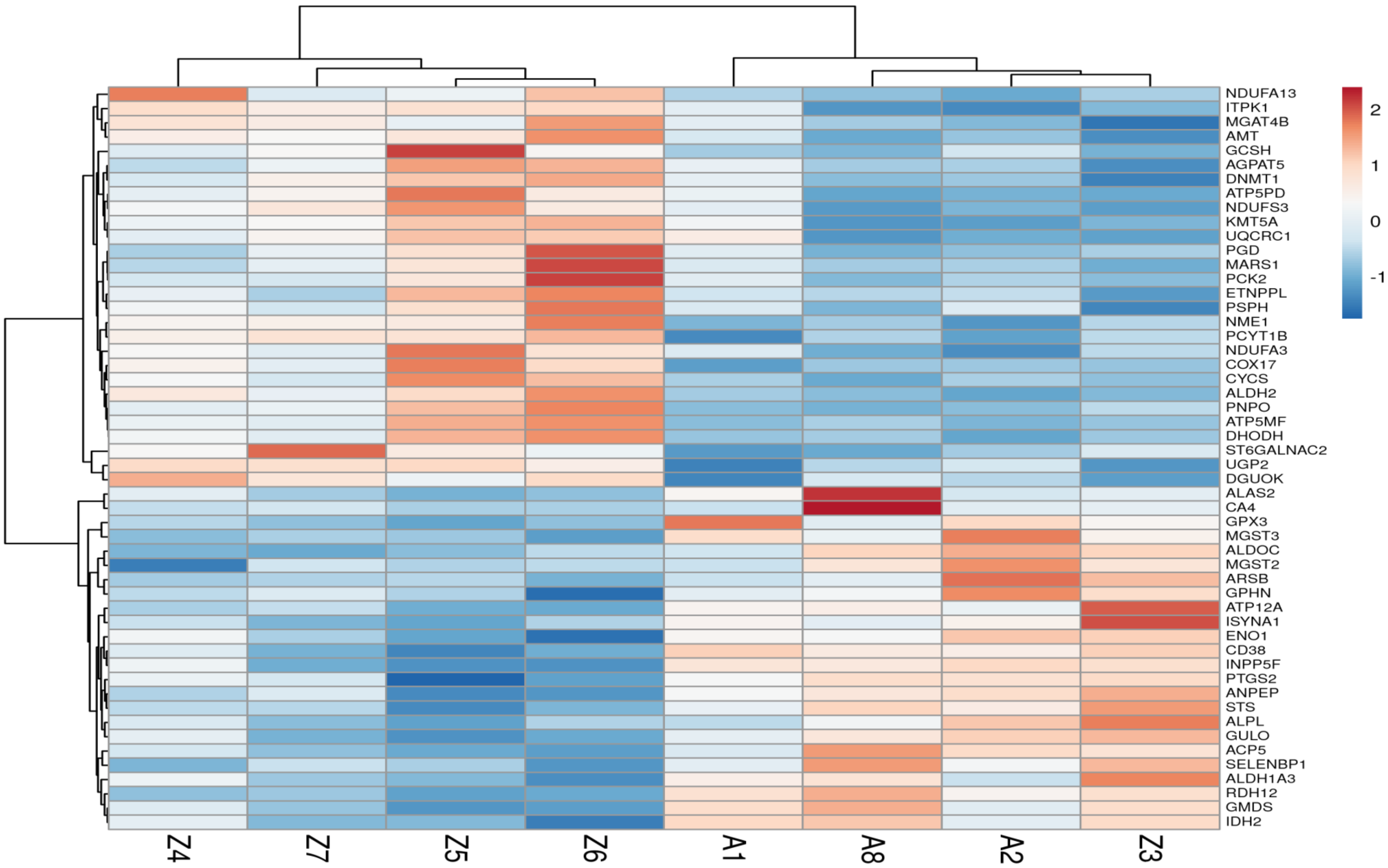


Supplemental Figure S3. The heatmap corresponding to the identified DEGs in metabolic pathways.

A

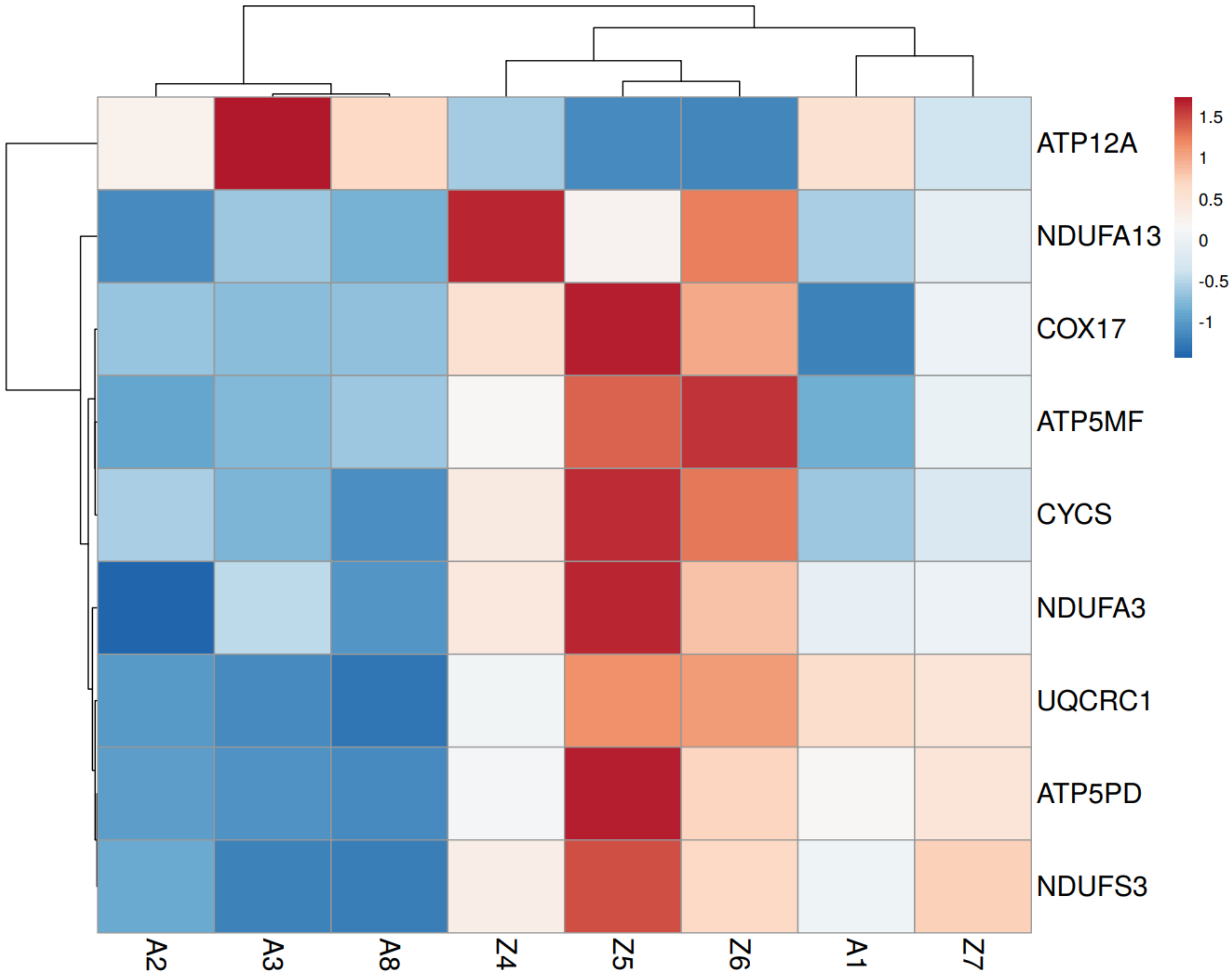

ARSB
GPHN
ALDOC
MGST2
GPX3
MGST3
ALPL
ISYNA1
ALAS2
SELENBP1
ACP5
CA4
ANPEP
PTGS2
STS
ENO1
GULO
CD38
INPP5F
ALDH1A3
GMDS
IDH2
ATP12A
RDH12
ST6GALNAC2
UGP2
DGUOK
NDUFA13
NME1
PCYT1B
ALDH2
PNPO
ATP5MF
DHODH
COX17
CYCS
MGAT4B
AMT
ETNPPL
PSPH
AGPAT5
DNMT1
PGD
MARS1
PCK2
GCSH
NDUFA3
ITPK1
ATP5PD
NDUFS3
KMT5A
UQCRC1
A1
A2
Z3
A8
Z4
Z7
Z5
Z6
1
0
-1

B



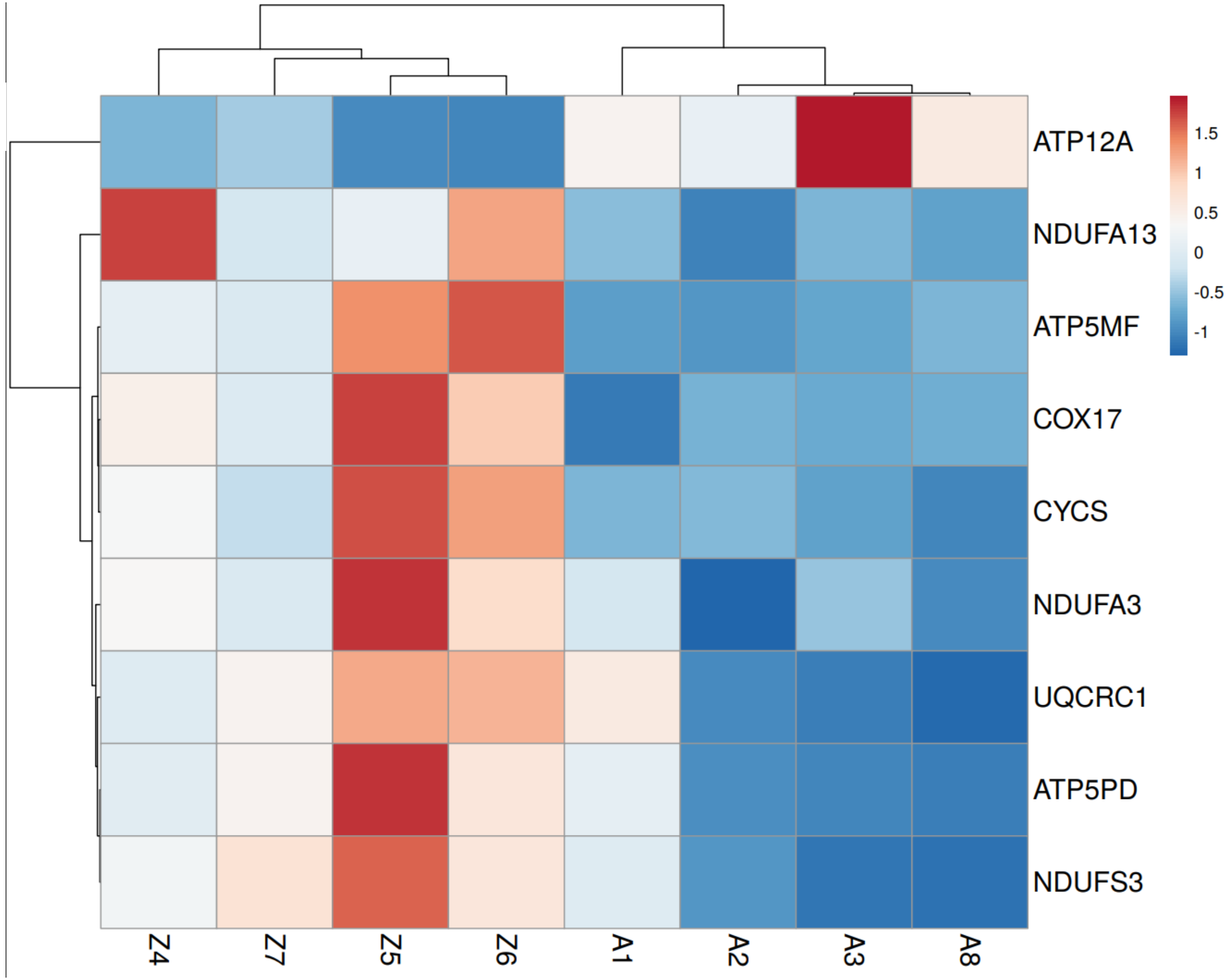
Supplemental Figure S4. (A, B) Heatmap showing identified DEGs associated with metabolic pathway.

A

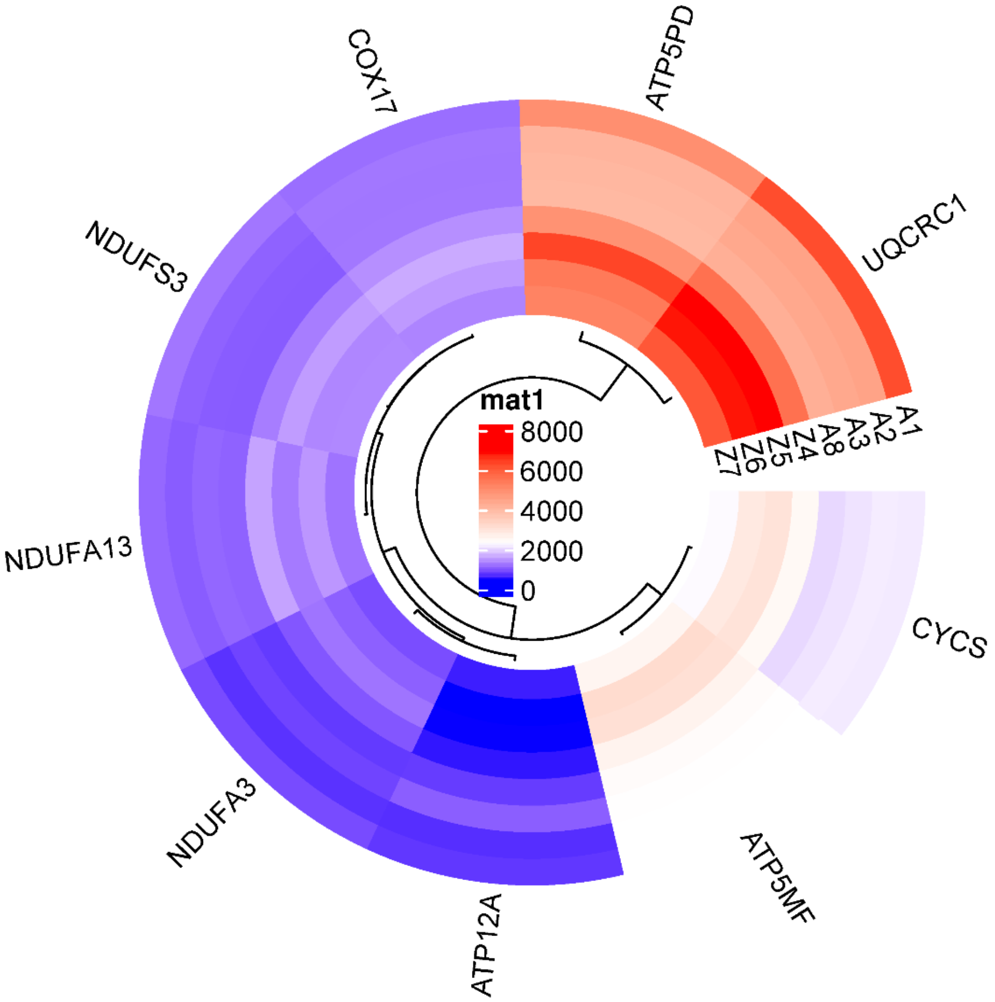


ATP12A
NDUFA13
COX17
ATP5MF
CYCS
NDUFA3
UQCRC1
ATP5PD
NDUFS3
A2
A3
A8
Z4
Z5
Z6
A1
Z7
1.5
1
0.5
0
-0.5
-1

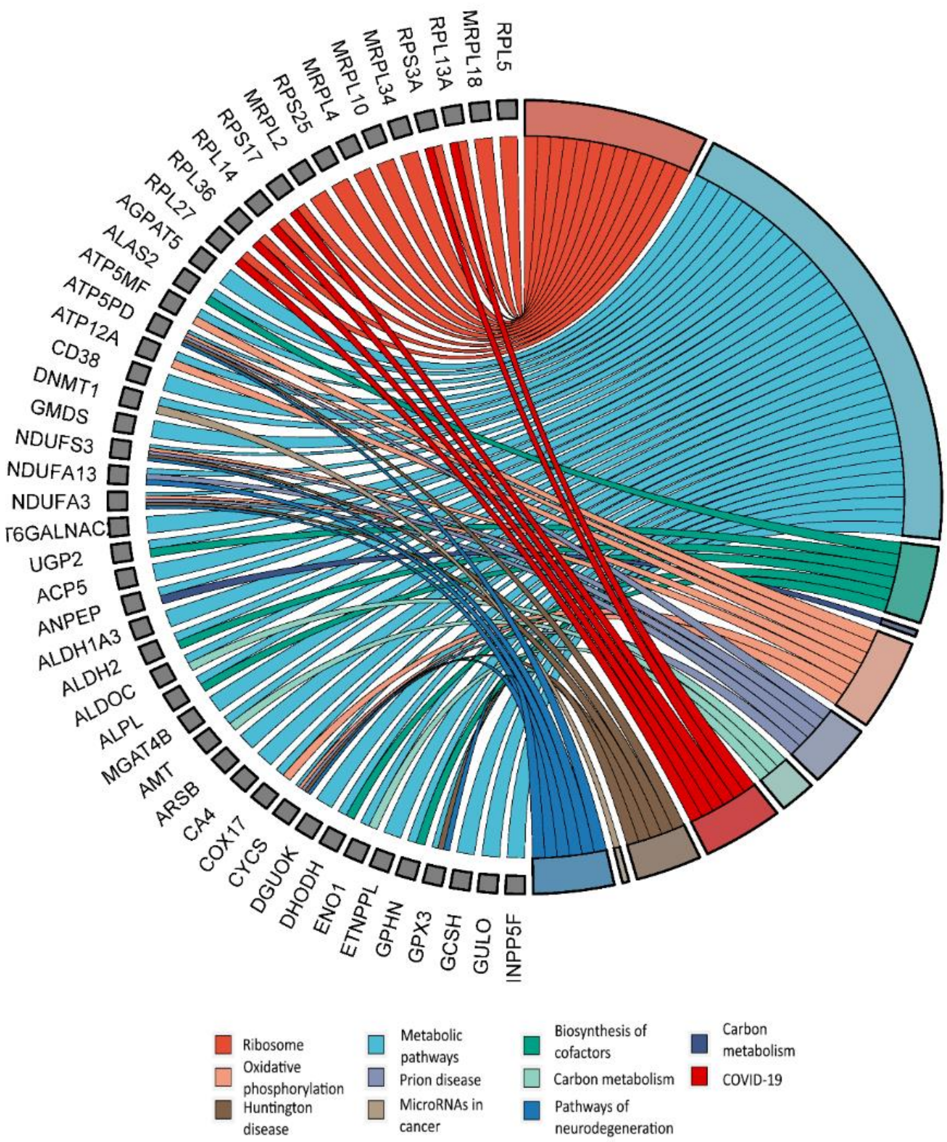
B



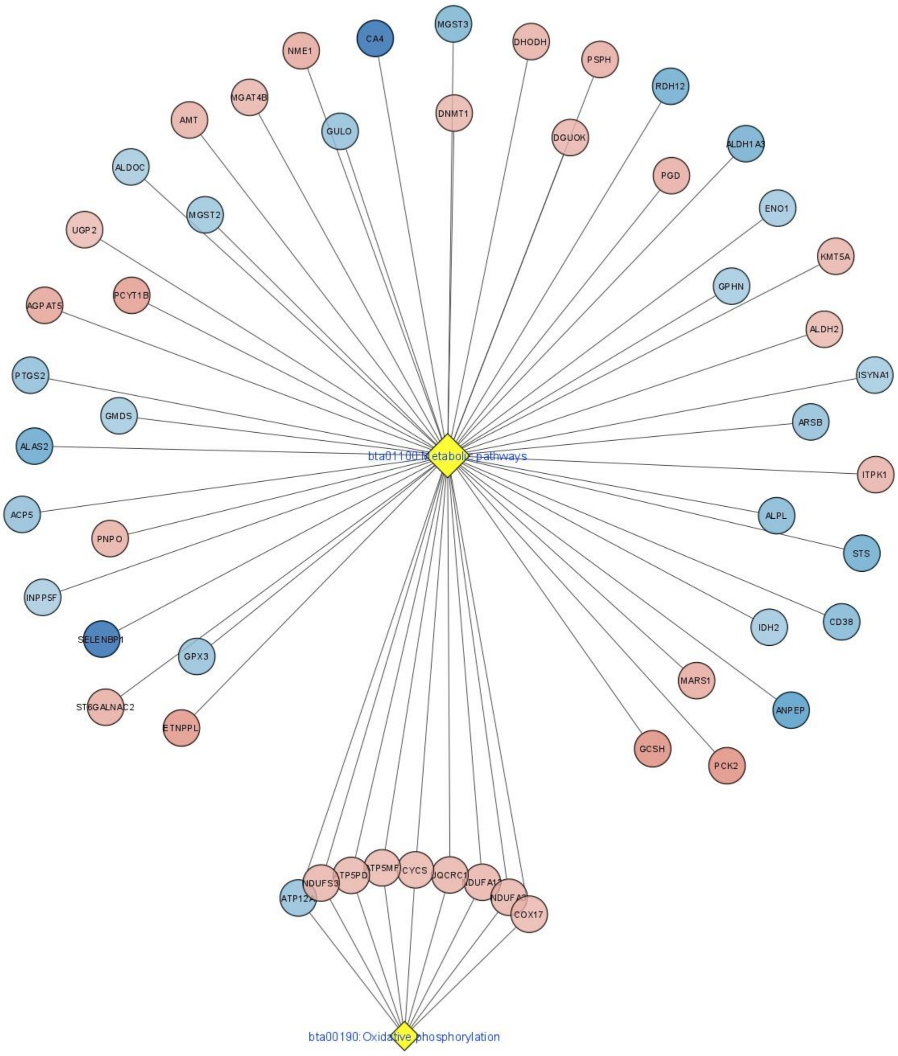
Supplemental Figure S5 (A,B). Heatmap showing identified DEGs associated with oxidative phoshorylation pathway.



Supplemental Figure S6. The heatmap corresponding to the identified DEGs in oxidative phoshorylation pathways.



Supplemental Figure S7. The chord diagram represents DEGs associated with the enriched KEGG pathways (Ribosome, Oxidative phosphorylation, Huntington disease, metabolic pathways, prion disease, MicroRnas in cancer, Biosynthesis of cofactors, Carbon metabolism, Pathways of neurodegeneration, Additional File 7. Carbon metabolism, COVID-19.



Supplemental Figure S8. Network chart showing DEGs involved in oxidative phosphorylation associated with metabolic pathways.

Supplementary Table S1. List of DEGs associated with the metabolic pathway

Kolumna1	gene	baseMear	log2FoldCl	Fc	FC2	znak zmia	lfcSE	stat	pvalue	padj	a1count.t	a2count.t	a3count.t	a8count.t	z4count.t	z5count.t	z6count.t	z7count.t	Gene nam	a - niedojr	z - dojrzałe
229	ENSBTAGC	1847,962	0,741286	1,671665	1,671665	up reg w d	0,23405	3,167214	0,001539	0,092333	1896,001	1398,882	872,0853	1366,066	1485,968	2973,553	2838,104	1953,04	AGPAT5	1383,259	2312,666
273	ENSBTAGC	113,1197	-1,24367	2,368009	2,368009	down reg	0,402915	-3,08669	0,002024	0,103298	144,4667	92,99218	112,3821	286,7405	111,9343	39,90693	52,83469	63,69994	ALAS2	159,1454	67,09396
387	ENSBTAGC	2529,366	0,296991	1,228579	1,228579	up reg w d	0,103243	2,876623	0,00402	0,14421	2248,698	2232,812	2276,862	2322,503	2563,944	2993,02	3087,307	2509,778	ATP5MF	2270,219	2788,512
285	ENSBTAGC	4795,947	0,361476	1,284739	1,284739	up reg w d	0,117834	3,067681	0,002157	0,105464	4875,999	4027,661	3960,346	3928,63	4820,475	6275,122	5333,662	5145,681	ATP5PD	4198,159	5393,735
39	ENSBTAGC	580,956	-0,73235	1,661338	1,661338	down reg	0,172588	-4,24331	2,2E-05	0,007868	661,5577	601,9494	949,6289	687,7961	464,7706	394,2026	388,335	499,4075	ATP12A	725,233	436,6789
17	ENSBTAGC	858,0662	-0,96168	1,947583	1,947583	down reg	0,189352	-5,07881	3,8E-07	0,000311	1206,546	1043,912	1215,975	1070,752	825,7182	406,856	538,0333	556,7375	CD38	1134,296	581,8362
389	ENSBTAGC	2056,343	0,362622	1,28576	1,28576	up reg w d	0,12634	2,870215	0,004102	0,14421	2067,368	1812,848	1550,873	1765,216	1978,317	2473,256	2558,08	2244,786	DNMT1	1799,076	2313,61
205	ENSBTAGC	630,4914	-0,46767	1,382877	1,382877	down reg	0,143816	-3,25187	0,001146	0,077921	747,2414	621,9477	749,5887	808,7796	618,0719	475,9632	483,4374	538,9015	GMDS	731,8894	529,0935
214	ENSBTAGC	1148,272	0,38295	1,304006	1,304006	up reg w d	0,119119	3,214849	0,001305	0,085299	1144,774	980,9175	933,8954	926,9053	1198,832	1446,383	1271,555	1282,917	NDUFS3	996,623	1299,922
269	ENSBTAGC	1145,283	0,393526	1,3136	1,3136	up reg w d	0,127354	3,090026	0,002001	0,102898	1029,2	931,9217	1018,182	983,1102	1506,246	1174,821	1402,761	1116,023	NDUFA13	990,6036	1299,963
401	ENSBTAGC	832,3881	0,407519	1,326403	1,326403	up reg w d	0,14221	2,865618	0,004162	0,14461	809,0134	625,9474	752,9602	675,412	887,3631	1124,207	962,472	821,7292	NDUFA3	715,8332	948,943
130	ENSBTAGC	653,1514	0,545887	1,459918	1,459918	up reg w d	0,156316	3,492201	0,000479	0,051735	462,2934	547,9539	620,3493	492,5077	712,9728	758,2317	675,4035	955,4991	ST6GALNA	530,7761	775,5268
407	ENSBTAGC	2653,034	0,250887	1,189938	1,189938	up reg w d	0,088025	2,850173	0,00437	0,148803	2274,603	2583,783	2320,691	2512,075	2915,158	2927,806	2802	2888,155	UGP2	2422,788	2883,28
52	ENSBTAGC	458,5592	-0,75035	1,682203	1,682203	down reg	0,184109	-4,07559	4,59E-05	0,012066	431,4074	603,9492	579,8917	685,8909	421,7814	310,4954	292,352	342,7057	ACP5	575,2848	341,8336
226	ENSBTAGC	44,45341	-1,42041	2,676624	2,676624	down reg	0,44573	-3,18671	0,001439	0,08883	51,80874	66,99437	77,54366	61,9207	30,82249	11,68008	14,08925	40,76796	ANPEP	64,56687	24,33994
242	ENSBTAGC	240,0641	-1,1241	2,17966	2,17966	down reg	0,356531	-3,15289	0,001617	0,092853	316,8304	187,9842	466,3858	345,8033	261,18	125,5608	65,16279	151,6059	ALDH1A3	329,2509	150,8774
282	ENSBTAGC	1080,58	0,321534	1,249659	1,249659	up reg w d	0,104644	3,072633	0,002122	0,104463	987,355	930,9218	959,7433	965,963	1176,121	1221,541	1311,181	1091,817	ALDH2	960,9958	1200,165
357	ENSBTAGC	366,19	-0,45546	1,371217	1,371217	down reg	0,155484	-2,9293	0,003397	0,131529	340,7421	467,9607	443,9094	442,0186	301,7359	308,5487	332,8586	291,7457	ALDOC	423,6577	308,7222
183	ENSBTAGC	252,346	-0,93673	1,914181	1,914181	down reg	0,282154	-3,31992	0,0009	0,068498	194,2828	391,9671	460,7667	280,0721	232,7909	120,6941	184,0408	154,1539	ALPL	331,7722	172,9199
326	ENSBTAGC	1484,295	0,374147	1,296073	1,296073	up reg w d	0,12569	2,976734	0,002913	0,123509	1480,534	1273,893	1095,726	1321,293	1691,181	1501,863	1879,154	1630,718	MGAT4B	1292,861	1675,729
67	ENSBTAGC	1560,765	0,488206	1,402699	1,402699	up reg w d	0,123792	3,943756	8,02E-05	0,016198	1492,49	1326,888	1144,05	1233,651	1739,037	1788,025	2090,493	1671,486	AMT	1299,27	1822,26
208	ENSBTAGC	296,9889	-0,7497	1,681444	1,681444	down reg	0,231953	-3,23212	0,001229	0,082308	250,0768	505,9575	439,4141	294,3615	224,6797	238,4683	191,0855	231,8678	ARSB	372,4525	221,5253
65	ENSBTAGC	64,56742	-2,64277	6,245285	6,245285	down reg	0,665176	-3,97303	7,1E-05	0,015282	28,89333	60,99487	65,18163	289,5984	23,52242	7,786719	6,164047	34,39797	CA4	111,1671	17,96779
399	ENSBTAGC	1262,096	0,307972	1,237967	1,237967	up reg w d	0,107409	2,86729	0,00414	0,144542	1073,039	1149,903	1141,802	1147,915	1345,645	1559,29	1423,014	1256,163	COX17	1128,165	1396,028
299	ENSBTAGC	2300,23	0,345309	1,270423	1,270423	up reg w d	0,113591	3,039948	0,002366	0,109932	2074,342	2086,825	2017,259	1928,115	2416,321	2918,073	2763,254	2197,648	CYCS	2026,635	2573,824
429	ENSBTAGC	545,5426	0,380154	1,301481	1,301481	up reg w d	0,134689	2,822467	0,004766	0,15494	422,4405	501,9578	446,157	525,8497	669,1724	556,7504	630,494	611,5194	DGUOK	474,1012	616,984
227	ENSBTAGC	460,0694	0,480779	1,395497	1,395497	up reg w d	0,150894	3,186193	0,001442	0,08883	388,5655	358,9698	394,4612	395,3399	480,993	591,7906	618,1659	452,2696	DHODH	384,3341	535,8048
139	ENSBTAGC	5148,209	-0,52326	1,437199	1,437199	down reg	0,151314	-3,4581	0,000544	0,054167	5647,152	6603,445	6487,82	5549,048	5443,413	3861,239	3198,26	4395,296	ENO1	6071,866	4224,552
178	ENSBTAGC	610,1098	0,944947	1,925119	1,925119	up reg w d	0,283113	3,337707	0,000845	0,065802	500,1536	474,9601	244,993	448,6869	632,6721	1011,3	1143,871	424,2416	ETNPPL	417,1984	803,0212
385	ENSBTAGC	852,6017	-0,47869	1,393477	1,393477	down reg	0,166133	-2,88136	0,00396	0,143298	849,8626	1182,901	1035,039	902,137	761,6398	738,7649	527,4663	823,0032	GPHN	992,4849	712,7186
384	ENSBTAGC	110,8025	-0,70502	1,630172	1,630172	down reg	0,244605	-2,8823	0,003948	0,143243	171,3674	145,9877	123,6203	108,5994	93,27857	74,94717	84,53551	84,08392	GPX3	137,3937	84,21129
20	ENSBTAGC	1959,831	1,029513	2,041335	2,041335	up reg w d	0,213712	4,817289	1,46E-06	0,001014	1336,068	1658,861	1058,64	1101,236	1878,549	3997,507	2340,577	2307,212	GCSH	1288,701	2630,961
136	ENSBTAGC	244,0507	-0,74286	1,67349	1,67349	down reg	0,214006	-3,47121	0,000518	0,05308	244,0989	330,9722	346,1369	301,9825	245,7688	145,0276	166,4293	171,9898	GULO	305,7976	182,3039
192	ENSBTAGC	1479,989	-0,375	1,296835	1,296835	down reg	0,11374	-3,29694	0,000977	0,07093	1653,894	1710,856	1687,979	1633,754	1522,469	1185,528	1190,542	1254,889	INPP5F	1671,621	1288,357
271	ENSBTAGC	686,3971	-0,46282	1,378229	1,378229	down reg	0,149702	-3,09158	0,001991	0,102898	749,2341	758,9362	994,5818	681,1277	628,6165	524,6302	604,9572	549,0935	ISYNA1	795,9699	576,8243
47	ENSBTAGC	2693,001	0,454178	1,370002	1,370002	up reg w d	0,109334	4,154033	3,27E-05	0,009684	2673,132	2040,828	2274,614	2102,446	3155,249	3116,634	3185,932	2995,171	ITPK1	2272,755	3113,247
134	ENSBTAGC	970,2159	-0,51479	1,428784	1,428784	down reg	0,147852	-3,48178	0,000498	0,051791	1194,59	964,9189	1181,136	1226,03	975,775	781,5919	651,6279	786,0573	IDH2	1141,669	798,763
304	ENSBTAGC	1016,567	0,414664	1,332988	1,332988	up reg w d	0,137149	3,023455	0,002499	0,114162	1057,098	797,9329	846,2374	784,964	1046,342	1241,008	1278,6	1080,351	KMT5A	871,558	1161,575
260	ENSBTAGC	2164,235	0,628047	1,545472	1,545472	up reg w d	0,201324	3,119592	0,001811	0,09705	2030,504	1694,858	1410,396	1666,143	1768,237	2757,472	3798,814	2187,456	MARS1	1700,475	2627,995
365	ENSBTAGC	338,7736	-0,59804	1,513656	1,513656	down reg	0,204644	-2,92233	0,003474	0,132962	302,8819	498,9581	414,69	414,3924	195,4794	285,1886	294,1131	304,4857	MGST2	407,7306	269,8167
81	ENSBTAGC	103,7236	-1,0161	2,022443	2,022443	down reg	0,265326	-3,82962	0,000128	0,021821	143,4704	179,9849	123,6203	107,6468	69,75615	73,00049	54,59585	77,71393	MGST3	138,6806	68,7666
45	ENSBTAGC	463,4019	0,640364	1,558722	1,558722	up reg w d	0,153993	4,158397	3,2E-05	0,009684	354,6906	311,9738	398,9565	383,9084	514,2488	539,2303	678,0452	526,1615	NME1	362,3823	564,4215
96	ENSBTAGC	141,4711	0,827204	1,774243	1,774243	up reg w d	0,221226	3,739178	0,000185	0,026795	79,70575	91,99227	120,2489	116,2204	165,4681	178,1212	201,6524	178,3598	PCYT1B	102,0418	180,9004
151	ENSBTAGC	595,12	1,012617	2,017568	2,017568	up reg w d	0,29576	3,423782	0,000618	0,056984	566,9071	399,9664	310,1747	301,0299	499,6487	843,8856	1343,762	495,5855	PCK2	394,5195	795,7206
340	ENSBTAGC	6142,194	0,555461	1,469638	1,469638	up reg w d	0,187708	2,959185	0,003085	0,126977	6032,729	4652,609	4939,194	4272,529	4964,042	7864,586	10131,05	6280,814	PGD	4974,265	7310,124
361	ENSBTAGC	1087,49	0,553455	1,467596	1,467596	up reg w d	0,189068	2,927273	0,003419	0,131977	1023,223	1042,912	654,0639	805,9218	1154,221	1367,542	1663,412	988,6231	PSPH	881,5302	1293,45
113	ENSBTAGC	15595,88	-0,78584	1,724093	1,724093	down reg	0,218963	-3,5889	0,000332	0,040586	17194,52	20153,31	20982,87	20634,84	16128,27	6112,574	9542,826	14017,81	PTGS2	19741,38	11450,37
181	ENSBTAGC	673,5466	0,477156	1,391997	1,391997	up reg w d	0,143173	3,332719	0,00086	0,066202	556,9439	557,9531	602,3682	536,3286	669,9835	857,5124	919,3237	687,9594	PNPO	563,3984	783,6947
3	ENSBTAGC	234,31	-1,17375	2,255966	2,255966	down reg	0,187188	-6,2704	3,6E-10	1,67E-06	320,8156	276,9767	324,7843	375,3347	155,7347	123,6142	130,3256	166,8938	RDH12	324,4779	144,1421
177	ENSBTAGC	34,23001	-1,60396	3,039774	3,039774	down reg	0,480174	-3,34038	0,000837	0,065802	33,87494	39,99664	62,93399	68,58909	14,60012	20,44014	7,925204	25,47998	SELENBP1	51,34866	17,11136
29	ENSBTAGC	190,6643	-1,1771	2,261217	2,261217	down reg	0,272254	-4,32353	1,54E-05	0,007377	196,2754	244,9794	330,4034	285,7879	148,4346	67,16045	108,3111	143,9619	STS	264,3615	116,967
424	ENSBTAGC	5529,244	0,397085	1,316845	1,316845	up reg w d	0,140604	2,824142	0,004741	0,15469	6176,199	4433,627	4322,216	4160,119	5515,603	6861,072	6776,049	5989,068	UQCRC1	4773,04	6285,448

Supplementary Table S2. List of DEGs associated with the oxidative phosphorylation pathway.

	gene	baseMean	log2FoldCl	Fc	FC	znak zmia	lfcSE	stat	pvalue	padj	a1count.t	a2count.t	a3count.t	a8count.t	z4count.tx	z5count.tx	z6count.tx	z7count.tx	Gene nam	a - niedojr	z - dojrzałe
387	ENSBTAGC	2529,366	0,296991	1,228579	1,228579	up reg w d	0,103243	2,876623	0,00402	0,14421	2248,698	2232,812	2276,862	2322,503	2563,944	2993,02	3087,307	2509,778	**ATP5MF**	2270,219	2788,512
285	ENSBTAGC	4795,947	0,361476	1,284739	1,284739	up reg w d	0,117834	3,067681	0,002157	0,105464	4875,999	4027,661	3960,346	3928,63	4820,475	6275,122	5333,662	5145,681	**ATP5PD**	4198,159	5393,735
39	ENSBTAGC	580,956	-0,73235	1,661338	1,661338	down reg	0,172588	-4,24331	2,2E-05	0,007868	661,5577	601,9494	949,6289	687,7961	464,7706	394,2026	388,335	499,4075	**ATP12A**	725,233	436,6789
214	ENSBTAGC	1148,272	0,38295	1,304006	1,304006	up reg w d	0,119119	3,214849	0,001305	0,085299	1144,774	980,9175	933,8954	926,9053	1198,832	1446,383	1271,555	1282,917	**NDUFS3**	996,623	1299,922
269	ENSBTAGC	1145,283	0,393526	1,3136	1,3136	up reg w d	0,127354	3,090026	0,002001	0,102898	1029,2	931,9217	1018,182	983,1102	1506,246	1174,821	1402,761	1116,023	**NDUFA13**	990,6036	1299,963
401	ENSBTAGC	832,3881	0,407519	1,326403	1,326403	up reg w d	0,14221	2,865618	0,004162	0,14461	809,0134	625,9474	752,9602	675,412	887,3631	1124,207	962,472	821,7292	**NDUFA3**	715,8332	948,943
424	ENSBTAGC	5529,244	0,397085	1,316845	1,316845	up reg w d	0,140604	2,824142	0,004741	0,15469	6176,199	4433,627	4322,216	4160,119	5515,603	6861,072	6776,049	5989,068	**UQCRC1**	4773,04	6285,448
399	ENSBTAGC	1262,096	0,307972	1,237967	1,237967	up reg w d	0,107409	2,86729	0,00414	0,144542	1073,039	1149,903	1141,802	1147,915	1345,645	1559,29	1423,014	1256,163	**COX17**	1128,165	1396,028
299	ENSBTAGC	2300,23	0,345309	1,270423	1,270423	up reg w d	0,113591	3,039948	0,002366	0,109932	2074,342	2086,825	2017,259	1928,115	2416,321	2918,073	2763,254	2197,648	**CYCS**	2026,635	2573,824

Note: Fc=fold change, padj=p adjusted

Supplementary Table S3. List of DEGs involved in pathways for the KEGG enrichment analysis.

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
KEGG_PATHWAY	bta03010:Ribosome	13	3.258145363408521	2.2261005159093366E-4	RPL5, MRPL18, RPL13A, RPS3A, MRPL34, MRPL10, MRPL4, RPS25, MRPL2, RPS17, RPL14, RPL36, RPL27	192	168	9038	3.6425471230158726	0.05708165982399771	0.058769053620006485	0.058769053620006485
KEGG_PATHWAY	bta01100:Metabolic pathways	52	13.032581453634084	0.0012649076387564813	**AGPAT5, ALAS2, ATP5MF, ATP5PD, ATP12A, CD38, DNMT1, GMDS, NDUFS3, NDUFA13, NDUFA3, ST6GALNAC2, UGP2, ACP5, ANPEP, ALDH1A3, ALDH2, ALDOC, ALPL, MGAT4B, AMT, ARSB, CA4, COX17, CYCS, DGUOK, DHODH, ENO1, ETNPPL, GPHN, GPX3, GCSH, GULO, INPP5F, ISYNA1, ITPK1, IDH2, KMT5A, MARS1, MGST2, MGST3, NME1, PCYT1B, PCK2, PGD, PSPH, PTGS2, PNPO, RDH12, SELENBP1, STS, UQCRC1**	192	1607	9038	1.5232057664384981	0.2840514654714916	0.16696780831585553	0.16696780831585553
KEGG_PATHWAY	bta01240:Biosynthesis of cofactors	10	2.506265664160401	0.0048739679699571666	GULO, ALAS2, UGP2, ALDH2, RDH12, PNPO, ALPL, GPHN, DHODH, NME1	192	152	9038	3.096902412280702	0.724694394290895	0.4289091813562307	0.4289091813562307
KEGG_PATHWAY	bta00480:Glutathione metabolism	6	1.5037593984962405	0.00977742144109	GPX3, MGST3, ANPEP, IDH2, MGST2, PGD	192	62	9038	4.555443548387097	0.9252750361186063	0.5703305394639948	0.5703305394639948
KEGG_PATHWAY	bta00190:Oxidative phosphorylation	9	2.2556390977443606	0.0108017147625756 6	NDUFA13, ATP5PD, NDUFA3, COX17, NDUFS3, UQCRC1, CYCS, ATP12A, ATP5MF	192	143	9038	2.9626311188811187	0.9431401539695495	0.5703305394639948	0.5703305394639948
KEGG_PATHWAY	bta05020:Prion disease	13	3.258145363408521	0.016409826864334887	RYR2, NDUFA13, ATP5PD, NDUFA3, HSPA2, TUBB4A, C8A, TUBA4A, IL6, TUBA1A, NDUFS3, UQCRC1, CYCS	192	282	9038	2.1700280732860517	0.987325121078983	0.722032382030735	0.722032382030735
KEGG_PATHWAY	bta01200:Carbon metabolism	7	1.7543859649122806	0.031166590739815394	GCSH, IDH2, AMT, ALDOC, ENO1, PGD, PSPH	192	112	9038	2.942057291666667	0.9997657043936397	1.0	1.0
KEGG_PATHWAY	bta05171:Coronavirus disease - COVID-19	12	3.007518796992481	0.0367596350753311	RPS25, RPL5, RPS17, IL6, STAT3, RPL14, RPL36, RPL13A, RPS3A, ISG15, RPL27, C8A	192	282	9038	2.0031028368794326	0.9999491894589877	1.0	1.0
KEGG_PATHWAY	bta05410:Hypertrophic cardiomyopathy	6	1.5037593984962405	0.04501079440062588	MYBPC3, RYR2, IL6, TPM3, TPM1, ITGB8	192	92	9038	3.0699728260869565	0.9999947568273908	1.0	1.0
KEGG_PATHWAY	bta00260:Glycine, serine and threonine metabolism	4	1.0025062656641603	0.06907580778766026	GCSH, ALAS2, AMT, PSPH	192	45	9038	4.184259259259259	0.9999999937858808	1.0	1.0
KEGG_PATHWAY	bta05016:Huntington disease	12	3.007518796992481	0.0753237395905246	NDUFA13, ATP5PD, TUBA1A, BDNF, GPX3, NDUFA3, NDUFS3, UQCRC1, CYCS, AP2A1, TUBB4A, TUBA4A	192	319	9038	1.7707680250783697	0.9999999989497736	1.0	1.0
KEGG_PATHWAY	bta05206:MicroRNAs in cancer	11	2.756892230576441	0.09201259610208914	NOTCH3, ZEB2, DNMT1, TNN, STMN1, STAT3, TPM1, PTGS2, SLC7A1, SOX4, CDC25A	192	293	9038	1.7672426052332193	0.9999999999914282	1.0	1.0
KEGG_PATHWAY	bta05022:Pathways of neurodegeneration - multiple diseases	16	4.010025062656641	0.09491912472092189	DUFA13, ATP5PD, BDNF, GPX3, NDUFA3, UBE2G2, PTGS2, TUBB4A, TUBA4A, IL6, TUBA1A, NDUFS3, UQCRC1, CY(	192	490	9038	1.5370748299319728	0.9999999999963232	1.0	1.0

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2) **Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji do rozprawy doktorskiej pani mgr Mileny Traut**

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Zakład Pielęgniarstwa Położniczo-Ginekologicznego

Wydział Nauk o Zdrowiu

z Instytutem Medycyny Morskiej i Tropikalnej

Gdański Uniwersytet Medyczny

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2) **Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji do rozprawy doktorskiej pani mgr Mileny Traut**

Z poważaniem,

prof. dr hab. Krzysztof Łukaszuk

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Olsztyn, 25.08.2024

Oświadczenie

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Z poważaniem,

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Z poważaniem,

dr n. med. Dorota Boruszewska

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Z poważaniem,

mgr Sandra Gąsiorowska

Sandra Gąsiorowska

prof. dr hab. Krzysztof Łukaszuk
Zakład Pielęgniarstwa Położniczo-Ginekologicznego
Wydział Nauk o Zdrowiu
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Z poważaniem,

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prof. dr hab. Katarzyna Ropka-Molik
Zakład Biologii Molekularnej Zwierząt
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Sarego 2, 31-047, Kraków

Kraków, 25.08.2024

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2. **Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji do rozprawy doktorskiej pani mgr Mileny Traut**

Katarzyna Ropka-Molik

Z poważaniem,

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Tomasz Szmatoła

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