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POPRAWA PARAMETRÓW JAKOŚCI SUROWCA I WSKAŹNIKÓW PRODUKCYJNYCH W ASPEKCIE EKOINTENSYFIKACJI PRODUKCJI KARPIA (*CYPRINUS CARPIO* L.)

Improvement of meat quality parameters and production indicators in the aspect of common carp (Cyprinus carpio L.) production ecointensification

Rozprawa doktorska

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Katedra Technologii Mięsa

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„Nie umiera ten, kto trwa w pamięci żywych”

Kochanej Mamie

PODZIĘKOWANIA

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WYKAZ SPÓJNYCH TEMATYCZNIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ:

P1. Eljasik P., Panicz R., Sobczak M., Sadowski J. 2022. Key performance indicators of common carp (*Cyprinus carpio* L.) wintering in a pond and RAS under different feeding schemes. Sustainability 14(7), 3724. Lista $MNiSW_{2021}$ = 100 pkt., IF_{2020} = 3,251. Udział doktoranta w pracy 60%.

P2. Eljasik P., Panicz R., Sobczak M., Sadowski J., Barbosa V., Marques A., Dias J. 2020. Plasma biochemistry, gene expression and liver histomorphology in common carp (*Cyprinus carpio*) fed with different dietary fat sources. Food and Chemical Toxicology 140, 111300. Lista $MNiSW_{2020}$ = 100 pkt., IF_{2020} = 6,023. Udział doktoranta w pracy 60%.

P3. Sobczak M., Panicz R., **Eljasik P.**, Sadowski J., Tórz A., Żochowska-Kujawska J., Barbosa V., Domingues V., Marques A., Dias J. 2020. Quality improvement of common carp (*Cyprinus carpio* L.) meat fortified with n-3 PUFA. Food and Chemical Toxicology 139, 111261. Lista $MNiSW_{2020}$ = 100 pkt., IF_{2020} = 6,023. Udział doktoranta w pracy 40%.

P4. Eljasik P., Panicz R., Sobczak M., Sadowski J., Tórz A., Barbosa V., Marques A., Dias J. 2021. Structural and molecular indices in common carp (*Cyprinus carpio* L.) fed n-3 PUFA enriched diet. Food and Chemical Toxicology 151, 112146. Lista $MNiSW_{2020}$ = 100 pkt., IF_{2020} = 6,023. Udział doktoranta w pracy 60%.

P5. Sobczak M., Panicz R., **Eljasik P**., Sadowski J., Tórz A., Żochowska-Kujawska J., Barbosa V., Dias J., Marques A. 2021. Nutritional value and sensory properties of common carp (*Cyprinus carpio* L.) fillets enriched with sustainable and natural feed ingredients. Food and Chemical Toxicology 152, 112197. Lista $MNiSW_{2020}$ = 100 pkt., IF_{2020} = 6,023. Udział doktoranta w pracy 40%.

Łączna ilość punktów = 500, IF = 27,343

STRESZCZENIE

Aktualna sytuacja demograficzna na świecie wskazuje na potrzebę intensyfikacji obecnych systemów produkcji żywności w celu zapewnienia bezpieczeństwa żywnościowego bez wywierania presji na środowisko naturalne. Akwakultura to jeden z sektorów produkcji żywności, który rozwija się niezwykle dynamicznie jednocześnie starając się postępować zgodnie z zasadami zrównoważonego rozwoju. W światowej produkcji żywności pochodzenia wodnego ważną rolę odgrywa akwakultura słodkowodna, której jednym z kluczowych gatunków jest karp (*Cyprinus carpio*). Ze względu na popularność tego gatunku, zwłaszcza w Europie centralnej i wschodniej, oraz plastyczność fenotypową, karp wydaje się idealnym gatunkiem do ekointensyfikacji produkcji i biofortyfikacji w celu pokrycia niedoborów wielonienasyconych kwasów tłuszczowych (omega-3 PUFA) populacji ludzkiej. Dlatego celem badań, wchodzących w skład rozprawy doktorskiej, było skrócenie cyklu produkcyjnego karpia oraz biofortyfikacja mięsa karpia w omega-3 PUFA, poprzez zastosowanie w żywieniu ryb wzbogaconych pasz, bez negatywnego wpływu na strukturę, teksturę oraz właściwości sensoryczne mięsa.

Przeprowadzone badania wykazały, że zastosowanie zimowania w recyrkulacyjnych systemach akwakultury (RAS) może doprowadzić do skrócenia cyklu hodowlanego z 33 do 19 miesięcy. Zaproponowana metoda pozwala na uzyskanie znacznie wyższej przeżywalności ryb w porównaniu do tradycyjnego zimowania w zimochowach, a dzięki monitorowaniu markerów molekularnych (np. genów związanych ze stresem oksydacyjnym) daje hodowcom możliwość wdrożenia żywienia interwencyjnego. Wykorzystanie zaproponowanego rozwiązania może przełożyć się na intensyfikację produkcji, a tym samym na zwiększenie bezpieczeństwa żywnościowego.

Wykazano również, że zastosowanie pasz wzbogaconych naturalnymi komponentami, tj. mikroglonami *Schizochytrium* sp. oraz olejem z produktów ubocznych przetwórstwa łososia atlantyckiego (*Salmo salar*), istotnie poprawia wartość żywieniową mięsa karpia. Mięso ryb biofortyfikowanych charakteryzowało się istotnie wyższą zawartością kwasów eikozapentaenowego (EPA) i dokozaheksaenowego (DHA) oraz lepszą jakością tłuszczu mięśniowego w porównaniu z wariantem kontrolnym. Zwiększona podaż EPA i DHA w paszy wpłynęła również na stymulację metabolizmu kwasów tłuszczowych na poziomie transkrypcyjnym, co było widoczne zwłaszcza w przypadku zastosowania mikroglonów w paszy. Ponadto, badanie profilu lipidowego krwi ryb potwierdziło intensywny transport

kwasów tłuszczowych we krwi karpi. Wzbogacenie pasz w naturalne komponenty nie wpłynęło negatywnie na parametry dobrostanu ryb (np. integralność jelita), co przełożyło się również na charakterystykę jakościową surowca. Badania wykazały, że parametry jakości (tekstura, cechy sensoryczne, barwa) filetów karpi żywionych paszami wzbogaconymi nie różniły się istotnie od filetów ryb żywionych paszą kontrolną.

Wszystkie powyższe aspekty potwierdzają możliwość przeprowadzenia skutecznej ekointensyfikacji i biofortyfikacji karpi, które istotnie poprawiają wartość odżywczą mięsa bez negatywnych zmian jakości surowca i kondycji ryby. Uzyskany produkt (ryba i jej mięso) powinien być w pełni akceptowalny przez dotychczasowych konsumentów, jak i zyskać uznanie nowych, co może poprawić bezpieczeństwo żywnościowe w Europie i uzupełnić niedobory EPA i DHA w diecie.

SUMMARY

The current global demographic situation indicates the need of current food production systems intensification to ensure food security without negatively affecting the environment. Aquaculture is one of the food production systems that is developing rapidly, while trying to follow the principles of sustainable development. Freshwater aquaculture plays an important role in the global production of aquatic food, of which common carp (*Cyprinus carpio*) is one of the key species. Due to the popularity, especially in Central and Eastern Europe, and its phenotypic plasticity, carp seems to be a potential candidate for eco-intensification of production and biofortification to cover the polyunsaturated fatty acids (omega-3 PUFA) deficiencies of the human population. Therefore, the aim of the studies included in the doctoral dissertation was to shorten the carp production cycle and biofortify carp meat with omega-3 PUFA, using enriched feeds, without adversely affecting the structure, texture and sensory properties of flesh.

The research has shown that the use of wintering in recirculating aquaculture systems (RAS) may shorten the production cycle from 33 to 19 months. The proposed method allowed to obtain a much higher survival rate of fish compared to traditional wintering in wintering ponds, and thanks to the monitoring of molecular markers (e.g. genes related to oxidative stress), gives farmers the possibility of applying intervention nutrition. The use of the proposed solution may intensify the production, and thus increase food security.

The research also showed that use of feed enriched with microalgae *Schizochytrium* sp. and with oil from Atlantic salmon (*Salmo salar*) processing by-products significantly improves the nutritional value of carp flesh. The flesh of biofortified fish had significantly higher content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and better fat quality compared to the control variant. The increased supply of EPA and DHA in the feed also stimulated the metabolism of fatty acids at the transcriptional level, which was visible especially in the case of microalgae utilization. Moreover, plasma lipid profile confirmed the intensive transport of fatty acids in the fish organisms. Enriching feeds with natural components did not adversely affect the welfare parameters of the fish (e.g. gut integrity), which also translated into the quality characteristics of the filets. The research showed that the quality parameters (texture, sensory traits, colour) of filets of fish fed enriched diets did not differ significantly from filets of fish fed a control diet.

The above aspects indicate the possibility of successful eco-intensification and biofortification of carp, which significantly improves nutritional value without compromising flesh quality and fish health. The obtained product (fish and its meat) should be fully acceptable to existing consumers and gain recognition of new ones, which may improve food security in Europe and cover EPA and DHA deficiencies in diet.

1. PRZEGLĄD PIŚMIENNICTWA

Wyznaczone w 2015 r. przez Organizację Narodów Zjednoczonych (United Nations 2015) Cele Zrównoważonego Rozwoju (ang. Sustainable Development Goals, SDGs) stały się podstawą wspólnego dążenia świata do zaspokojenia potrzeb obecnej populacji bez narażania na niedobory przyszłych pokoleń (Sachs i in. 2021). Wobec nieustannie rosnącej liczby ludności jednym z kluczowych SDG (cel nr 2) jest osiągnięcie bezpieczeństwa żywnościowego (ang. food security), które wymaga wdrażania zrównoważonej produkcji żywności (cel nr 12) bez pogarszania stanu środowiska naturalnego (cel nr 14). Systemem produkcji żywności, który ma ogromny potencjał do zapewnienia wysokiej jakości pożywienia i osiągnięcia bezpieczeństwa żywnościowego dla stale rosnącej populacji ludzi na świecie, jest dynamicznie rozwijająca się akwakultura (Garlock i in. 2020). Żywność pochodzenia wodnego, nazywana również niebieską żywnością (ang. blue foods), pomimo że jest powszechnie dostępna i stanowi ważne źródło składników odżywczych dla wszystkich grup społecznych (Belton i in. 2018), wciąż nie jest w pełni wpisana w ramy zrównoważonego rozwoju systemów żywnościowych (Tlusty i in. 2019, Naylor i in. 2021a, Garlock i in. 2022). Ponadto, znaczenie żywności pochodzenia wodnego, a szczególnie tej produkowanej w różnych systemach akwakultury, w zapewnieniu bezpieczeństwa żywnościowego jest mniejsze w porównaniu z lądową produkcją zwierzęcą i roślinną (Stetkiewicz i in. 2022), pomimo że jej negatywny wpływ na środowisko jest mniejszy (Gephart i in. 2021).

Światowa produkcja żywności pochodzenia wodnego w 2020 r. wyniosła 177,8 mln ton, z czego blisko połowę (49,2%) stanowiła akwakultura. Udział akwakultury słodkowodnej w całkowitej globalnej hodowli organizmów wodnych wynosił 61% (FAO 2022). Zdecydowana większość produkcji słodkowodnych gatunków jest realizowana w Azji (95%), a w samych tylko Chinach produkowane jest 30,9 mln ton organizmów z tej grupy. W Europie udział akwakultury słodkowodnej w całkowitej produkcji akwakultury stanowi ok. 17% (555 tys. ton), (FAO 2022). Według danych Organizacji Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (ang. Food and Agriculture Organisation, FAO) największym europejskim producentem ryb słodkowodnych jest Polska, która w 2020 r. wyprodukowała 47,7 tys. ton tego surowca (FAO 2022). Globalnie, w strukturze gatunkowej ryb produkowanych w akwakulturze dominują ryby słodkowodne, tj.: amur biały (*Ctenopharyngodon idella*), tołpyga biała (*Hypophthalmichthys molitrix*), tilapia nilowa (*Oreochromis niloticus*), karp (*Cyprinus carpio*) oraz tołpyga pstra (*Hypophthalmichthys nobilis*), których łączna produkcja w 2020 r. wyniosła 22,6 mln ton, przy czym 16,7 mln ton

wyprodukowano w samych Chinach (FAO 2022). W Europie głównym gatunkami akwakultury są ryby łososiowate, tj. łosoś atlantycki (*Salmo salar*) i pstrąg tęczowy (*Oncorhynchus mykiss*), których roczna produkcja w 2020 r. wyniosła odpowiednio 1,7 oraz 0,35 mln ton. Trzecim gatunkiem pod względem udziału w akwakulturze europejskiej jest karp (165 tys. ton). W Unii Europejskiej produkcja tego gatunku w 2020 r. wyniosła 72 tys. ton, z czego polska akwakultura dostarczyła na rynek 31% karpia (FAO 2022).

W świetle najnowszych badań dalszy wzrost znaczenia akwakultury w zapewnieniu bezpieczeństwa żywnościowego będzie możliwy jedynie dzięki jej intensyfikacji, rozumianej jako zwiększenie produkcji na jednostkę produkcyjną, tj. m^3 wody lub m^2 lądu (Belton i in. 2020). Ponadto intensyfikacja będzie dotyczyła głównie sektora akwakultury słodkowodnej, której potencjał produkcyjny do tej pory nie został w pełni wykorzystany (Belton i in. 2020, Zhang i in. 2022). Należy jednak pamiętać, że intensyfikacja poprzez wzrost zagęszczenia obsad, wprowadzenie intensywnego żywienia paszami ekstrudowanymi czy też horyzontalną ekspansję tradycyjnej produkcji, może negatywnie wpłynąć na stan środowiska oraz przyspieszyć zmiany klimatu (Foley i in. 2005). Te obawy podziela również Unia Europejska, która w zasadach Zielonego Ładu (ang. Green Deal) wskazuje na konieczność zaniechania działań nastawionych jedynie na intensyfikację akwakultury i popiera ekointensyfikację, czyli taki wzrost produkcji organizmów wodnych, który jednocześnie nie pogarsza stanu środowiska (Naylor i in. 2021b) oraz wpisuje się w zasady cyrkularnej ekonomii (Little i in. 2018). Dlatego też sektor akwakultury poszukuje możliwości ekointensyfikacji, m.in. poprzez zwiększenie produkcji takich gatunków, których hodowla nie jest w pełni uzależniona od pasz ekstrudowanych zawierających mączkę oraz olej rybny (Belton i in. 2020). Jednym z takich gatunków jest właśnie karp, będący jednym z głównych gatunków światowej i europejskiej akwakultury. Jest to również gatunek o wieloletniej i ugruntowanej pozycji na rynku, którego konsumpcja jest zakorzeniona w państwach centralnej i wschodniej Europy oraz wielu krajach azjatyckich. Dodatkowo, jak wykazały ostatnie badania, zmiany klimatu mogą paradoksalnie wpłynąć na poprawę warunków hodowlanych tej ryby w państwach takich jak Polska (Panicz i in. 2022) oraz Węgry (Varga i in. 2020). W związku z powyższym, działania zmierzające do zintensyfikowania zarówno produkcji, jak i spożycia mięsa karpia, mają większą szansę powodzenia w porównaniu z gatunkami nowymi, które nie mają ugruntowanej pozycji na rynku rybnym i znane są jedynie ograniczonej grupie konsumentów.

Ryby oraz inne produkty pochodzenia wodnego są pokarmem bogatym w składniki odżywcze i stanowią ważny element diety człowieka. Żywność pochodzenia wodnego, bez względu na środowisko pozyskania (słodkowodne, morskie), jest bogatym źródłem m.in. składników mineralnych (np. cynk, wapń), jak również długołańcuchowych wielonienasyconych kwasów tłuszczowych (omega-3 PUFA), szczególnie eikozapentaenowego (EPA) i dokozaheksaenowego (DHA). EPA oraz DHA są kluczowe między innymi dla rozwoju neuropoznawczego płodu i prawidłowego funkcjonowania układu krążenia osób dorosłych (Hibbeln i in. 2019, Mohan i in. 2021). Rekomendowane dzienne spożycie EPA i DHA powinno wynosić 250-1000 mg dla osób zdrowych, przy czym kobietom w ciąży oraz karmiącym rekomenduje się większe spożycie DHA (Kris-Etherton i in. 2009). Globalnie średnie spożycie ryb przeliczone na mieszkańca wynosi 20,5 kg i rośnie średnio o 3,1% rocznie (FAO 2020). Jednakże, spożycie ryb różni się znacząco pomiędzy regionami i państwami, przykładowo w krajach rozwijających się spożycie to jest często dwa razy większe niż średnia światowa (FAO 2020). Niestety, aktualny poziom konsumpcji ryb nie jest wystarczający, aby w pełni pokryć zapotrzebowanie populacji ludzkiej na EPA i DHA. Jak pokazały badania Hamilton i in. (2020) obecne pokrycie zapotrzebowania na te niezbędne kwasy tłuszczowe wynosi zaledwie około 30% (150 mg/dzień/osobę), co więcej niedobór jest dwa razy większy niż wynika z wcześniejszych szacunków (Tocher 2015). Hamilton i in. (2020) wskazują również, że aktualny problem dotyczący niedostatecznej realizacji zapotrzebowania ludzi na EPA i DHA może w dalszym ciągu rosnąć i należy go rozwiązać poprzez wprowadzenie innowacji na każdym etapie produkcji, przetwórstwa, dystrybucji oraz sprzedaży ryb i owoców morza. Jednym z rozwiązań, stosowanym do poprawy zawartości składników odżywczych, jest fortyfikacja, czyli wzbogacanie surowca w składniki niezbędne z punktu widzenia żywieniowego (Allen i in. 2006). Innowacyjnym sposobem, który może przyczynić się do realizacji zapotrzebowania na EPA i DHA, jest biofortyfikacja lub bio-wzbogacanie (ang. bio-enrichment), czyli proces wzbogacania surowca w biodostępne składniki odżywcze pochodzenia organicznego, już na etapie jego produkcji (Bouis i Saltzman 2017). Takie rozwiązanie może być zaadaptowane przez akwakulturę słodkowodną do biofortyfikacji mięsa ryb słodkowodnych, które zawiera mniejsze ilości EPA i DHA w porównaniu do ryb morskich. Przykładowo udział procentowy EPA oraz DHA w tłuszczu tkanki mięśniowej karpia wynosi odpowiednio 2,3 oraz 2,1% i jest znaczne niższy w porównaniu do tłustych ryb morskich, tj. śledzia (*Clupea harengus membras*) oraz dorsza

(*Gadus morhua callarias*), u których poziom EPA i DHA wynosi odpowiednio 6,2 i 20,4% oraz 7,6 i 50,8% (Kaliniak i in. 2015).

Karp jest jednym z najbardziej popularnych gatunków ryb słodkowodnych hodowanych zgodnie z wieloletnią tradycją w stawach ziemnych zarówno w Europie, jak i na świecie (Billard 1999, Wojda 2004). Zaliczany jest od ryb średniotłustych (2–7% tłuszczu), przy czym poziom tłuszczu w mięsie ryb handlowych (1,2–1,5 kg) wyprodukowanych tradycyjnie zależy od sposobu żywienia (dostępności pokarmu naturalnego, żywienia uzupełniającego zbożami lub paszami ekstrudowanymi) oraz od podłoża molekularnego hodowanej linii (Kestemont 1995). W przypadku stosowania żywienia paszami ekstrudowanymi poziom tłuszczu w mięsie karpi można zwiększyć nawet do 9% (Eljasik i in. 2020). W typowej paszy ekstrudowanej źródłem tłuszczu jest zwykle olej rybny, jednakże naturalne zasoby ryb, z których jest produkowany, narażone są na stałą presję ze strony akwakultury (Naylor i in. 2000). Dlatego w akwakulturze poszukuje się nowych źródeł tłuszczu, które nie tylko zastąpią olej rybny i przyczynią się do opracowania nowych kompozycji paszowych zgodnie z zasadami cyrkularnej ekonomii (Stevens i in. 2018), ale również pozwolą profilować skład chemiczny filetów ryb, wzbogacając tkankę mięśniową o bioaktywne omega-3 PUFA (Ramos i in. 2008, Neill i in. 2021) i w konsekwencji przyczynią się do zmniejszenia niedoborów EPA i DHA w diecie ludzi. Potencjalnym surowcem do produkcji paszy mogą być produkty uboczne akwakultury, które charakteryzują się wysoką wartością odżywczą (Malcorps i in. 2021). Pomimo to poziom ich zagospodarowywania jest w dalszym ciągu niski w porównaniu z produktami otrzymywanymi podczas uboju i przetwórstwa zwierząt rzeźnych (Newton i in. 2014). Również mikro- oraz makroglony stanowią cenne źródło omega-3 PUFA, np. zawartość DHA w mikroglonach z gatunku *Schizochytrium* sp. może sięgać nawet 27% (Allen i in. 2019). Glony ze względu na swoje duże możliwości produkcyjne mogą także przyczynić się do redukcji uzależnienia akwakultury od oleju rybnego (Cottrell i in. 2020, Malcorps i in. 2021).

W ostatnich latach wykorzystanie alternatywnych źródeł tłuszczu do produkcji pasz przeznaczonych do żywienia ryb i tym samym do wzbogacania ich tkanki mięśniowej było szeroko opisywane w przypadku popularnych gatunków, tj. łosoś atlantycki (*Salmo salar*), (Wilke i in. 2015, Norambuena i in. 2015), dorada (*Spratus aurata*), (Ganuza i in. 2008, Ribeiro i in. 2015), czy krewetka biała (*Litopenaeus vannamei*), (Kumar i in. 2018, Allen i in. 2019), jak również karp (Mráz 2011). Jednakże publikacje te nie obejmują kompleksowej oceny wpływu żywienia ryb paszami (zawierającymi innowacyjne składniki) na dobrostan ryb oraz na jakość ich mięsa. Dlatego też podejście do tego tematu musi być wieloaspektowe

i na początku obejmować ocenę wpływu mieszanek paszowych na odpowiedź organizmu ryb określoną za pomocą biometrycznych, biochemicznych, histologicznych oraz molekularnych wskaźników kondycji. Dopiero uzyskane w ten sposób informacje pozwolą określić efektywność biofortyfikacji, jak również umożliwią prowadzenie produkcji w zrównoważony sposób z jednoczesnym poszanowaniem aspektów dobrostanu ryb, których mięso będzie charakteryzowało się wysoką wartością odżywczą oraz jakością kulinarną i przetwórczą. Karp, ze względu na swoją plastyczność fenotypową, wydaje się być gatunkiem o dużym potencjale do biofortyfikacji. W literaturze opisane zostały jedynie próby wykorzystania naturalnego szlaku syntezy omega-3 PUFA karpia poprzez zwiększenie w diecie ilości kwasu alfa-linolenowego oraz kwasu linolowego jako substratów w tym szlaku metabolicznym (Mráz i in. 2012). Nie znaleziono natomiast wyników badań dotyczących biofortyfikacji tego gatunku za pomocą omega-3 PUFA (EPA i DHA) pochodzących ze zrównoważonych źródeł. Rozwiązanie problemów badawczych w tym zakresie może być szansą dla akwakultury słodkowodnej, jak również dla producentów żywności. Dlatego uzasadnione jest przeprowadzenie badań poświęconych ocenie możliwości wzbogacenia mięsa karpia w EPA i DHA pochodzących ze zrównoważonych źródeł, co może odblokować potencjał produkcyjny europejskiej akwakultury słodkowodnej, producentów żywności oraz zwiększyć dostęp konsumentów do żywności o polepszonej wartości odżywczej i w konsekwencji przyczynić się wzrostu bezpieczeństwa żywnościowego i żywieniowego ludności. Z jednej strony zastosowanie alternatywnych źródeł tłuszczu, pochodzących z produktów ubocznych akwakultury innych gatunków, przyczyni się do zrównoważonej, ekologicznej i cyrkularnej produkcji karpia. Z drugiej zaś strony – umożliwi zwiększenie pokrycia zapotrzebowania populacji ludzkiej na EPA i DHA. Aby te cele osiągnąć konieczne będzie zacieśnienie współpracy pomiędzy sektorem akwakultury słodkowodnej a producentami żywności pochodzenia wodnego.

2. HIPOTEZY I CELE BADAWCZE

Dążenie świata do osiągnięcia zrównoważonego rozwoju, stale rosnące zapotrzebowanie na żywność pochodzenia wodnego, jak i zmniejszająca się dostępność omega-3 PUFA (szczególnie EPA i DHA) w populacji, wskazują na konieczność ekointensyfikacji produkcji ryb, z uwzględnieniem możliwości profilowania składu chemicznego ich mięsa.

Założono, że karp ze względu na jego kluczowe znaczenie w światowej akwakulturze oraz dużą plastyczność fenotypową jest gatunkiem, którego okres produkcji można znacząco

skrócić, a przy zastosowaniu pasz biofortyfikujących wzbogacić filety w omega-3 PUFA, bez negatywnego wpływu na kondycję ryb oraz na strukturę, teksturę i właściwości sensoryczne ich mięsa.

W celu weryfikacji przyjętej hipotezy:

- sprawdzono możliwość skrócenia okresu produkcji karpia z tradycyjnych 33 do 19 miesięcy, poprzez zastąpienie dwóch etapów zimowania w stawach jednym etapem zimowania w recyrkulacyjnych systemach akwakultury (RAS);
- przeprowadzono biofortyfikację mięsa karpia za pomocą pasz wzbogaconych w omega-3 PUFA (EPA i DHA) pochodzących z naturalnych źródeł;
- oceniono wpływ biofortyfikacji karpia omega-3 PUFA (EPA i DHA) na kluczowe biometryczne, biochemiczne, histologiczne oraz molekularne wskaźniki kondycji ryb, jak również na wartość odżywczą i wybrane parametry jakości surowca (mięsa).

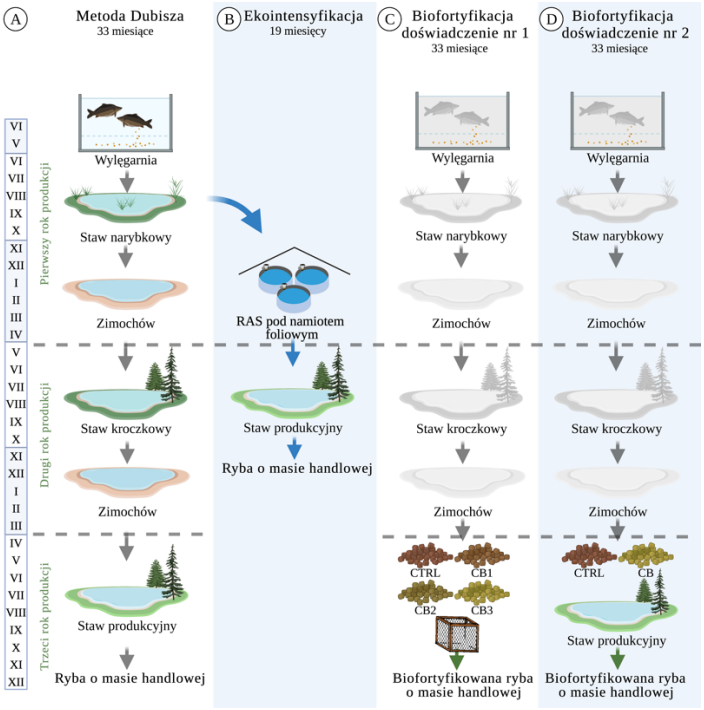
3. MATERIAŁY I METODY BADAWCZE

Materiał badawczy stanowiły karpie pozyskane z gospodarstwa rybackiego w Malińcu zlokalizowanego w województwie zachodniopomorskim (53° 42′ 5.99″ N, 15° 21′ 22.19″ E). Ryby wykorzystano do przeprowadzenia doświadczeń żywieniowych (Rys. 1), których celem była ekointesyfikacja produkcji oraz biofortyfikacja ryb i ich mięsa.

Ekointensyfikacja (**P1)** polegała na skróceniu czasu produkcji karpi z 33 do 19 miesięcy, poprzez zastąpienie dwóch etapów zimowania w stawach (tradycyjna metoda hodowli Dubisza), (Rys. 1, A) jednym etapem zimowania w RAS znajdującym się w tunelu foliowym chroniącym przed warunkami atmosferycznymi (Rys. 1, B). Podczas zimowania karpie utrzymywane w RAS były żywione paszami ekstrudowanymi, podczas gdy w tradycyjnym zimowaniu nie stosuje się żywienia. Na pozostałych etapach tradycyjnej hodowli ryby żywiły się pokarmem naturalnym i były dokarmiane zbożem. Biofortyfikacja została przeprowadzona poprzez żywienie ryb w końcowym etapie ich produkcji paszami finiszującymi wzbogaconymi w składniki (omega-3 PUFA) pochodzące z naturalnych i zrównoważonych źródeł (mikro-, makroglony, produkty uboczne przetwórstwa łososia atlantyckiego). Skład komponentowy pasz wykorzystanych w doświadczeniach żywieniowych zamieszczono w Tabeli S2 (Aneks). Biofortyfikację przeprowadzono w dwóch doświadczeniach. W pierwszym doświadczeniu (Rys. 1, C) karpie żywiono 3 paszami eksperymentalnymi (CB1–CB3) o zróżnicowanym składzie. Celem doświadczenia była ocena wpływu żywienia na kondycję ryb, wartość odżywczą i kulinarną mięsa karpia (**P2**, **P3**). Na podstawie uzyskanych wyników oraz analiz

ekonomicznych (dane poufne) opracowano skład paszy finiszującej (CB), która została następnie wykorzystana do żywienia karpi w drugim doświadczeniu przeprowadzonym w typowych warunkach hodowlanych (Rys. 1, D). Celem tego doświadczenia była ocena wpływu żywienia paszą CB na kondycję ryb, wartość odżywczą i kulinarną mięsa karpia (**P4**, **P5**).

Na podstawie uzyskanych wyników wyliczono przewidywaną ilość ton EPA i DHA, jaką w ciągu roku mogłaby dostarczyć krajowa akwakultura karpia stosując ekointensyfikację, biofortyfikację i ich połączenie. Oszacowano również poziom pokrycia zapotrzebowania polskiej populacji na EPA i DHA uwzględniając każdą z tych strategii hodowlanych. Opis scenariuszy hodowlanych i założenia niezbędne do wyliczeń zamieszczono w Tabelach S3 i S4 (Aneks).



Rys. 1. Schemat doświadczeń żywieniowych.

W przeprowadzonych badaniach oznaczono:

- parametry zootechniczne (np. FCR, SR, *K*) (**P1**, **P2**, **P4**);
- aktywność genów w skrzelach, nerce głowowej (**P1**), wątrobie (**P1**, **P2**, **P4**) oraz w jelicie (**P4**) za pomocą techniki real-time PCR (Higuchi i in. 1992, Bustin i in. 2009);
- wskaźniki histomorfologiczne preparatów mikroskopowych skóry, skrzeli (**P1**), wątroby (**P2**, **P4**), mięśni (**P3**) i jelita (**P1**, **P4**) za pomocą programu NIS-Elements Basic Research,

- profil lipidowy (TC, LDL, HDL, TAG, nie-HDL) osocza krwi (**P2**) metodą chemiluminescencji,
- podstawowy skład chemiczny (Latimer 2019), w tym:
  - zawartość białka, metodą Kjeldahla (**P3**, **P5**),
  - zawartość tłuszczu, metodą Soxhleta (**P2**, **P3**, **P4**, **P5**),
  - zawartość wody, metodą suszarkowo-wagową (**P5**),
  - zawartość popiołu, metodą spopielenia suchej masy (**P3**, **P5**).

Na podstawie oznaczonego składu chemicznego obliczano wartość energetyczną mięsa (**P5**).

- profil kwasów tłuszczowych (**P2**, **P3**, **P4**, **P5**), metodą chromatografii gazowej z detektorem płomieniowo-jonizacyjnym (Douglas i in., 2007) zgodnie z normą PN-EN ISO 12966-1:2015-01. Na podstawie uzyskanych wyników wyliczono parametry wartości odżywczej tłuszczu:
  - n3:n6 - proporcję kwasów tłuszczowych omega-3 i omega-6 (**P2**, **P3**, **P5**),
  - h:H - proporcję kwasów tłuszczowych hipocholesterolemicznych i hipercholesterolemicznych (Fernández i in. 2007), (**P3**, **P5**),
  - IT - indeks trombogenny (Fehily i in. 1994), (**P3**, **P5**),
  - IA - indeks aterogenny (Ulbricht i Southgate 1991, Fehily i in. 1994), (**P3**, **P5**),
  - UFA:SFA - proporcję kwasów tłuszczowych nienasyconych i nasyconych (**P5**),
  - PUFA:SFA - proporcję kwasów tłuszczowych wielonienasyconych i nasyconych (**P3**, **P5**).
- profil aminokwasów (**P5**), metodą wysokosprawnej chromatografii cieczowej w trzech grupach: aminokwasy po hydrolizie kwaśnej w roztworze kwasu solnego (Davies i Thomas 1973), aminokwasy po hydrolizie kwaśnej z utlenianiem (Schramm i in. 1954) oraz tryptofan po hydrolizie zasadowej w roztworze $Ba(OH)_2$ (Sławiński i Tyczkowska 1974). Na podstawie uzyskanych wyników wyliczono wskaźniki wartości odżywczej białka:
  - CS - współczynnik chemiczny białka (FAO/WHO/UNU 2007),
  - EAAI -indeks aminokwasów niezbędnych (Shahidi i Synowiecki 1993).
- jakość kulinarną (**P3**, **P5**) mięsa obejmującą oznaczenia:
  -pH, pomiar bezpośredni za pomocą pH-metru,
  - barwy, pomiar bezpośredni w systemie L*a*b*,
  - parametrów tekstury za pomocą testu TPA (Bourne 1982),
  - wyróżników w ocenie sensorycznej zgodnie z normą PN-ISO 11036:1999,

- ubytków cieplnych oraz rozmrażalniczych metodą wagową.

Uzyskane w badaniach wyniki poddano analizie statystycznej za pomocą programu STATISTICA v. 13.1 (Kraków, Polska). Wyniki przedstawiono w postaci średnich i odchyleń standardowych. Normalność rozkładów danych sprawdzano testem Shapiro–Wilka ($P \leq 0,05$). W zależności od normalności rozkładu, istotność różnic oszacowano za pomocą testu RIR Tukeya przy $P = 0,05$ lub testem t. W celu oceny wpływu żywienia paszami wzbogaconymi w omega-3 PUFA na badane parametry jakości mięsa przeprowadzono również jedno- i dwuczynnikową analizę wariancji (ANOVA). Istotność wpływu tych czynników oceniono przy $P = 0,05$ i $P = 0,01$. Rysunki przygotowano używając programu R i pakietu ggplot2 (Wickham 2016) oraz narzędzia biorender.com.

4. OMÓWIENIE WYNIKÓW

4.1. Ekointensyfikacja produkcji karpia

W przeprowadzonych badaniach oceniano wpływ ekointensyfikacji produkcji karpia, poprzez skrócenie czasu hodowli tego gatunku z 33 do 19 miesięcy, na kluczowe zootechniczne, histologiczne i molekularne wskaźniki kondycji ryb (**P1**). Markery molekularne stresu oksydacyjnego (*sod1*, *cat*, *gpx*, *gst*, *hsp70*, *hsp90*) umożliwiły oszacowanie kondycji ryb po zimowaniu, dzięki czemu wykazano, że ryby zimujące w RAS charakteryzowały się znacznie lepszą kondycją (np. brak procesów zapalnych w jelicie, prawidłowy obraz histologiczny skóry) w porównaniu do tych utrzymywanych w warunkach tradycyjnych. Opracowany zestaw wskaźników molekularnych może być również wykorzystany na innych etapach hodowli karpia, między innymi do oceny wpływu warunków hodowlanych oraz środowiskowych na organizm ryb, a w konsekwencji pośrednio do zapobiegania niepożądanym zmianom jakości surowca. Przykładowo, poziom stresu u ryb można zredukować poprzez wprowadzenie żywienia interwencyjnego z wykorzystaniem pasz zawierających zwiększony poziom antyoksydantów (Ciji i Akhtar 2021). Podobnie jak stres oksydacyjny, również stany zapalne mogą wpływać negatywnie na funkcjonowanie ryb oraz jakość i ilość surowca (Serhan i in. 2008). W pracy **P1** po raz pierwszy scharakteryzowano odpowiedź układu immunologicznego karpi po siedmiomiesięcznym okresie zimowania, w trakcie którego ryby nie były żywione. Analiza ekspresji genów związanych z odpowiedzią immunologiczną wskazała obecność procesów zapalnych u ryb pochodzących z tradycyjnych zimochowów,

co potwierdziły również zmiany w obrazie histologicznym skrzeli (hiperplazja komórek nabłonkowych), skóry (zredukowana warstwa *stratum spongiosum*) oraz jelita (niewidoczne wakuole trawienne). Zmiany te w połączeniu z trudnymi warunkami podczas zimowania determinują poziom śmiertelności karpi w stawach, który wynosi zazwyczaj około 10% (Wojda 2004). Natomiast, jak pokazał przeprowadzony eksperyment (**P1**), zimowanie w RAS, dzięki częściowo kontrolowanym warunkom i odpowiedniemu żywieniu, pozwoliło zminimalizować śnięcia (do poziomu poniżej 1%). Ponadto, nieinwazyjna metoda oceny kondycji za pomocą współczynnika *K* Fultona wykazała, iż karpie po zimowaniu w RAS charakteryzowały się lepszą kondycją (poprawa o 9,8–14,6%) w porównaniu do karpi z zimochowu (spadek o 5,7%). Wobec przewidywanych zmian dla sektora słodkowodnej akwakultury związanych z ociepleniem klimatu (Panicz i in. 2022), wykorzystanie RAS w tunelu foliowym umożliwia dalszy wzrost ryb w okresie zimowym, odmiennie niż w przypadku zimowania w stawach, w których ryby tracą masę, pogarsza się ich kondycja i w konsekwencji sną (Wojda 2004). Uzyskany przyrost masy ryb w RAS pozwala uzyskać materiał obsadowy, który w kolejnym roku produkcyjnym osiągnie masę handlową (1,2–1,5 kg) w stawie towarowym. Uzyskane wyniki dowodzą zatem, że możliwe jest skrócenie cyklu produkcji karpia do 19 miesięcy z klasycznych 33 miesięcy (metoda Dubisza) dzięki zastosowaniu zimowania w RAS w tunelu foliowym. Zastosowanie ekointensyfikacji może przełożyć się na zwiększenie bezpieczeństwa żywnościowego, ze względu na wyższą przeżywalność ryb w RAS oraz uwolnienie potencjału produkcyjnego, tj. produkcję ryb w skróconym okresie, który pozwala na ponowne wykorzystanie stawów hodowlanych o rok wcześniej.

Wyniki badań są pierwszym odnotowanym w literaturze dla karpia kompleksowym opisem wpływu warunków zimowania w stawach oraz w RAS na zestaw kluczowych wskaźników kondycji (KPI), które mogą być pomocne w optymalizacji produkcji tego gatunku na wszystkich etapach produkcji w celu uzyskania maksymalnej wydajności i maksymalnego wykorzystania użytych zasobów przy zachowaniu odpowiedniego stanu zdrowia ryb. Jest to ogromnie ważne, ponieważ dobrostan ryb przekłada się bezpośrednio na wielkość produkcji (Stien i in. 2020) oraz na parametry jakościowe mięsa (Daskalova 2019). Dlatego monitorowanie dobrostanu z wykorzystaniem nowoczesnych metod inwazyjnych i nieinwazyjnych jest niezbędne, aby intensyfikować produkcję z poszanowaniem hodowanych zwierząt i bez pogorszenia cech jakościowych surowca (Barreto i in. 2022).

4.2. Biofortyfikacja

Na podstawie przeprowadzonych badań wykazano, że ekointensyfikacja nie miała istotnego wpływu na podstawowy skład chemiczny mięsa karpia (praca w przygotowaniu). Zatem w celu poprawy wartości odżywczej surowca (mięsa), który mógłby zapewnić m.in. wystarczające pokrycie zapotrzebowania ludności na EPA i DHA, konieczna jest jego biofortyfikacja. Biofortyfikację mięsa ryb przeprowadzono za pomocą żywienia karpi paszami wzbogaconymi w wielonienasycone kwasy tłuszczowe omega-3 (**P2**–**P5**).

W pierwszym doświadczeniu do biofortyfikacji ryb wykorzystano 3 pasze eksperymentalne, różniące się zawartością mączki z mikroglonów *Schizochytrium* sp. oraz oleju z produktów ubocznych przetwórstwa łososia i olejów roślinnych (sojowego i rzepakowego), (Tabela S2, Aneks). Wyniki badań przedstawionych w pracach **P2** i **P3** wskazują, że zastosowanie w żywieniu karpi pasz zawierających alternatywne dla oleju rybnego źródła tłuszczu w postaci surowców bogatych w wielonienasycone kwasy tłuszczowe omega-3, tj. mikroglonów z gatunku *Schizochytrium* sp. (Shah i in. 2018, Wang i in. 2021) oraz oleju z produktów ubocznych przetwórstwa łososia atlantyckiego (Wu i Bechtel 2008, Routray i in. 2018) pozwala na istotne zwiększenie zawartości EPA i DHA w mięsie karpia. Największą ilość EPA i DHA stwierdzono w tłuszczu filetów ryb żywionych paszą wzbogaconą mikroglonami *Schizochytrium* sp. (3,78–4,17% kwasów tłuszczowych), podczas gdy w tłuszczu mięśniowym ryb żywionych paszą z olejem z produktów ubocznych przetwórstwa łososia zawartość tych kwasów była istotnie niższa (2,3% kwasów tłuszczowych), a najniższa w mięsie ryb z grupy kontrolnej żywionych paszą komercyjną (1,08% kwasów tłuszczowych). Zwiększone odkładanie długołańcuchowych kwasów tłuszczowych w mięśniach ryb żywionych paszami wzbogaconymi to prawdopodobnie konsekwencja obserwowanego podwyższonego stężenia cholesterolu całkowitego oraz poszczególnych jego frakcji w plazmie krwi karpi (**P2**), co świadczy o wzmożonym metabolizmie lipidów w organizmach ryb. Habte-Tsion i in. (2020) zaobserwowali podobną zależność u bassa wielkogębowego (*Micropterus salmoides*) żywionego mączką z mikroglonów *Schizochytrium*. Zwiększona podaż EPA i DHA w wzbogaconych paszach wpłynęła również na aktywność genów (*elovl5a*, *fads6a*) kodujących białka zaangażowane w metabolizm tłuszczów, a szczególnie te odpowiedzialne za biosyntezę długołańcuchowych wielonienasyconych kwasów tłuszczowych. Potwierdzeniem tego był obraz histologiczny wątroby karpi biofortyfikowanych, w którym wyraźnie widoczne były powiększone hepatocyty

(**P2**), co sugeruje intensywne odkładanie lipidów w komórkach wątrobowych, podobnie jak wykazano wcześniej u dorady (Caballero i in. 1999).

Efekt biofortyfikacji karpi żywionych paszą zawierającą *Schizochytrium* sp. w postaci zwiększonej ilości EPA i DHA w filetach, był podobny do tych uzyskanych dla innych, ważnych gatunków europejskiej akwakultury, m.in. dla dorady (Ferreira i in. 2022) czy pstrąga tęczowego (Bélanger i in. 2021). Natomiast włączenie mikroglonów do diety karpi w pierwszym doświadczeniu okazało się znacznie skuteczniejsze niż w przypadku żywienia nimi łososia atlantyckiego (Sprague i in. 2015), prawdopodobnie ze względu na większe wymagania żywieniowe łososia. Ponadto, w pracy **P2** i **P3** u karpia o masie handlowej, podobnie jak w przypadku młodocianego stadium karpia (Xiao i in. 2021) oraz tilapii nilowej (Sarker i in. 2016), efektywność biofortyfikacji (rozumiana jako wzrost ilości EPA i DHA w mięsie) zależna była od dawki mikroglonów w paszy, co wskazuje na dużą plastyczność fenotypową tego gatunku oraz możliwość rozwiązania problemu niedoborów EPA i DHA w diecie wielu osób na świecie. Oprócz zwiększonego poziomu EPA i DHA w filetach ryb karmionych paszami wzbogaconymi (**P2**, **P3**), stwierdzono również poprawę wskaźników jakościowych tłuszczu. Wartości proporcji kwasów tłuszczowych omega-3 do omega-6 (n-3:n-6), omega-6 do omega-3 (n-6:n-3) oraz wielonienasyconych kwasów tłuszczowych do nasyconych kwasów tłuszczowych (PUFA:SFA) mieściły się w rekomendacjach FAO/WHO (odpowiednio > 0,25; < 4; > 0,4), (FAO/WHO 2008). Potwierdziły również lepszą jakość żywieniową tłuszczu mięśniowego ryb żywionych paszami wzbogaconymi w EPA i DHA pochodzącymi ze *Schizochytrium* niż z oleju z łososia. Uzyskane dla karpia wyniki są zgodne z wynikami dotyczącymi wykorzystania mikroglonów w diecie różnych gatunków ryb (Nagappan i in. 2021).

Przeprowadzona biofortyfikacja wpłynęła na podstawowy skład chemiczny mięsa karpi, wielkość włókien mięśniowych i grubość tkanki łącznej, parametry barwy i wyczuwalności smaku rybnego, podczas gdy nie miała wpływu na parametry tekstury mierzonej instrumentalnie i sensorycznie (**P3**). Mięso karpi biofortyfikowanych charakteryzowało się mniejszą zawartością białka i wody oraz większą ilością tłuszczu niż mięso ryb z grupy kontrolnej. W biofortyfikowanym surowcu obserwowano także większe włókna mięśniowe i grubszą tkankę łączną, jaśniejszą i bardziej czystą barwę z większym natężeniem barwy czerwonej i żółtej. Smak rybny był również wyraźniej wyczuwalny we wszystkich wariantach biofortyfikowanych. Dane literaturowe dotyczące wpływu żywienia ryb paszą z dodatkiem mikroglonów na parametry jakości mięsa nie są jednoznaczne. W przypadku łososia

atlantyckiego zastosowanie mikroglonów w diecie nie wpłynęło na jakość kulinarną filetów, w tym także na wysoce cenioną w przypadku tego gatunku barwę (Kousoulaki i in. 2016). W innych badaniach żywienie łososia atlantyckiego paszą z udziałem *Schizochytrium* wpłynęło pozytywnie na atrakcyjność barwy filetów oraz nie pogorszyło innych właściwości sensorycznych (Kousoulaki i in. 2020). Liao i in. (2022) wykazali, że 4% dodatek *Schizochytrium* w diecie bassa wielkogębowego, oprócz profilowania składu kwasów tłuszczowych mięśni, poprawia również teksturę filetów.

Na podstawie wyników pierwszego doświadczenia (**P2** i **P3**) wykazano, że lepszą efektywność biofortyfikacji mięsa karpia w EPA i DHA uzyskano przy żywieniu ryb paszą wzbogaconą w mączkę z mikroglonów niż w olej z produktów ubocznych przetwórstwa łososia. Biorąc jednak pod uwagę prawie 5-krotnie wyższy koszt produkcji pasz z mączką ze *Schizochytrium* sp. (dane zastrzeżone), który stanowi ok. 30% kosztu produkcji karpia, opracowano skład paszy finiszującej (CB), w której źródłem tłuszczu był wyłącznie olej z produktów ubocznych przetwórstwa łososia. Jednak w celu poprawy efektywności biofortyfikacji udział tego komponentu w paszy został zwiększony (Tabela S2, Aneks). Tak skomponowana pasza została następnie wykorzystana w drugim doświadczeniu jako pasza finiszująca do żywienia karpi w typowych warunkach hodowli.

Zastosowanie oleju z łososia w paszy wykorzystanej do żywienia karpi pozwoliło uzyskać surowiec o większej zawartości EPA i DHA (2,58% kwasów tłuszczowych) w porównaniu do próby kontrolnej (0,79% kwasów tłuszczowych), (**P4**, **P5**), przy zawartości tłuszczu mięśniowego odpowiednio 1,18 i 1,88% (**P4**). Poziom tłuszczu oznaczony w mięsie karpi z drugiego doświadczenia (**P5**) był zatem niższy w porównaniu z rybami pochodzącymi z pierwszego doświadczenia (**P3**), co sugeruje, że warunki środowiskowe, w tym temperatura, mają fundamentalny wpływ na metabolizm lipidów ryb (Sun i in. 2019). Potwierdzają to wyniki przeprowadzonych badań molekularnych, z których wynika, że szlak biosyntezy długołańcuchowych kwasów tłuszczowych nie został pobudzony przez paszę o podwyższonej zawartości EPA i DHA, z wyjątkiem genu *elovl2* (**P4**), odpowiedzialnego m.in. za wydłużanie EPA do kwasu dokozapentaenowego (Xie i in. 2021). Również w obrazie histologicznym wątroby ryb żywionych paszą wzbogaconą nie zaobserwowano oznak odkładania się lipidów (powiększonych hepatocytów), jak miało to miejsce w przypadku pierwszego doświadczenia żywieniowego (**P2**). W pracy **P4** wykazano również brak negatywnych skutków żywienia paszą wzbogaconą w EPA i DHA na integralność jelita na poziomie histologicznym (brak oznak

nekrozy, pyknozy, kariolizy), a wyniki te potwierdzono na poziomie ekspresji genów kodujących białka ścisłego połączenia oraz enzymy absorpcyjne w jelicie.

Pasza finiszująca (CB) została stworzona na potrzeby hodowców, dlatego w celu oceny możliwości jej implementacji, badania jakościowe zostały rozszerzone o ocenę jakości białka mięsa ryb. Jest to również niezbędne do określenia wartości odżywczej fortyfikowanego mięsa karpia, która jest jednym z ważniejszych elementów jakości branych pod uwagę przy podejmowaniu przez konsumentów decyzji o wyborze i zakupie produktu żywnościowego. Wykazano, że żywienie karpi paszą wzbogaconą wpłynęło głównie na profil kwasów tłuszczowych i jakość tłuszczu, a w mniejszym stopniu na profil aminokwasów oraz jakość białka w ich mięsie (**P5**). Wskaźniki jakościowe tłuszczu u karpi biofortyfikowanych spełniały wspomniane wcześniej rekomendacje FAO/WHO (FAO/WHO 2008), analogicznie jak w przypadku ryb biofortyfikowanych w pierwszym doświadczeniu żywieniowym (**P3**). Nie stwierdzono natomiast istotnych różnic w badanych parametrach jakości kulinarnej (pH, parametry barwy L*a*b*, parametry testu TPA, sensoryczne wyróżniki tekstury i smakowitości) pomiędzy grupą kontrolną i eksperymentalną (**P5**).

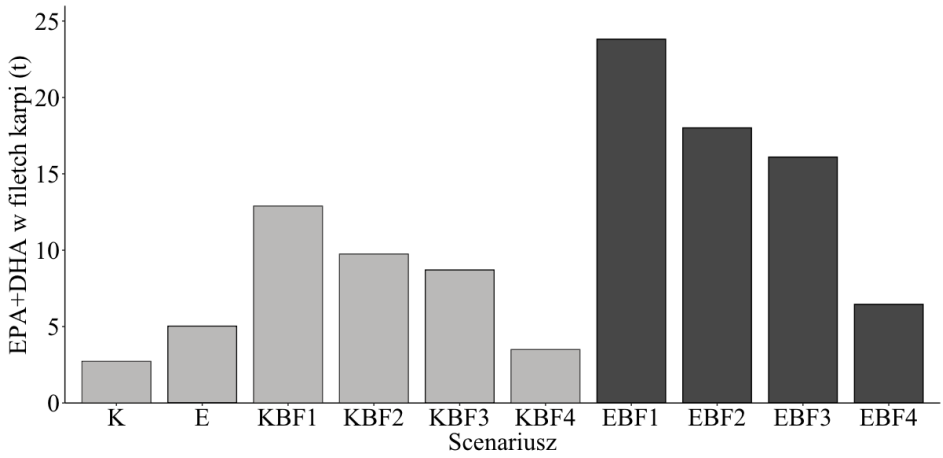
Na podstawie przeprowadzonych badań, obejmujących oba doświadczenia żywieniowe, można wykazać, że biofortyfikacja mięsa karpi za pomocą omega-3 PUFA, pochodzących z naturalnych i zrównoważonych źródeł, jest możliwa i nie powoduje pogorszenia kondycji ryb i jakości kulinarnej uzyskanego surowca. Zaobserwowane pomiędzy pierwszym (**P2**, **P3**) i drugim (**P4**, **P5**) doświadczeniem żywieniowym różnice we wpływie żywienia paszami wzbogaconymi w omega-3 PUFA na poziom tłuszczu, zawartość EPA i DHA w mięsie karpi oraz ekspresję genów świadczących o odpowiedzi organizmu, pokazują jak ważne z punktu widzenia praktycznego jest przeprowadzanie doświadczeń żywieniowych w typowych warunkach produkcyjnych. Dopiero weryfikacja uzyskanych w pierwszym doświadczeniu wyników, poprzez ocenę wpływu warunków środowiskowych na stwierdzone zależności, zwiększa charakter aplikacyjny opracowanych rozwiązań, dzięki czemu możliwe jest ich wykorzystanie w praktyce produkcyjnej akwakultury.

Dodatkowo, istotnym efektem opisanej biofortyfikacji karpi za pomocą kwasów omega-3 PUFA może być uzyskanie większego poziomu tych kwasów tłuszczowych w narządach ryb. Przykładem mogą być powiększone hepatocyty zaobserwowane w wątrobie ryb (**P2),** co może sugerować zwiększoną akumulację tłuszczu w tym organie. Badania Liao i in. (2022) wykazały, że wątroba jest cennym źródłem kwasów tłuszczowych, w tym szczególnie DHA. Można zatem przypuszczać, że mogłaby być wykorzystywana

jako naturalne źródło niezbędnych nienasyconych kwasów tłuszczowych w produkcji pasz dla zwierząt. Wymaga to jednak prowadzenia kolejnych badań, niezbędnych do poprawy efektywności wykorzystania produktów ubocznych akwakultury, które mają wysoką wartość żywieniową (Malcorps i in. 2021), a ich zagospodarowanie mogłoby ograniczyć straty cennych komponentów takich jak EPA i DHA (Hamilton i in. 2020).

4.3. Biofortyfikowane mięso karpia a zmniejszenie niedoborów EPA i DHA w diecie Polaków

Badania konsumenckie przeprowadzone w Europie wykazały, że konsumenci ryb mogą płacić więcej za żywność pochodzenia wodnego oznaczoną jako "bio" lub pochodzącą ze zrównoważonych źródeł (Menozzi i in. 2020). Pomimo że produkty słodkowodne nie są pierwszym wyborem europejskich konsumentów, to powinny być uwzględniane w badaniach, ponieważ w niektórych rejonach naszego kontynentu, w tym m.in. w Polsce, stanowią ważny element diety (Pounds i in. 2022). Dlatego populację Polski wybrano do stworzenia modelu pokazującego jak ekointensyfikacja, czyli skrócenie cyklu produkcji oraz biofortyfikacja karpia EPA i DHA lub połączenie obu rozwiązań może skutkować większą podażą EPA i DHA zgodnie z przyjętymi scenariuszami (Tabela S3, Aneks). Stworzony model określa ilość wyprodukowanego EPA i DHA w ciągu roku przez polską akwakulturę karpia. Przyjęto, że 500 mg/osobę EPA i DHA stanowi niezbędną podstawową dawkę pokrywającą dzienne zapotrzebowanie człowieka, którą wykorzystano do wyliczenia pokrycia dziennego zapotrzebowania całej populacji naszego kraju (Flock i in. 2013, Hamilton i in. 2020). Wszystkie założenia niezbędne do opracowania modelu zamieszczono w Tabeli S4 (Aneks).



Rys. 2. Ilość EPA i DHA w filetach karpi dostarczana rocznie przez polską akwakulturę przy zastosowaniu poszczególnych scenariuszy ekointensyfikacji i biofortyfikacji. Objaśnienia skrótów zamieszczono w Tabeli S3 (Aneks).

Wyniki stworzonego modelu (Rys. 2) pokazały, że przy obecnym krajowym poziomie produkcji tradycyjną metodą Dubisza, filety z karpi dostarczają 2,72 ton EPA i DHA rocznie, co przekłada się na pokrycie zalecanej dziennej dawki EPA i DHA populacji Polski na poziomie 0,14 (14%). Zastosowanie ekointensyfikacji (**P1**) pozwoliłoby na wyprodukowanie większej ilości surowca i dzięki temu niemal podwojenie ilości dostarczanego EPA i DHA rocznie (5,0 ton). Z kolei efektywność biofortyfikacji EPA i DHA mięsa karpi zależała od rodzaju i ilości składnika wzbogacającego paszę oraz od poziomu tłuszczu w mięsie. Wykorzystanie pasz zawierających 1,563% i 3,125% mączki z mikroglonów *Schizochytrium* sp. mogłoby potencjalnie zwiększyć dostarczaną ilość EPA + DHA do odpowiednio 9,7 ton i 12,9 ton rocznie (**P2**, **P3**). Zastąpienie *Schizochytrium* sp. olejem z produktów ubocznych przetwórstwa łososia atlantyckiego w paszy użytej do żywienia karpi mogłoby dostarczyć 8,71 ton EPA i DHA rocznie (**P2**, **P3**). Natomiast zastosowanie oleju z produktów ubocznych przetwórstwa łososia atlantyckiego jako jedynego źródła tłuszczu w paszy mogłoby dostarczyć jedynie 3,5 ton EPA i DHA rocznie (**P4**, **P5**). Analizując wyliczone wskaźniki pokrycia zalecanej dziennej dawki EPA i DHA wykazano, że roczna produkcja karpia przy wykorzystaniu paszy wzbogaconej w mączkę z mikroglonów *Schizochytrium* sp. pokrywa to zalecenie na poziomie 51 i 67%, podczas gdy biofortyfikacja olejem z produktów ubocznych przetwórstwa łososi na poziomie 18 i 46%. Zwiększenie udziału mączki z mikroglonów w paszy podnosi efektywność biofortyfikacji, natomiast oleju z przetwórstwa produktów ubocznych prowadzi do jej obniżenia. Ze stworzonego modelu wynika jednocześnie, że zastosowanie połączenia obu strategii hodowanych w polskiej akwakulturze karpi może znacząco zwiększyć produkcję i pokrycie zalecanej dziennej dawki EPA oraz DHA populacji. Jest to szczególnie widoczne w przypadku połączenia ekointensyfikacji i biofortyfikacji mikroglonami *Schizochytrium* sp. na poziomie 3,125% w paszy. Taki scenariusz może potencjalnie dostarczyć 23,8 ton EPA i DHA rocznie oraz pokryć zalecaną dla polskiej populacji dawkę tych składników w 125%. Należy jednak pamiętać, żeby uzyskać taki poziom pokrycia niezbędne jest znaczące obniżenie kosztów produkcji mikroglonów, jak również pasz z udziałem tych składników.

5. WNIOSKI

1. Ekointensyfikacja produkcji karpia poprzez zastosowanie zimowania w RAS w tunelu foliowym pozwala skrócić cykl hodowlany z 33 do 19 miesięcy oraz wykorzystać inne typy stawów w gospodarstwie, co może istotnie wpłynąć na zwiększenie produkcji, a w konsekwencji poprawę bezpieczeństwa żywnościowego w Polsce i Europie.

2. Zimowanie w RAS nie wywołuje negatywnych reakcji organizmu karpia w przeciwieństwie do tradycyjnego zimowania, gdzie chroniczny stres spowodowany limitowanym żywieniem wywołuje odpowiedź immunologiczną organizmu, która z kolei może przełożyć się na dłuższy okres dochodzenia do optymalnej kondycji.

3. Zastosowanie wyłącznie ekointensyfikacji w hodowli karpia może dostarczyć więcej surowca w krótszym czasie, ale nie jest wystarczające do pokrycia niedoborów EPA i DHA w populacji Polski przez krajową akwakulturę.

4. Biofortyfikacja karpi za pomocą pasz wzbogaconych w omega-3 PUFA z naturalnych źródeł przyczynia się do poprawy wartości odżywczej mięsa ryb w postaci zwiększenia zawartości EPA i DHA oraz wskaźników jakościowych tłuszczu mięśniowego. Uzyskane efekty biofortyfikacji surowca uzależnione były od zastosowanego składnika i jego ilości w paszy.

5. Wykorzystanie oleju z produktów ubocznych przetwórstwa łososia atlantyckiego do biofortyfikacji omega-3 PUFA (EPA i DHA) mięsa karpia jest mniej efektywne niż wykorzystanie mączki z mikroglonów *Schizochytirum* sp., jednak nie wpływa na zwiększenie kosztów produkcji paszy i ryb. Olej z produktów ubocznych przetwórstwa łososia może być wykorzystany do produkcji paszy stosowanej do biofortyfikacji pod warunkiem zwiększenia jego udziału w składzie komponentowym w paszy.

6. Biofortyfikacja omega-3 PUFA (EPA i DHA) nie powoduje pogorszenia jakości kulinarnej mięsa karpia. Brak różnic w badanych parametrach jakości kulinarnej (pH, parametry barwy L*a*b*, parametry testu TPA, sensoryczne wyróżniki tekstury i smakowitości) mięsa ryb żywionych paszami wzbogaconymi i ryb z grupy kontrolnej może zachęcić konsumentów do sięgania po produkt o wyższej wartości żywieniowej.

7. Ekointensyfikacja poprzez zimowanie w RAS oraz biofortyfikacja omega-3 PUFA mięsa karpi nie wpłynęły negatywnie na kluczowe wskaźniki kondycji ryb, co potwierdzono na podstawie parametrów biometrycznych, biochemicznych, histologicznych oraz molekularnych.

8. Wykazana skuteczność stworzonego zestawu wskaźników zootechnicznych, histologicznych i molekularnych do oceny kondycji ryb pokazuje, że może on być wykorzystany przez akwakulturę na każdym etapie hodowli, co potwierdza aplikacyjny charakter tego rozwiązania.

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7. ANEKS

Tabela S1. Wykaz skrótów wykorzystanych w autoreferacie

Skrót	Wyjaśnienie
AI	współczynnik aterogenny
cat	katalaza
DHA	kwas dokozaheksaenowy
elovl2	elongaza kwasów tłuszczowych 2
elovl5a	elongaza kwasów tłuszczowych 5a
EPA	kwas eikozapentaenowy
fads6a	desaturaza kwasów tłuszczowych 6a
FCR	współczynnik konwersji paszy
gpx	peroksydaza glutationowa
gst	S-transferaza glutationu
h:H	współczynnik kwasów tłuszczowych hipocholesterolemicznych do hipercholesterolemicznych
HDL	cholesterol o dużej gęstości
hsp70	białko szoku cieplnego 70
hsp90	białko szoku cieplnego 90
K	współczynnik kondycji Fultona *K*
KPI	kluczowe wskaźniki efektywności
LDL	cholesterol o niskiej gęstości
PER	współczynnik wykorzystania białka
PUFA	wielonienasycone kwasy tłuszczowe
RAS	recyrkulacyjny system akwakultury
PCR	reakcja łańcuchowa polimerazy
SDG	Cele Zrównoważonego Rozwoju
SFA	nasycone kwasy tłuszczowe
sod1	dysmutaza ponadtlenkowa
SR	współczynnik przeżywalności
TAG	triacyloglicerole
TC	całkowity cholesterol
TI	współczynnik trombogenny
UFA	nienasycone kwasy tłuszczowe

Tabela S2. Skład komponentowy pasz wykorzystanych w doświadczeniach żywieniowych.

		Pierwsze doświadczenie żywieniowe			Doświadczenie w warunkach stawowych
Składniki, %	**kontrola**	**CB1**	**CB2**	**CB3**	**CB**
Mączka rybna 60	5.000	2.500	2.500	2.500	2.500
Mączka z krwi wieprzowej	2.000	2.000	2.000	2.000	2.000
Mączka z glonów (*Spirulina* sp.)		1.000	1.000	1.000	1.000
Mączka z glonów (*Chlorella* sp.)		1.000	1.000	1.000	1.000
Mączka z glonów (*Schizochytrium* sp.)		3.125	1.563		
Koncentrat białek sojowych	2.500	2.500	2.500	2.500	2.500
Mączka z glutenu kukurydzianego	4.000	4.000	4.000	4.000	4.000
Mączka sojowa 40	25.000	25.000	25.000	25.000	25.000
Mączka rzepakowa	7.000	7.000	7.000	7.000	7.000
Mączka słonecznikowa	12.500	12.500	12.500	12.500	12.500
Mączka pszenna	22.500	21.224	21.786	22.349	22.329
Otręby pszenne	5.000	5.000	5.000	5.000	5.000
Mączka kukurydziana	2.500	2.500	2.500	2.500	2.500
Olej z produktów ubocznych przetwórstwa łososia				2.100	6.100
Olej sojowy	3.000			2.000	
Olej rzepakowy	3.000	4.100	5.100	2.000	
Mieszanka witamin i minerałów	1.000	1.000	1.000	1.000	1.000
Chlorowodorek betainy	0.100	0.100	0.100	0.100	0.100
Substancje spajające	1.000	1.000	1.000	1.000	1.000
Mączka z glonów (*Laminaria digitata*)		0.541	0.541	0.541	0.541
Antyoxydanty	0.200	0.200	0.200	0.200	0.200
Propionian sodu	0.100	0.100	0.100	0.100	0.100
Fosforan sodu	2.100	2.100	2.100	2.100	2.100
Drożdże selenowe		0.010	0.010	0.010	0.030
L-Lizyna	0.700	0.700	0.700	0.700	0.700
L-Tryptofan	0.200	0.200	0.200	0.200	0.200
DL-Metionina	0.600	0.600	0.600	0.600	0.600

Tabela S3. Opis scenariuszy wykorzystany do stworzenia modelu pokazującego potencjał pokrycia zapotrzebowania na EPA i DHA populacji Polski przez akwakulturę przy zastosowaniu ekointensyfikacji produkcji karpia i biofortyfikacji jego mięsa.

Skrót	Scenariusz	Składnik wykorzystany do biofortyfikacji
K	obecna sytuacja, karp produkowany metodą Dubisza	-
KBF1	karp produkowany metodą Dubisza, biofortyfikowany paszą CB1 z *Schizochytrium* sp.	3,125% udział *Schizochytrium* sp.
KBF2	karp produkowany metodą Dubisza, biofortyfikowany paszą CB2 z *Schizochytrium* sp.	1,563% udział *Schizochytrium* sp.
KBF3	karp produkowany metodą Dubisza, biofortyfikowany paszą CB3 z olejem z produktów ubocznych przetwórstwa łososia atlantyckiego	2,1% udział oleju z produktów ubocznych przetwórstwa łososia atlantyckiego;
KBF4	karp produkowany metodą Dubisza, biofortyfikowany paszą CB z olejem z produktów ubocznych przetwórstwa łososia atlantyckiego	6,1% udział oleju z produktów ubocznych przetwórstwa łososia atlantyckiego
E	karp produkowany metodą ekointensywną z zastosowaniem zimowania w RAS	-
EBF1	karp produkowany metodą ekointensywną, biofortyfikowany paszą CB1 z *Schizochytrium* sp.	3,125% udział *Schizochytrium* sp.
EBF2	karp produkowany metodą ekointensywną, biofortyfikowany paszą CB2 z *Schizochytrium* sp.	1,563% udział *Schizochytrium* sp.
EBF3	karp produkowany metodą ekointensywną, biofortyfikowany paszą CB3 z olejem z produktów ubocznych przetwórstwa łososia atlantyckiego	2,1% udział oleju z produktów ubocznych przetwórstwa łososia atlantyckiego
EBF4	karp produkowany metodą ekointensywną, biofortyfikowany paszą CB z olejem z produktów ubocznych przetwórstwa łososia atlantyckiego	6,1% udział oleju z produktów ubocznych przetwórstwa łososia atlantyckiego

Tabela S4. Założenia przyjęte do stworzenia modelu pokazującego potencjał pokrycia zapotrzebowania na EPA i DHA populacji Polski przez akwakulturę przy zastosowaniu ekointensyfikacji produkcji karpia i biofortyfikacji jego mięsa.

Parametry modelowania	**Przyjęte założenia**
Poziom krajowej produkcji karpia	22500 ton (FAO 2022)
Średnia masa ryb handlowych	1,5 kg
Śmiertelność ryb	dane literaturowe dla poszczególnych etapów produkcji metodą Dubisza (Wojda 2004)
Liczba ludności Polski	38 179,8 tys. (dane GUS, stan na 31 marca 2021)
Forma produktu	filety, które są formą najbardziej wygodną dla konsumentów w Polsce (Pounds i in. 2022)
Śmiertelność w RAS, zawartość tłuszczu, poziom EPA i DHA oraz wydajność filetów	dane empiryczne zebrano podczas doświadczeń prowadzonych w ramach prac **P1**, **P2** oraz **P4**. Dane empiryczne pochodzące z **P2** skorygowano współczynnikiem hodowli stawowej wyliczonym na podstawie wyników próby kontrolnej
Zdolność sektora akwakultury słodkowodnej do implementacji rozwiązań	wyliczona na podstawie całkowitej produkcji ryb słodkowodnych w akwakulturze, powierzchni ewidencyjnej stawów w Polsce (Lirski 2021) oraz wynikającej z ustawy maksymalnej możliwej produkcji ryb nie przekraczającej 1500 kg przyrostu rocznego z ha (Dz. U. 2017 poz. 1566, Ustawa z dnia 20 lipca 2017 r. Prawo wodne)

8. SPÓJNE TEMATYCZNIE PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ ORAZ OŚWIADCZENIA WSPÓŁAUTORÓW

OŚWIADCZENIE DO PRACY P1

W pracy **P1** byłem zaangażowany w przygotowanie koncepcji i przeprowadzenie doświadczenia, pobór i zabezpieczenie prób biologicznych, wykonanie analiz molekularnych oraz histologicznych, analizę i interpretację uzyskanych wyników, przygotowanie grafik, przygotowanie manuskryptu artykułu, udzielenie odpowiedzi na recenzje, naniesienie zasadnych poprawek do zrecenzowanej pracy. Swój udział określam na 60%.

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Tytuł publikacji: Key performance indicators of common carp (*Cyprinus carpio* L.) wintering in a pond and RAS under different feeding schemes. Sustainability 2022, 14(7), 3724. doi: 10.3390/su14073724

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Jacek Sadowski	10%	

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Article

Key Performance Indicators of Common Carp (*Cyprinus carpio* L.) Wintering in a Pond and RAS under Different Feeding Schemes

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Abstract: Overwintering impacts common carp performance, yet the nature of changes is not known. The aim of the study was to compare the zootechnical and key performance indicators (KPI) of *Cyprinus carpio* wintering in a pond with no supplementary feeding (MCF), in a Recirculating Aquaculture System (RAS) fed typical (30% of protein and 8% of fat) carp diet (AFC), and in a RAS fed high protein (42%) and fat (12%) diet (ABF). The analysis showed that ABF fish had the highest final body weight and the Fulton's condition factor, as well as the lowest food conversion rate compared with AFC and MCF fish. Histomorphological assessment revealed that MCF fish had thinner skin layers, a depleted population of mucous cells in skin, an excessive interlamellar mass in the gills, and no supranuclear vacuoles in the intestine compared to fish from RAS. At the molecular level, higher transcript levels of *il-1β* and *il-6* transcripts were found in the gills of MCF than in fish from RAS. The transcript level of the intestinal *muc5b* was the highest in ABF fish. Relative expression of *il-1β* and *il-6* in gills were presumably the highest due to lamellar fusions in MCF fish. Described KPIs may assist carp production to ensure sustainability and food security in the European Union.

Keywords: gene expression; gills; intestine; *mucin 5b*; performance

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1. Introduction

Aquaculture is a key food production sector globally [1]. In 2018, its total production was 114.5 Mt in live weight, with a total farmgate sale value of USD 263.6 billion. A considerable part of this production was represented by freshwater aquaculture (51.3 Mt, 62.5%), of which 47 Mt (91.5%) consisted of finfish production. The freshwater production of finfish is forecasted to reach 60% of global aquaculture production by 2030, according to the report, State of World Fisheries and Aquaculture (SOFIA) [2]. Among the five most important aquaculture species, four belong to Cyprinidae: the herbivorous grass carp, *Ctenopharyngodon idella* (5.7 Mt, 10.5%); the omnivorous common carp, *Cyprinus carpio* (4.2 Mt, 7.7%); and two planktivorous: silver carp, *Hypophthalmichthys molitrix* (4.8 Mt, 8.8%), and bighead carp, *Hypophthalmichthys nobilis* (3.1 Mt, 5.8%) [2]. The latest report published by the Food and Agriculture Organisation (FAO) on the top 10 species groups in global aquaculture in 2019 showed that "carps, barbels and other cyprinids" are the main species group in freshwater aquaculture, amounting to nearly 24.8% (29.8 Mt) of 120 Mt of world production. The publication also showed that production of this group has increased 1.5% from the 2018 level and, consequently confirming the upward trend predicted in the report SOFIA [3]. However, as emphasized by Belton et al. [4], this trend was possible mainly due to intensification rather than horizontal expansion, i.e., increase in production per unit land and water.

For decades, the main farmed cyprinid species in Europe has been common carp (hereafter referred to as carp) that was the mainstay, both traditionally and commercially, for fisheries production in "land-locked" central European Union (EU) [5]. Total production of *C. carpio* in the EU in 2019 was 65,715 tonnes, and the Czech Republic followed by Poland, Hungary and Germany (ranked in order of production size) provided nearly 80% of carp production in the region [6]. In the EU, carp is produced with the conventional Dubisch method, which involves multiple fish transfers into different stage ponds throughout production. A crucial part of carp farming is wintering (October through April) that happens twice, i.e., after the first and second year of production. In this period, fish do not receive feed and are relatively active; hence, their growth and condition parameters undergo gradual deterioration [7,8]. However, with the existing farming model, carp production has continued its declining trend in the EU (by 10% between 2009 and 2018) due to numerous circumstances, such as predation by protected wildlife (cormorants, great egrets and otters), increasing cost, lower subsidies, and consumer preference for carnivorous finfish such as trout or salmon [6,9]. Carp farmers in the EU are seeking opportunities that would increase the consumption of carp throughout the year, not only in the Christmas period, in which sales reach up to 90% of their annual values [10]. Moreover, consumer awareness of animal welfare causes decreased interest in purchasing live fish and, consequently, a gradual increase in carp processing into a more convenient form, i.e., carcasses, slices, sheets and fillets. These circumstances, but also global indicators, such as climate change (e.g., water shortages, eutrophication), force carp farmers to quickly update their business models accordingly. One way in which the economic profitability and sustainability of carp production can be improved is its eco-intensification, as assessed in the GAIN (Green Aquaculture Intensification) project, which includes shortening the conventional production time (from 33 to 19 months) by running part of the rearing process (first wintering) in a closed recirculating aquaculture system (RAS) decoupled with aquaponics (Figure 1).

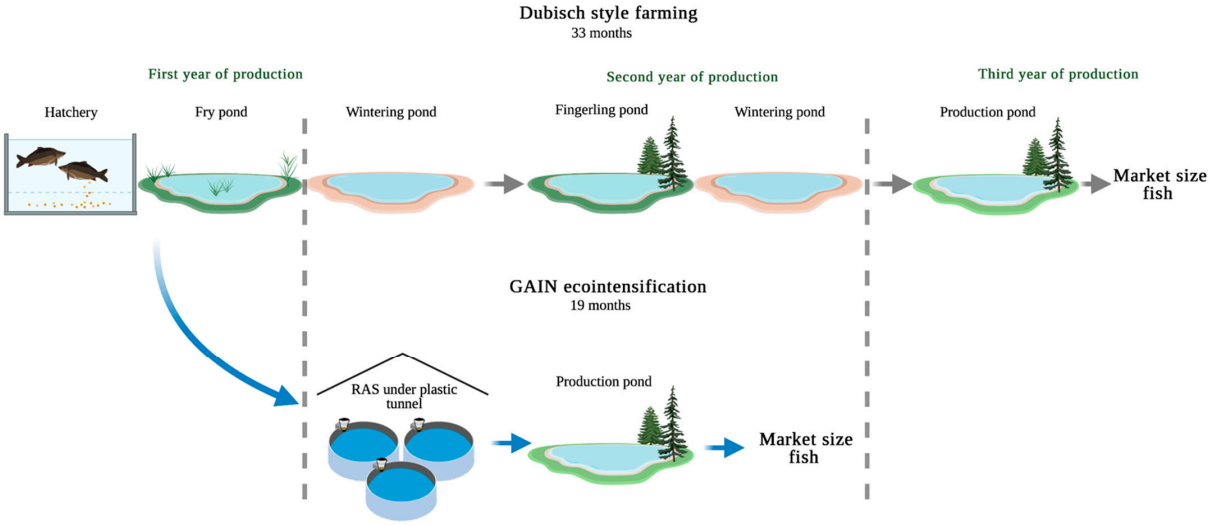

Figure 1. Overview of the traditional (Dubisch style) and eco-intensive (GAIN) culture of common carp.

Eco-intensification, however, requires verification by assessing a set of key performance indicators (KPI), i.e., growth efficiency (weight gain, feed intake), resource utilisation (food conversion ratio), health and welfare issues (survival rate, enteritis), and in market-size fish, quality (fillet yield, taste). For *C. carpio*, various bioindicators in muscle [11], liver and intestine [12], and skin [13] have been identified. Moreover, several performance indicators such as total growth rate, survival rate, protein efficiency ratio and feed conversion ratio were commonly used for common carp farmed in cages [14] and ponds [12] during

production season. However, to the best of our knowledge, the KPIs in common carp overwintering have never been assessed and are currently not available for this species. Therefore, the aims of the study were to compare the histological and molecular KPI of *C. carpio* wintering in a pond with no supplementary feeding (MCF), in a RAS fed typical (30% of protein and 8% of fat) carp diet (AFC), and in a RAS fed high protein (42%) and fat (12%) diet (ABF), and to assess whether wintering common carp in RAS can be a viable option to shorten culture time and contribute to the eco-intensification of common carp production in Europe.

2. Materials and Methods

2.1. The Experimental Trial and Fish

The ethical committee of the Faculty of Food Sciences and Fisheries, West Pomeranian University of Technology in Szczecin approved the fish trial. "Guidelines for the treatment of animals in behavioural research and teaching," published in *Animal Behaviour*, were adhered to during the trial [15].

The experimental setup was designed to tighten the production cycle from the traditional 33 months to 19 months with subsequent welfare improvement. The fish trial was performed at the Fisheries Research Station (FRS), Nowe Czarnowo, Poland (53°120′36″ N 14°270′48″ E) in a RAS system under a plastic tunnel in ambient, i.e., weather-dependent conditions. One week prior to the trial start, 1620 fish (47.18 ± 1.82 g) were obtained from the carp farm in Maliniec and distributed for acclimatisation (n = 270 per tank) in two sets of three tanks (n = 6), with a tank capacity of 2.7 m^3 each (Figure 2).

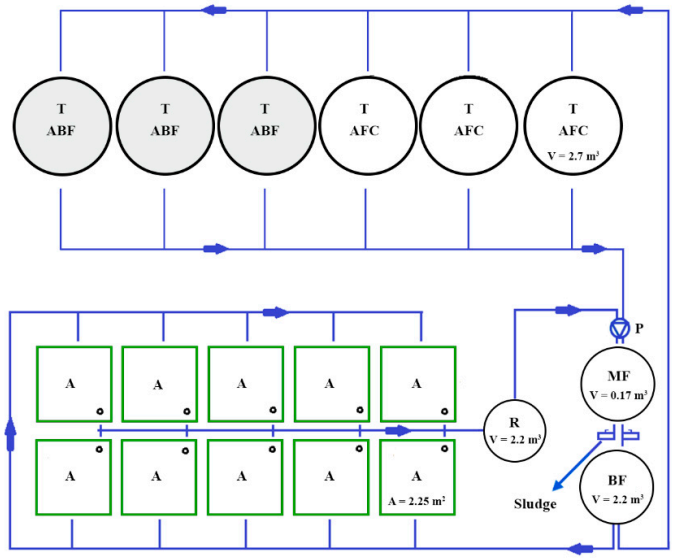

Figure 2. Schematic representation of the recirculating aquaculture system (RAS) used in this study. Abbreviations: T, fish tank; ABF, fish fed ABF diet; AFC, fish fed AFC diet; A, aquaponic tray; R, reservoir; P, pump; MF, mechanical filter; BF, biological filter. Blue arrows indicate water flow direction.

The 203-day trial (October–May 2019/2020) was performed in triplicate (n = 3 $diet^{-1}$). Fish were automatically fed with two commercial feed blends, with different levels of protein and fat, i.e., typical Agro-Fish Carp (AFC, 30% and 8%, respectively) and high energetic Aller Bona Float (ABF, 42% and 12%, respectively). Fish were fed diets at a rate of 1.5% body weight using automatic feeders. The rate of each diet was calculated based on information from biweekly bulk weighing of fish. During the trial, the temperature of the water in the RAS tanks was between 6.0 (January) and 26.2 °C (May), oxygen saturation between 40% and 82%, and pH value between 5.75 and 6.75. Traditional wintering was

performed in the Maliniec carp farm (MCF), (53°42′5″ N 15°21′22″ E) in an approx. 2.5 m deep wintering pond with a total surface area of 3 ha. During that period, the temperature in the wintering pond was between 4 and 8 °C until May, and feeding was not applied.

2.2. Sample Collection

At the end of the wintering trial, fish (n = 6) from each dietary treatment in RAS (n = 2 per tank) were sacrificed using a lethal dose of 2-phenoxyethanol (2 mL L^{-1}), (Sigma-Aldrich, Saint Louis, MO, USA). Simultaneously, fish from the wintering pond (n = 6) were sacrificed with the same procedure. Briefly, fish gills (2nd and 3rd arches), proximal intestine and head kidney samples were collected immediately and secured in DNA/RNA Shield™ (Zymo Research, Irvine, CA, USA) and, until RNA extraction, were stored at −80 °C. Additionally, a piece of proximal intestine bulb (approx. 5 mm), skin (1 × 1 × 0.5 cm) from the mid-dorsal epaxial body and 2nd and 3rd gill arch samples were collected and washed with deionized water and covered with 10% buffered formalin solution in 50 mL glass jars at room temperature for 5 h [16]. To assess the wintering and nutritional effects on carp in RAS, the following zootechnical parameters were calculated: final body weight (FBW) as an average weight of the carp at the end of the wintering period, and feed conversion ratio (FCR), Fulton's condition factor (K) and survival rate (SR) using the Equations (1)–(3):

$$FCR = FC \times WG^{-1} \quad (1)$$

$$SR = FN \times IN^{-1} \times 100 \quad (2)$$

$$K = (W \times 100) \times L^{-3} \quad (3)$$

where:

FC—feed consumed (g);
WG—weight gain (g);
FN—final number of individuals;
IN—initial number of individuals;
W—fish weight (g);
L—fish length (cm).

The data for MCF on Fulton's condition factor (n = 20), final body weight (bulk weighting of 100 fish) and survival rate (based on total biomass) were collected as part of the standard Maliniec carp farm screening after wintering, and none of the fish were harmed for the purpose of this study.

2.3. Total RNA Extraction and Synthesis of the cDNA

All preserved samples were homogenised in 750 µL Tri Reagent® (Zymo Research, Irvine, CA, USA) for 60 s using Minilys® homogenizer (Bertin Corp., Rockville, MD, USA). Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA), with DNase I treatment, to avoid contamination with genomic DNA. The extracted DNA was quantified and quality checked using NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and electrophoresis on 2% agarose gel. The 260/280 ratio of all RNA extracts was 1.8–2.1, and signs of RNA degradation were not observed. Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) and 1 µg of RNA were used for reverse transcription, following the manufacturer's instructions, using anchored oligo(dT)18 primers.

2.4. Assessment of Gene Expression in Gills, Intestine and Kidney

Real-time PCR was conducted on LightCycler® 480 II (Roche, Switzerland). LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland), 0.1 µM of each primer and 5 µL of 10× diluted cDNA templates were used for the reaction in the final volume of 20 µL. All reactions were performed under the following thermal profile: initial activation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and

extending at 72 °C for 15 s. At the end of each qPCR melting curve analysis (65–95 °C) was conducted to ensure the specificity of amplification. To ensure the absence of genomic DNA contamination, a random RNA sample was tested accordingly. Relative expression of the common carp genes in the 2nd and 3rd gill arches [*immunoglobulin M* (*IgM*), *interleukin 1 beta* (*il-1β*), *interleukin 6* (*il-6*), *interleukin 8* (*il-8*), *tumour necrosis factor alpha* (*tnf-α*), *mucin 5b* (*muc5b*), *lysozyme C* (*lysC*), *superoxide dismutase 1* (*sod1*), *catalase* (*cat*), *glutathione peroxidase* (*gpx*) and *glutathione S-transferase* (*gst*)], in the proximal intestine [*IgM*, *il-1β*, *il-6*, *il-8*, *tnf-α*, *muc5b*, *lysC*, *heat shock protein 70* (*hsp70*) and *heat shock protein 90* (*hsp90*)] and in the head kidney [*il-6*, *il-8*, *il-1β*, *tnf-α*, *IgM*, *muc5b*, *lysC*, *inducible nitric oxide synthase* (*inos*), *nuclear factor-erythroid 2-related factor 2* (*nrf2*)] were measured as well as two reference genes: *60S ribosomal protein L8* (*rpl8*) and *40S ribosomal protein S11* (*40sRNA*) (Table 1).

Decimal dilutions (ranging from 0.92 to 1.10) were performed to evaluate and correct the efficiency of qPCR reactions. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression using the GeneEx (MultiD Analyzes, Göteborg, Sweden) software [17].

2.5. Histomorphology of Gills, Skin and Intestine

Fixed in 10% buffered formalin samples of 2nd and 3rd gill arches, skin from the mid-dorsal epaxial body and proximal intestine fragments were dehydrated using alcohol and were saturated in intermediate solutions (benzene, benzene: paraffin) [16]. Next, the samples were embedded in paraffin blocks and then trimmed and serial sectioned (6 ± 1 µm, Rotary Microtome MPS-2, Opta-Tech, Warsaw, Poland). Gill and skin samples were stained with alcian blue and periodic acid–Schiff (AB/PAS, pH 2.5) [29]. Intestine samples were stained with haematoxylin and eosin (H&E) and alcian blue (AB) [30]. All samples were mounted with DPX mounting medium and covered by coverslips. Twelve glass slides (3 fish × 4 slides) for both RAS diets and traditional wintering and all tissue types were randomly selected and examined using an Eclipse E600 microscope (Nikon, Tokyo, Japan) with 100× objective and the NIS-Elements Basic Research software (Nikon Instruments Europe B.V, Amsterdam, The Netherlands). Gill samples were examined to assess the thickness of the epithelial tissue, and the number and area of the mucous cells (MC). In the skin samples, the thickness of the epidermis was assessed, and the ratio between the thickness of the epidermis (E) and the outer part (*stratum spongiosum*, SS) and the deeper part (*stratum compactum*, SC) of the dermis was calculated to compare the share of each layer between ABF, AFC and MCF fish. Moreover, the area of MC and the number of MC per 100 µm of epithelium were counted manually. The total number of acidic, neutral and mixed (acidic and neutral) mucin-containing cells per 100 µm of epidermis was counted. Intestine samples were examined to assess the size of supranuclear vacuoles (SNV), width of lamina propria (LP), number and area of goblet cells (GC) per villus, and thickness of sub-epithelial mucosa (SM). Gill, skin, and intestine samples were also checked against the presence of pathological changes. Measurements of histological section characteristics were made according to the methodology described earlier for different fish species [31–33].

2.6. Statistical Analysis and Data Visualisation

Throughout this paper, data are shown as mean ± standard deviation unless otherwise specified. The Shapiro–Wilk test (significance level $p < 0.05$) was used to assess the normal distribution of data. Depending on the normality of distribution, the ANOVA or the Kruskal–Wallis test and subsequently the Dunnett's, Tukey HSD and Dunn's post hoc test were used to assess significance of differences, using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). Visualisation of data was performed using R-package ggplot2 [34] and bio-render.com (access on 6 March 2022).

Table 1. Sequences of *C. carpio* primers used for qPCR analysis.

Gene		Primer Sequence 5′ -> 3′	Tm (°C)	Function	Reference
IgM [1]	F	TCGTATTAGCACCCCCAGAG	53.8	First line of host defence against infections	[18]
	R	TCATCAGCAAGCCAAGACACA	52.4		
il-1β [2]	F	CCTGAAGAAGAGGAGGCTGTCA	56.7	Mediator of inflammatory response	[19]
	R	AAGGAGGCCAGTGGCTCTGT	55.9		
il-6 [3]	F	CCGCACATGAAGACAGTGAT	51.8	Stimulating acute phase protein synthesis	[20]
	R	GGGTATATTTGGCTGCAGGA	51.8		
il-8 [4]	F	TGGAGCTCTTCCCTCCAAG	53.2	Attracting and activating neutrophils	[20]
	R	AGGGTGCAGTAGGGTCCAG	55.4		
tnf-α [5]	F	CCTTGGAAGTGACATTTGCTTTT	51.7	Signalling events within cells	[19]
	R	GCTGTCTGCTTCACGCTCAA	53.8		
muc5b [6]	F	CAGCCCTCTTCCTCTTTCATC	54.4	Ensure normal mucus clearance	[20]
	R	CCACTCATCTTTCCTTTCTCTTC	53.5		
hsp70 [7]	F	TGAGAACATCAACGAGCCCA	51.8	Protein maturation, re-folding and degradation	[21]
	R	TTGTCAAAGTCCTCCCCACC	53.8		
hsp90 [8]	F	AAAGACCAGGTCGCCCACTC	55.9	Protein maturation, re-folding and degradation	[22]
	R	AGTACTCGTCGATGGGCTCG	55.9		
sod1 [9]	F	TGGTCCACCGTGAGCTTTATT	52.4	Antioxidant enzyme	[23]
	R	GACAACACAAACGGCGGCAT	53.8		
gpx [10]	F	TGCAACCAGTTCGGACATCA	51.8	Catalyses the reduction of hydrogen peroxide	[24]
	R	GAAGCCATTTCCAGGACGGA	53.8		
cat [11]	F	CTGGAAGTGGAATCCGTTTG	51.8	Maintaining the cellular redox homeostasis	[25]
	R	CGACCTCAGCGAAATAGTTG	51.8		
gst [12]	F	TACAATACTTTCACGCTTTCCC	51.1	Protect cellular macromolecules	[26]
	R	GGCTCAACACCTCCTTCAC	53.2		
rpl8 [13]	F	CTCCGTCTTCAAAGCCCATGT	54.4	Ribosomal protein coding	[27]
	R	TCCTTCACGATCCCCTTGATG	54.4		
40sRNA [14]	F	CCGTGGGTGACATCGTTACA	53.8	Ribosomal RNA gene	[28]
	R	TCAGGACATTGAACCTCACTGTCT	55.7		

[1] *Immunoglobulin M*; [2] *interleukin 1 beta*; [3] *interleukin 6*; [4] *interleukin 8*; [5] *tumour necrosis factor alpha*; [6] *mucin 5b*; [7] *heat shock protein 70*; [8] *heat shock protein 90*; [9] *superoxide dismutase 1*; [10] *catalase*; [11] *glutathione peroxidase*; [12] *glutathione S-transferase*; [13] *60S ribosomal protein L8*; [14] *40S ribosomal protein S11*.

3. Results

3.1. Basic Performance and Welfare Indices of Carp Wintering in Pond and RAS

Final body weight of MCF fish (56.44 ± 9.80 g) did not differ significantly compared with the initial weight of the fish (47.18 ± 1.82 g). The final body weight of fish from RAS differed significantly ($p = 0.01$) between AFC (91.01 ± 3.05 g) and ABF (113.30 ± 4.71 g). Fulton's condition factor *K* was significantly ($p = 0.03$) lower for MCF fish (1.81 ± 0.20) compared with fish before overwintering (1.92 ± 0.12). Additionally, *K* was significantly higher ($p = 0.003$) for AFC (2.11 ± 0.17) and ABF (2.20 ± 0.19) fish compared with MCF fish and those before experimental RAS. The survival rate did not differ significantly between AFC (99.91 ± 0.19%) and ABF (99.54 ± 0.47%) fish but was higher than in traditional pond wintering (survival rate 70%, pers. comm. M. Gzyl). The feed intake was significantly

higher (p = 0.05) for ABF (129.91 ± 3.04 g) than for AFC (120.54 ± 4.61 g), and consequently FCR was significantly lower (p = 0.01) for ABF (1.98 ± 0.09) than for AFC (2.72 ± 0.07).

3.2. Histomorphology of the Gills, Skin and Intestine of Carp Wintering in Pond and RAS

Gill histology of carp demonstrated that the thickness of the epithelial tissue between the secondary lamellae in MCF was lower than in AFC and ABF (p = 0.01), as the ratio between the length of the secondary lamellae and the interlamellar cell thickness was 1.43, 3.96 and 2.92, respectively (Figure 3A–C).

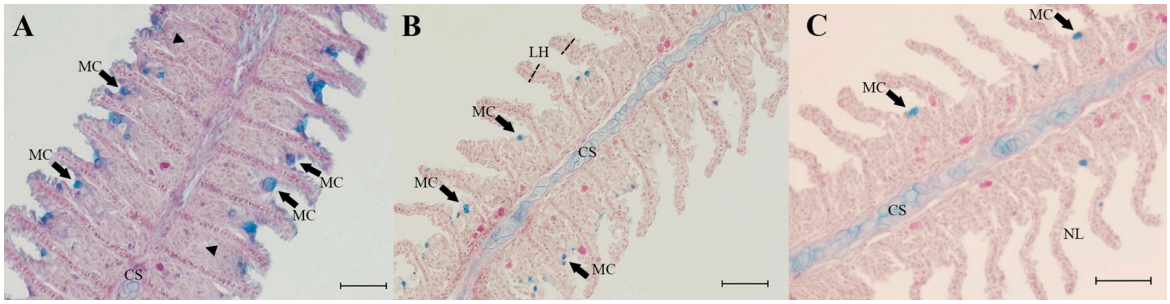

Figure 3. Histological sections of gills from *C. carpio* wintering in different conditions. (**A**) Fish from ponds (MCF); note the epithelial cell hyperplasia and numerous mucous cells (MC) seen in the interlamellar space. (**B**) Fish from RAS fed with typical carp diet (AFC); note less numerous MC and thickened lamellae caused by epithelial cell hyperplasia (LH). (**C**) Fish from RAS fed with high fat and protein diet (ABF); note low number of MC and normal appearance of lamellae (NL). CS, cartilaginous structure. AB-PAS (pH 2.5) reaction. Bar = 50 µm.

In some cases, in the gills of carp wintering in ponds, extensive epithelial cell hyperplasia led to complete lamellar fusions (Figure 4A). In carp wintering in the RAS, the gills of AFC carp had thicker secondary lamellae than in ABF carp due to epithelial cell hyperplasia (Figure 4B,C) and an increased number of mucous cells (Figure 4D). The number of mucous cells (MC) was significantly higher (p = 0.01) in the MCF group (n = 54 ± 3) than in the AFC (n = 17 ± 3) and ABF groups (n = 10 ± 2). Moreover, the size of MC was the largest (p = 0.01) in MCF (112.6 ± 26.96 µm^2) compared with AFC (44.8 ± 9.37 µm^2) and ABF (59.1 ± 28.01 µm^2) fish. Sections of skin were compared between fish wintering in RAS (ABF, AFC) and ponds (MCF) (Figure 5). The epidermis was thicker in AFC fish (64.71 ± 3.89 µm) than the other two groups, which were also different from each other (ABF 53.39 ± 3.32 µm, MCF 48.47 ± 3.05 µm). The measurements of the epidermis, *stratum spongiosum* and *stratum compactum*, and subsequent calculation of the ratio between the thickness of each layer (ABF 1:3.4:4.6, AFC 1:1.2:2.9, MCF 2.3:1:4) showed that, in all fish, SC was the thickest part in the ABF, AFC and MCF fish. The SS was the thickest layer both in ABF and AFC, except the MCF fish, in whom thickness of SS was the least according to the calculated ratio (Figure 5A–C). The overall skin morphology showed higher amounts of subcutaneous fat (adipose tissue) deposits in ABF fish (Figure 5B) than in AFC (Figure 5C). Lack of subcutaneous adipose tissue was observed between SS and muscle tissue in the skin of MCF fish (Figure 5A). The area of MC was the largest in ABF fish (153.5 ± 25.01), (Figure 5D) than in AFC (84.96 ± 19.43) and MCF (46.18 ± 21.43) carp (p < 0.001). The total number of acidic, neutral and mixed (acidic and neutral) mucin-containing cells per 100 µm of epidermis was the lowest in MCF fish (2.8 ± 1.1), (Figure 5E) and between fish from RAS fed typical carp (AFC 4 ± 0.7) and high (ABF 7.2 ± 0.8) fat and protein diets (p < 0.01), (Figure 5F).

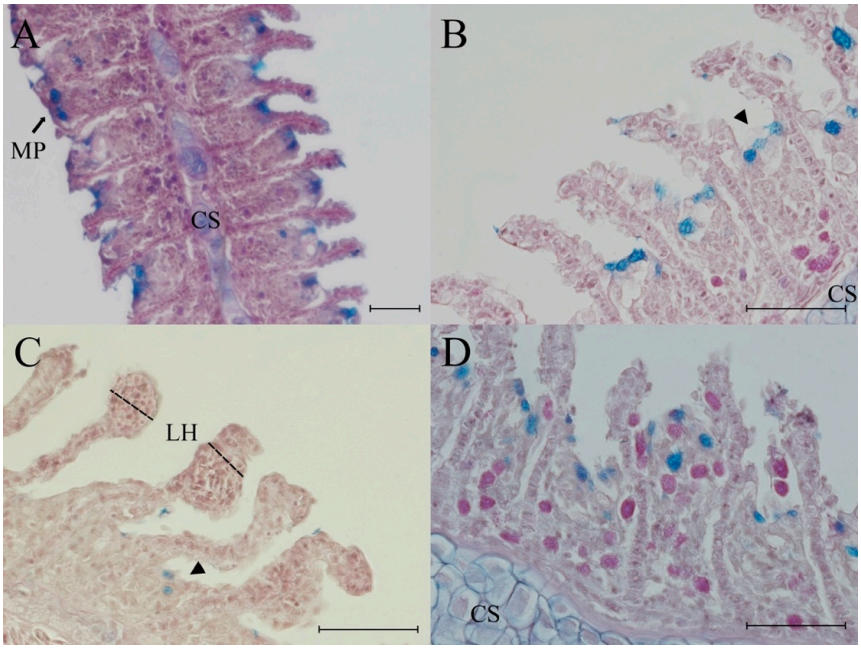

Figure 4. Histological changes in gills from *C. carpio* wintering in different conditions. (**A**) Extensive epithelial cell hyperplasia and metaplasia (MP) in the gills of fish from ponds (MCF). (**B**,**C**) Thicker secondary lamellae due to epithelial cell hyperplasia (arrowheads) and (**D**) numerous MC in the gills of fish from RAS fed with typical carp diet (AFC). CS, cartilaginous structure. AB-PAS (pH 2.5) reaction. Bar = 50 μm.

Results from the histological evaluation of the intestine samples showed that AFC fish had a significantly (p = 0.01) larger size of supranuclear vacuoles, smaller size of lamina propria, an intermediate number of goblet cells and lower thickness of submucosa compared with ABF and MCF. The GC area was significantly (p = 0.01) larger among AFC and MCF fish compared with ABF fish (Figure 6, Table 2).

3.3. Gene Expression in the Gills and Intestine of Carp Wintering in Pond and RAS

Gene expression in the gills showed that fish from traditional wintering (MCF) in the pond had a significantly higher relative mRNA expression of *il-6* and a high level (over 13-fold change) of *il-1ß* compared with fish from RAS (Figure 7). In contrast, the *IgM* and *gpx* activities were significantly lower in MCF compared with AFC and ABF. The expression of *sod1* was similar between both RAS groups and significantly higher in ABF compared with MCF. A difference between AFC and ABF was found in the expression of other oxidative stress-related genes (*cat* and *gst*). Expression in MCF was similar to that in AFC and ABF for cat and similar to that in AFC for *gst*.

The effects of wintering in the pond and RAS on the expression of genes in the intestine of carp are shown in Figure 8. Gene expression analysis in the intestine showed that fish overwintering traditionally in the pond had a significantly lower relative mRNA expression of *IgM*, *il-6*, *il-8* and *hsp90* than fish wintering in the experimental RAS (AFC, ABF). Additionally, the level of *il-6* transcripts in the intestine of fish fed AFC diet was significantly lower than in ABF carp. The transcript level of the mucin gene *muc5b* was the highest in the intestine of ABF fish compared with MCF and AFC carp. Gene expression of the third interleukin *il-1ß* and the chaperon *hsp70* were similar in the intestine of ABF and MCF fish, and significantly higher compared with AFC fish. No difference in the expression of *tnf-α* between fish wintering in the pond and RAS and between fish from RAS fed AFC and ABF diets were observed.

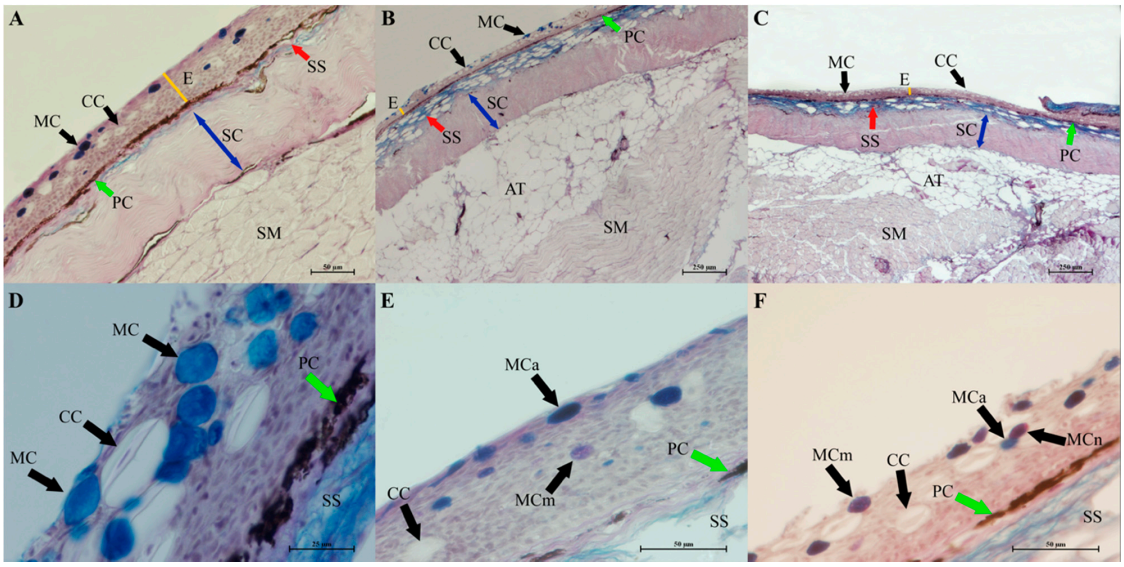

Figure 5. Histological changes in skin from *C. carpio* wintering in different conditions. Differences in skin layer structure of fish from ponds (MCF) (**A**), fish from RAS fed with high fat and protein diet (ABF), (**B**) and fish from RAS fed with typical carp diet (AFC). (**C**) Common carp and changes in abundance and morphology of different mucin containing cells in ABF (**D**), MCF (**E**) and AFC (**F**) fish. Abbreviations: E, epidermis; SS, outer part (stratum spongiosum) of dermis; SC, deeper part (stratum compactum) of dermis; PC, pigment cells; AT, adipose tissue; SM, smooth muscle; MC, mucous cells; MCa, acidic mucous cells; MCn, neutral mucous cells; MCm, mixed mucous cells; CC, club cells. AB-PAS (pH 2.5).

Analysis of relative mRNA gene expression in the kidney samples showed higher expression of *IgM* in the ABF than AFC and MCF carp. The transcript level of *il-1β* was the highest in the MCF fish to the other two groups, which were not different from each other. The expression of the *tnf-α* and *nrf2* genes was the lowest in AFC carp compared to ABF and MCF, which had similar level of transcripts in the kidney samples (Figure 9).

The study also compared relative mRNA expression of *lysC*, *IgM*, *il-1β*, *il-6*, *il-8*, *tnf-α* and *muc5b* between the samples of gills, intestine and kidney individually for the ABF, AFC and MCF carps. In most of the instances, analysed genes in MCF fish had the lowest expression in the intestine (*il-6*, *il-8*, *tnf-α*) or was similar either to the expression level in gills (*IgM*) or kidney (*lysC*). Only in the case of the *il-1β* there was an intermediate level of expression in the intestine found compared to the two other samples (Figure 10A). In the AFC, all the genes had the lowest expression in the intestine samples for the gills and kidney, which were not different from each other (Figure 10B). Fish fed a high fat and protein diet (ABF) had the lowest transcript levels of *lysC*, *IgM*, *il-8* and *tnf-α* in intestine samples. The relative mRNA expression of *il-1β* and *il-6* was higher in gills and intestine than kidney, while the expression of *muc5b* was significantly lower in kidney than gills (Figure 10C).

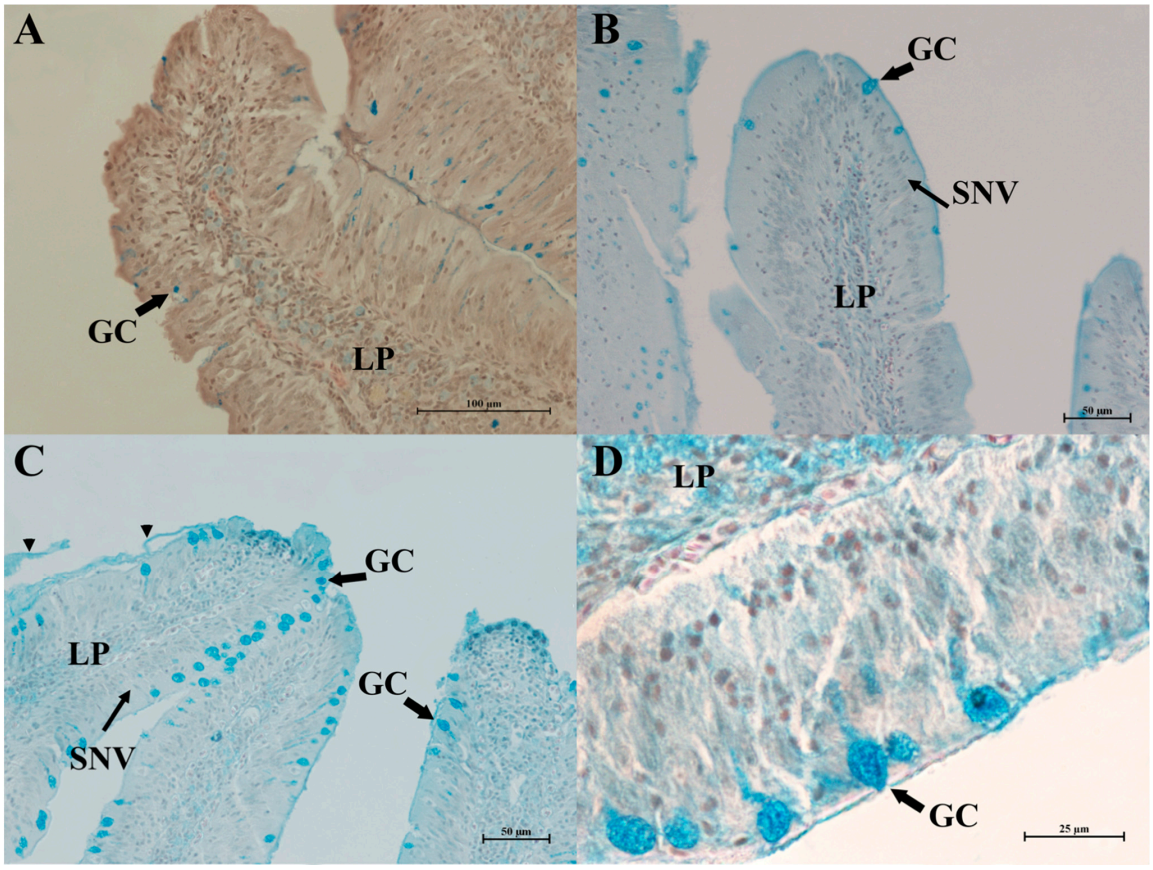

Figure 6. Histological sections of intestinal fold from *C. carpio* wintering in different conditions. (**A**) Fish from ponds (MCF); note low number of goblet cells (GC), lack of supranuclear vacuoles (SNV) and widened lamina propria (LP). (**B**) Fish from RAS fed with typical carp diet (AFC); note increased number of GC and presence of SNV. (**C**) Fish from RAS fed with high fat and protein diet (ABF); note numerous GC, copious amounts of mucin (arrowheads), and presence of SNV. (**D**) ABF fish; note numerous GC.

Table 2. Intestine histological parameters of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets.

Parameter/Fish Group	MCF	AFC	ABF
SNV (µm)	ND	23.2 ± 3.13^{a}	18.8 ± 1.67^{b}
LP (µm)	42.4 ± 11.69^{a}	19.3 ± 3.93^{b}	24.5 ± 5.16^{c}
GC (n)	28.0 ± 2.45^{a}	57.6 ± 3.44^{b}	73.5 ± 7.77^{c}
SM (µm)	72.9 ± 9.68^{a}	45.1 ± 8.31^{b}	58.9 ± 4.56^{c}
GC (μm^2)	50.7 ± 13.54^{a}	50.7 ± 9.95^{a}	41.7 ± 10.63^{b}

Abbreviations: SNV, supranuclear vacuoles; LP, lamina propria; GC, number of goblet cell per villus; SM, sub-epithelial mucosa; ND, not detected. Different lowercase letters in rows indicate significant differences between values ($p \leq 0.01$). Values were compared with parametric tests.

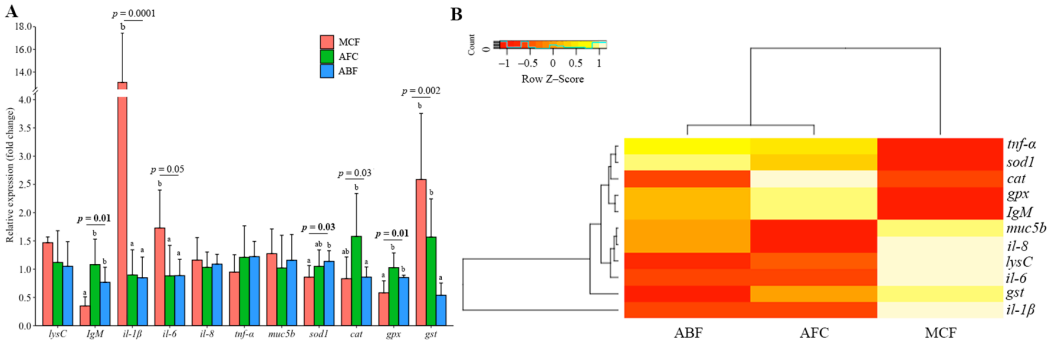

Figure 7. Gene expression profiles (**A**) and heatmap analysis (**B**) for gills of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets. Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

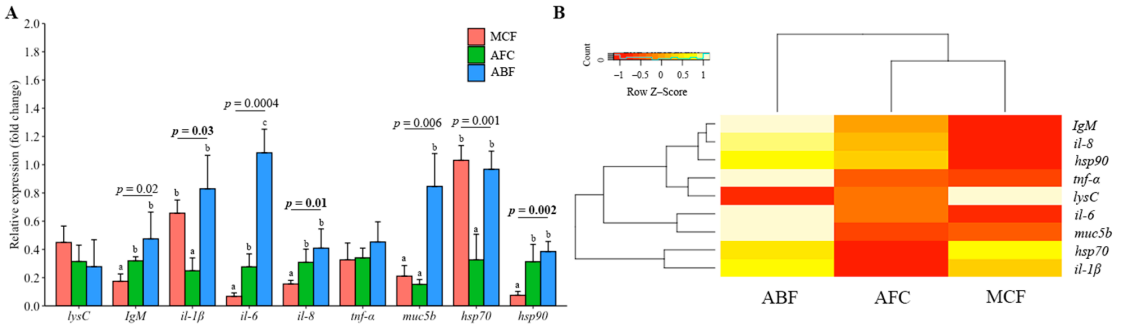

Figure 8. Gene expression profiles (**A**) and heatmap analysis (**B**) for intestine of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets. Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

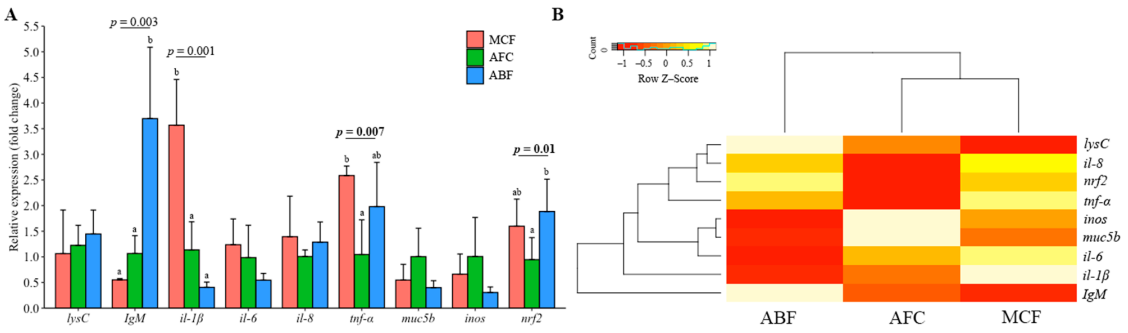

Figure 9. Gene expression profiles (**A**) and heatmap analysis (**B**) for kidney of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets. Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

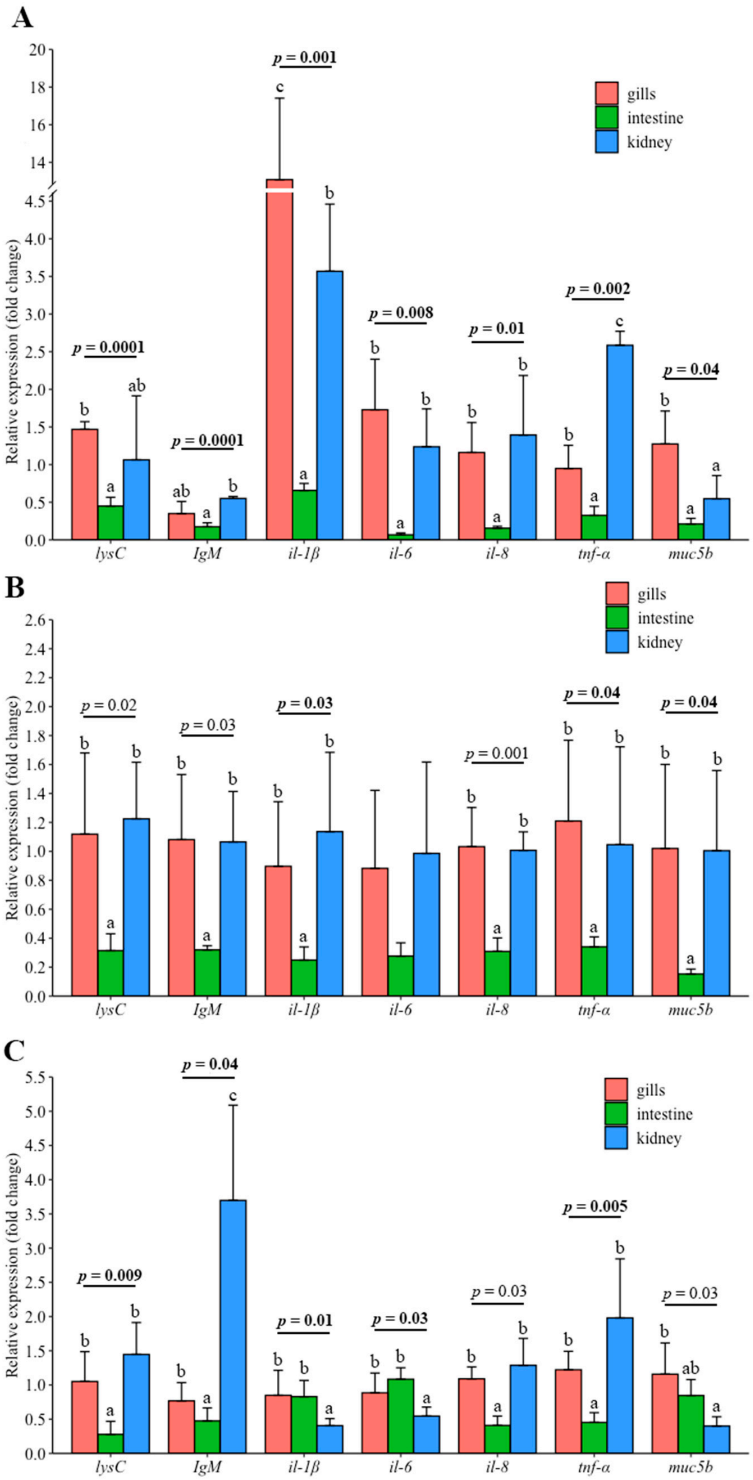

Figure 10. Tissue-specific (gills, intestine, kidney) gene expression profiles of common carp wintering in ponds (**A**, MCF); RAS fed with typical carp diet (**B**, AFC); RAS fed a high fat and protein (**C**, ABF). Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

4. Discussion

4.1. Differences in Weight Gain, Condition Factor and Mortality of Carp Overwintering in Earthen Ponds and RAS

Multi-stage farming of common carp in Europe, here referred to as the Dubisch style, since the early beginning, has included two wintering periods in >2 m deep earthen ponds until spring when the temperature profile and primary production in ongrowing ponds are favourable to continue production. During wintering that lasts usually from late October to May fish were restricted from feeding and their activity, including metabolic, depending on water temperature [7]. Our study showed minor weight loss and lowered (5.7%) condition in the MCF fish after wintering. According to Lukowicz and Gerstner [35], overwintering of common carp is considered successful if condition factor *K* does not decrease more than 15–20%. Additionally, Geldhauser and Gerstner [36] showed that weight loss of 5–10% is a typical observation for conditions of Central European aquaculture in winter. The fish from RAS (AFC, ABF) had significantly higher weight and better conditions than those wintering in the pond (MCF), which may be due to more favourable thermal conditions and adapted feeding programme. The differences in the final weight between fish wintering in RAS could be attributed to the composition of the feeds, mainly the content of the lipids. In ectothermic common carp the energy is primarily stored in the form of lipids that must be assimilated before the winter period when animals are decoupled from the resource base [37]. In RAS, the ABF carp were fed with the diet containing a higher content of lipids (12%) than AFC (8%), therefore efficiently utilising this compound to meet metabolic demands and continuing to build body mass.

Fish overwintering in earthen ponds may face stress from starvation, cold, and predators which together or individually may disturb physiology and lead to huge economic losses in aquaculture due to elevated mortality rates [38]. Common carp farming includes two wintering stages (W1 and W2) in the colder months (October through April) in subsequent years. Survival rate of fish after W1 and W2, even concerning severity of winter conditions, is significantly lower in one-summer-old carp (50%) than two-summer-old carp (80–95%) [39]. In our study, carp after W1 reached a much higher survival rate of 70% that presumably stems from the efficient accumulation of lipid reserves during warmer, more productive months and low impact of common stressors such as too high stocking density, low water quality and injuries caused by handling, parasites, and diseases [37,40]. The higher survival rate of MCF fish, as well as insignificant weight loss and decreased condition factor, surprise even more, as small-bodied fish have a smaller capacity for energy storage and a higher mass-specific metabolic rate [41]. Thus, they are more susceptible to over-winter starvation and mortality than larger bodied individuals, i.e., two-summer-old carp which can accumulate larger absolute and relative lipid levels, and in the case of the Maliniec carp farm, experience 10% mortality during W2 ([42], pers. comm. M. Gzyl). Survival rate of AFC (99.91 $\pm$ 0.19%) and ABF (99.54 $\pm$ 0.47%) carp wintering in RAS was significantly higher than in the fish from earthen pond but was also better in relation to *C. carpio* cultured in experimental RAS (93.33 $\pm$ 5.70) and comparable to the two biofloc technologies (BFT1 97.57 $\pm$ 3.70, BTF2 96.67 $\pm$ 5.70) and the system with 50% water exchange method (98.67 $\pm$ 1.20) [43]. Such low mortality rate of AFC and ABF fish might be contributed to high quality of water, lack of pathogens and feeding that directly influenced the growth and health state of carp in RAS.

4.2. Influence of Wintering Practice on the Histological and Molecular Indices of Carp Gills

The relative mRNA expression of two pro-inflammatory cytokines, namely *il-1β* and *il-6*, was upregulated in MCF, while the relative mRNA expression of *tnf-α* remained comparable to that in AFC and ABF. A high number of *il-1β* transcripts and, consequently, *il-6* may suggest an ongoing inflammation in MCF fish [44]. Moreover, the epithelial cell hyperplasia visible in the histological picture of MCF gills has been previously linked with inflammation processes caused by different pathogens in Atlantic salmon, *Salmo salar* [45] and rainbow trout, *Oncorhynchus mykiss* [46]. However, no difference in the expression of

il-8 suggests the absence of acute inflammation in favour of chronic inflammation [47]. It is also plausible that changes in cytokine expression are a result of prolonged starvation [48], which in turn creates chronic stress that also influences the immune response of common carp [49]. Moreover, the increased number of MC, and their size, in the gills of MCF fish compared with AFC and ABF suggests more stressful conditions (e.g., water quality) in the wintering pond [50], despite the lack of differences in the transcript level of *muc5b*, encoding a major gel-forming mucin in mucus.

The upregulation of *IgM* in ABF and AFC compared with MCF found in our study may be a result of a higher bacteria load (especially of those pathogenic for carp) present in RAS [51,52]. Such conditions result from higher water temperature in RAS compared with the wintering pond, since both bacterial activity and fish productivity depend on water temperature [53]. In contrast, similar *muc5b* levels in MCF, AFC and ABF may indicate the absence of pathogenic bacteria in both wintering systems, since in channel catfish (*Ictalurus punctatus*), microbial infection has been seen to upregulate the activity of *muc5b* in both the gills and the intestine [54]. To comprehensively understand the influence of wintering on carp, high throughput methods (e.g., RNA-seq) combined with histological observations should be implemented.

We revealed differences in the expression levels of oxidative stress response-related genes (*sod1*, *cat*, *gpx* and *gst*). The higher expression of *gst* in MCF compared with that in ABF could be explained by prolonged starvation of fish in the wintering pond [55]. Moreover, the significant difference between AFC and ABF, and no difference between AFC and MCF, could be related to the lower energy content in the AFC feed, which presumably did not fully cover the needs of fish during the wintering period [56]. The lower expression of *sod1* and *gpx* in MCF compared with that in ABF suggests a more complex response to winter and different winter-related conditions such as starvation and multiple factors influencing the expression level. For instance, differences in the number of *sod1*, *gpx* and *cat* transcripts could be related to the level of minerals in formulated feeds [57,58]. Additionally, the presence of Zn can inhibit the GST activity in vitro [59], which we observed in vivo at a molecular level, since ABF feed contained the highest Zn level (50 mg kg^{-1}). Water flow could be another factor influencing the downregulation of *gpx* in MCF, since there is no water flow in the wintering pond, while in RAS, water flows constantly (1.7 m^3 h^{-1}), thus creating relatively stable conditions in terms of oxygen level [60]. The lack of water flow in the wintering pond may result in hypoxic and high ammonia conditions [61], which also alters oxidative stress response in fish [62]. Suboptimal conditions in the wintering pond could potentially decrease the expression of *sod1* and *gpx* and cause pathological changes in the gills (complete lamellar fusions), as reported for largemouth bass (*Micropterus salmoides*) exposed to hypoxia and high ammonia levels [63]. However, this does not explain the higher expression of cat in AFC compared with that in ABF, and the difference is most probably caused by dietary differences, i.e., lipid and protein levels [64,65].

4.3. Influence of Wintering Practice on the Histological and Molecular Indices of Carp Intestine

A lower number of *IgM* transcripts in the intestine of MCF fish than those from RAS might result from low water temperature (4–8 °C) and ceased feeding during the wintering period. The relatively stable conditions in deep earthen ponds and fish thriving in suboptimal conditions [42] shaped a relatively constant diversity and richness of the gut microbiota [66]. Some of the gut residents are commensal microbiota populations that have been described as recognizable by immunoglobulins [67], thus having a beneficial effect on the immune system of fish [68]. According to Bisht et al. [69], the intestinal bacterial count, including those that maintain gut health, in *C. carpio* was higher in the winter compared with the summer season. In contrast, the increased expression of *IgM* in the intestine of AFC and ABF fish wintering in the experimental RAS was enriched by more complex internal and environmental stimulants (microbiota, feeds, water temperature up to 20 °C). As evidenced by Eichmiller et al. [70], differences in the microbiota of common carp may be due to a combination of the effects of diet, habitat usage, temperature and physiology,

and the populations of gut residents may adapt via a specific and dynamic interplay with immunoglobulins [67].

Analysis of gene expression of pro-inflammatory cytokines to some extent showed a relatively consistent picture and explained observations identified in the MCF, AFC, and ABF intestine slides during histomorphological evaluation. The relative mRNA expression of *il-1β* in the intestine of fish from the wintering pond and from RAS fed the ABF diet was the highest and coincided with the highest severity of enteritis found in these fish compared with AFC carp. However, the source of these observations seems to be different, as MCF fish had enteritis due to prolonged starvation during overwintering in earthen ponds. ABF fish were fed diets with a high amount of energy coming from the increased portion of fat and protein in the administered diet. In the case of fish from the pond, seasonal wintering of fish is a typical procedure in carp farming in Central and Eastern Europe [8], and the species is habituated to tolerate winter temperatures close to 0 °C [71]. Liang et al. [72] reported that adaptation of *C. carpio* to low temperatures is the result of long-term evolution, but differences in the mechanisms of survival at cold temperature have been found among various populations or subspecies. In our study, the high transcript level of the *il-1β* gene in the intestine of MCF fish is directly related to the regulation of the inflammatory processes that developed due to specific conditions during overwintering. As the inflammatory response of the immune system progresses, *il-1β* induces expression of subsequent pro-inflammatory genes, such as *il-6*, *il-8* and *tnf-α* [73]. Apparently, in the case of MCF fish, the severity of physiological changes in the intestine remained at the "typical-for-wintering" level, and further exacerbation of the immune response was unjustified. In contrast, the limited stimulatory relative mRNA expression of the *il-1β* gene in MCF was not observed in RAS fish, as the expression of both *il-6* and *il-8* was ramped up in the latter, specifically in ABF carp fed a high fat and protein diet. The cause of the observed differences in the transcript levels of *il-1β*, *il-6* and *il-8* between AFC and ABF fish arose directly from the feed composition, as fish in the RAS were managed according to the same procedures. ABF fish fed a more energetic diet reached higher final body weight (113.30 ± 4.71 g) compared with AFC carp (91.01 ± 3.05 g). Furthermore, histological evaluation showed symptoms of diet-induced enteritis (thickened lamina propria and sub-epithelial mucosa, altered supranuclear vacuolization, increased number of goblet cells in the epithelium) in the intestine of ABF carp. Despite the presence of "non-infectious sub-acute enteritis", as named by Baeverfjord and Krogdahl [74], Fulton's body condition factor *K* as well as survival rate did not differ significantly between AFC and ABF fish. These results appear promising, as the RAS fish, heavier and in better condition compared with those wintering in the pond, will be transferred to ongrowing earthen ponds in the spring to reach market size (~1.2 kg) within the following 6–7 months (until November) to be harvested before Christmas. The lack of difference in the relative mRNA expression of the *tnf-α* gene between fish groups may suggest that in the intestine, neither acute infection nor ongoing pathogenesis of several chronic diseases were present. The plausible explanation for the comparable number of *tnf-α* transcripts among variants is that this cytokine plays a key role in regulating inflammation, but some of its functions may overlap with *il-1β* [73].

Significant differences, during both the molecular and histomorphological assessments, were noticed regarding mucin production in the intestine of carp. At the molecular level, the transcript level of the *muc5b* gene was the highest in ABF fish, and the histological assessment showed apparently large amounts of mucin found in the intestine of these fish. However, in addition to *muc5b*, the production of mucins is dependent on the expression level of *muc2b*, and according to Marel et al. [75], the latter gene is the main one expressed in the first and second intestinal segments of *C. carpio*. Therefore, the massive amounts of mucins in the intestine of ABF fish could also be due to the high activity of *muc2b*. Nevertheless, our findings show that *muc5b* transcripts were, in addition to the brain, liver, skin and gills [75], detected in the intestine of common carp. The expression of *muc5b* in the gills was significantly higher than in the intestine of AFC ($p = 0.04$) and MCF ($p = 0.04$) carp, but not in ABF. As the relative mRNA expression of *muc5b* in the intestine of MCF and

AFC fish was similar, the upregulation of this gene in ABF fish was stimulated by the feed. Similar results were obtained by Smith et al. [76] who showed that feeds with different inclusion levels of laminarin, a seaweed-derived ß-D-glucan, led to a significant increase in *muc2* and *muc4* expression in the intestine. Further studies in carp should also include the assessment of other mucin genes, as the abundance per mucosal fold of the goblet cells was the highest in the intestine of MCF carp, and surface area was the largest in MCF and AFC fish, while the number of *muc5b* was similarly the lowest.

Tissue damage and the release of danger signals, such as heat shock proteins, may trigger the expression of pro-inflammatory cytokines [77]. According to this, in our study, the high number of *hsp70* transcripts in the intestine of ABF and MCF fish may have influenced the expression of the *il-1β gene*, which subsequently induced the expression of subsequent pro-inflammatory genes, as shown for *il-6* and *il-8*. This mechanism was also described by Li et al. [78] in the various tissues, including the intestine, of common carp after exposure to glyphosate-based herbicide (GBH). Their results also showed that, in addition to *hsp70*, the number of *hsp90* transcripts also increased in response to GBH. In the case of our study, the expression profile of *hsp90* differed from that of *hsp70*, indicating that conditions in the RAS induced higher expression of *hsp90* in the intestine of *C. carpio* compared with fish wintering in the pond. The *hsp70* and *hsp90* genes collaborate in numerous cellular remodelling reactions, but according to Genest et al. [79], each of them carries out some chaperone activities independently.

4.4. Influence of Wintering Practice on the Skin Histology of Carp from Pond and RAS

Lower thickness of the epithelium of the MCF fish than those from RAS resulted from an adaptive response to wintering conditions, allowing this species to withstand 203-day food limitation and lower temperatures. Similarly, Caruso et al. [80] in European eel (*Anguilla anguilla* L.) and Somejo et al. [81] in Nile tilapia found that the average thickness of the epidermis was lower in starved fish than those fed. Additionally, Landeira-Dabarca et al. [82] showed that Atlantic salmon deprived from food for 18 days significantly reduced epithelial tissue turnover and activity. This is in line with our other observations showing that AFC fish fed a less energetic diet had significantly thinner epithelium than ABF due to presumably reduced metabolic rate. The thickness of fish skin is also determined by factors such as species, age, and body region [83]. Therefore, to assess the overall influence of the wintering method on the epidermis, *SS* and *SC* of the MCF, AFC and ABF carp, the ratio between thickness of each layer was calculated. Here, the lowest thickness of the *SS* in the MCF carp showed that overall conditions in the wintering directly reduced activity of this layer. Among all the layers, *SS* is composed of loosely arranged connective tissue and holds fine blood capillaries, nerves, pigment cells and in some species or fish lines, scales [83]. The lower activity and thus the reduced thickness of the *SS* and epidermis may be restored or even increased after exposure to stressors as shown in the studies with common carp and rainbow trout [84,85]. Feeding ABF carp with a diet containing higher levels of fat and protein increased the area of subcutaneous adipose tissue in those fish. Wang et al. [86] demonstrated that feeding the Nile tilapia with high-fat diets increased the deposits of visceral and subcutaneous fat, whereas starved fish increased ß-oxidation of monounsaturated fatty acids in the subcutaneous layer to cover current metabolic demands and reduced the content of adipose tissue in general. The observation is also in line with the results of image analysis that showed a lower fat density in the subcutaneous region in the starved gilthead seabream (*Sparus aurata*) [87].

Limited food availability in the wintering pond presumably reduced both the number and the size of the MC in the skin of the MCF fish. Mucous cell population in the skin shrinks in consequence of limited availability of dietary carbohydrates that are essential to synthetise O-glycosylated glycoproteins, i.e., mucins [82]. During warmer months (May through October), unlike during the winter period, carp utilise a combination of natural food and supplementary feeding with carbohydrate-rich (up to 35–45% of the total diet) cereal grains such as wheat, triticale, maize, barley and rye [88]. Kideys and Hartnoll [89]

described a decline in mucus layer production as an energy-saving mechanism under adverse environmental conditions. In RAS, carp were fed with diets containing different, i.e., 26% (AFC) and 28.4% (ABF), levels of carbohydrates. The difference in the number and size of MC between AFC and ABF carp only to some extent may have resulted from the level of carbohydrate in the feeds itself. Presumably, the ABF feed with higher digestible energy (16.8 MJ kg^{-1}) than AFC (15.29 MJ kg^{-1}) more efficiently covered nutritional requirements of ABF carp and thus had a positive impact on the population of skin MC. In a study on Atlantic salmon fed feeds based on marine- or plant-derived ingredients and characterised by different energy digestibility, two ways by which plant-based diets can alter volumetric density and size of MC were identified [90]. Reduced production and secretion of skin mucins may expose fish to a higher risk from pathogenic microorganisms, viruses or parasites [91]. In our study, no clinical signs of disease caused by ectoparasites were observed; however, in our opinion, approx. 30% mortality of common carp during overwintering to some extent might have been caused by pathogens such as a ciliated protozoan, *Ichthyophthirius multifilis* [92], whereas in RAS, the health and welfare of fish were checked during routine daily maintenance, and almost no common carp mortalities were observed in the feeding groups. Losses of the ABF and AFC fish were minimised as a consequence of a well-adjusted feeding programme, and it is difficult to point out the cause.

4.5. Gene Expression in Kidney under Carp Wintering in Ponds and RAS

The increased expression of *il-1β* in the MCF carp is a commonly observed case of immunometabolism, an interplay between metabolic and immunological processes that was induced by prolonged chronic stress resulting from restricted feeding [93]. According to Liao et al. [94] and Reuter et al. [95] restricted feeding activates the antioxidant system, and once it is insufficient to clear the damage caused by starvation, numerous transcription factors can be activated, such as nuclear factor kappa light chain enhancer of activated B cells (NF-κB). In line with the current biological understanding, NF-κB can induce expression of the *il-1β* gene, leading to induction of inflammation and subsequent expression of the other pro-inflammatory gene *tnf-α* [96,97]. However, upregulation of the *tnf-α* in the kidney of the ABF fish wintering in RAS was presumably triggered by other or additional mechanisms than by the *il-1β* itself. The plausible explanation for the observed difference between the expression level on the *tnf-α* in the kidney of the ABF and AFC carp is the composition of the diets. Tumour necrosis factor-α emerged as a key cytokine that influences intermediary metabolism, and in the study on Swiss mice fed a high-carbohydrate and high-fat diet, only the former was able to increase TNF-α concentration in the liver [98]. Diets rich in various carbohydrates were also found to acutely activate NF-κB and subsequently the synthesis of pro-inflammatory cytokines, namely IL-1, IL-6 and TNF-alpha [99]. In the study on blunt snout bream (*Megalobrama amblycephala*), hepatic NF-κB, TNF-α, IL-1β and IL-6 expression in fish fed diets were all significantly increased, with increasing dietary carbohydrate levels [100]. Our study showed that expression of *nrf2*, similarly to *il-1β*, was higher in MCF and ABF fish compared to AFC. However, upregulation of the *nrf2* in the MCF and ABF may have different backgrounds. In MSC fish, overwintering in pond with limited access to food decreased the energy state of an organism and plasma glucose levels, leading to activation of the AMPK/SIRT1 axis that enhanced the expression of the *nrf2*, an oxidative stress regulator [101,102], whereas feeding ABF fish with a diet containing higher content of carbohydrates than AFC could cause physiological stresses and increased renal transcription of the *nrf2* in the kidney [103].

A carbohydrate-rich ABF diet possibly also increased synthesis of *IgM* transcripts in the kidney of carp since NF-κB controls the expression of numerous downstream genes that control cell proliferation, survival, stress responses, and immune functions [104]. Furthermore, a study on grass carp head kidney leukocytes showed that the TNF-α-mediated NF-κB pathway is an important signalling involved in protective immune and inflammatory responses [105]. Higher expression of *IgM* in the kidney of the ABF carp was exclusively impacted by feed composition, as both AFC from RAS and MCF overwintering

in the pond showed similar expression of the gene. Krogdahl et al. [106] showed that feeding Atlantic salmon with diets containing soybean molasses, an alcohol extract of soybean meal, causes an inflammatory response in intestinal mucosa as evidenced by the increased level of *IgM*. Other studies showed that the difference in the *IgM* expression in the intestine of gilthead seabream fed a vegetable and fish oil diet further increased when animals were exposed to adverse conditions, e.g., infection with the *Enteromyxum leei* [107]. However, the underlying mechanisms involved in *IgM* activity, especially in the kidney of common carp under different wintering conditions, still remain obscure and warrant further studies.

4.6. Differences in Gene Expression between Gill, Intestine and Kidney in Fish under Different Wintering Conditions

Differences in gene-relative mRNA expression emerge directly from the type of tissue that expresses inherent functions and are additionally challenged by internal and external stimuli. Regardless of the fish group (wintering conditions) the lowest gene expression of the seven analysed genes in most cases was observed in intestine samples. It was especially evident in the samples of AFC fish, whereas in MCF and ABF, the differences were more diverse. All the genes are involved in an array of defence mechanisms, and as evidenced in our study, the gills and the kidney are the primary site for their expression. Gills have a large epithelial surface and are constantly exposed to external milieu; thus, its function goes beyond respiratory gas exchange. To respond to all these challenges actively and accurately, the gills are equipped with their own immune system, called the gill-associated lymphoid tissue (GIALT), thereby substantially contributing to overall fish health and survival [108]. In the case of the kidney, a primary lymphoid organ, elevated expression of the genes in the ABF, AFC and MCF *C. carpio* is a common observation that results from the nature of this organ. Wu et al. [109] showed that comparing other studied organs, including intestine, gene expression of *il-1β* in the kidney and gills was significantly higher, and the observed difference has dramatically increased in the large yellow croaker (*Larimichthys crocea*) upon bacterial infection. Our results were also consistent with some other previous studies on Atlantic halibut, *Hippoglossus hippoglossus* [110], rainbow trout [111] and sea bass, *Dicentrarchus labrax* [112], where the primary role of gills in immunological responses was evidenced. The lower level of expression of most of the genes in the intestine might be related to the concentration and composition of intestinal commensal bacteria [113]. McEntee et al. [114] underlined that homeostasis in the gut depends on the activity of specialised immune cells that are regulated by a cross-talk with symbiotic microbes in the lumen. The overall composition of the microbiota in the intestine of the ABF, AFC and MCF carp is another variable which likely differed due to food/feed quality and availability and the environment (e.g., water temperature) in which the animals were overwintering. For example, the unique influence of multiple and synergic overwintering parameters on gene expression was shown for *il-1β* and *il-6*, which had similar patterns of their transcript level in the gill, kidney and intestine samples from MCF and AFC carps, but not from ABF. The underlying molecular mechanisms in common carp wintering in different conditions still require further and more detailed studies to define the role of symbiotic microbes and environmental factors that determine the performance of fish at the beginning of the next ongrowing stage in spring.

5. Conclusions

Despite being an important stage in a common carp farming, little is known regarding the response of fish to wintering conditions. In this communication, for the first time, we have identified that fish wintering in ponds due to limited access to natural food base and cold-water wintering conditions experienced loss of weight and mortality of 30% compared to common carp from RAS that maintained weight gain and in which only a low number of mortalities was observed during the trial. This observation confirmed the hypothesis that fish overwintering in RAS have better conditions to presumably reach

market size in the second, instead of the third, year of production. The histomorphological examination confirmed that fish adapted physiological responses to the wintering conditions by: (i) adjusting the thickness of layers (epithelium, *stratum spongiosum*, subcutaneous adipose) and changing the population of mucous cells in the skin; (ii) thickening of the interlamellar mass (metaplasia) in the gills; (iii) altering the size of, e.g., supranuclear vacuoles and lamina propria in the intestine. At the molecular level, the relative mRNA expression of the *muc5b* gene was the highest in ABF fish, and the histological assessment showed apparently copious amounts of mucin found in the intestine of these fish, whereas the transcript level of pro-inflammatory cytokines in gills, namely *il-1β* and *il-6*, was up-regulated and correlated with severe lamellar fusions in MCF fish. Here, we described that prolonged chronic stress resulting from restricted feeding increased expression of *il-1β* in the kidney of the MCF common carp, while feeding ABF fish with a diet containing a higher content of carbohydrates than AFC caused physiological stresses and increased renal transcription of the *tnf-α* and *nrf2* in the kidney.

Implementation of eco-intensification can potentially bring economic benefits to carp farmers. Wintering in RAS can save resources required for pond maintenance, fuel and fish for stocking, as well as solve problems with piscivorous predators and water shortages occurring due to climate change. However, investment in RAS facilities can be high, especially for large carp farms; therefore, a true economic analysis should be performed site specific to ensure that eco-intensification of *C. carpio* will bring financial benefits and sustainability to the carp sector.

This study has generated novel findings, insights and ideas which represent an important milestone for the common carp model and paves the way for more focused studies to be performed in the future with regard to the key performance indicators. The histomorphological changes and expression of the genes can be used as biomarkers to further delineate the influence of wintering conditions on fish performance that is crucial to shorten the time to produce market-sized carp, which can help to ensure both sustainability and food security in the EU.

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OŚWIADCZENIE DO PRACY P2

W pracy **P2** byłem zaangażowany w przeprowadzenie doświadczenia, pobór i zabezpieczenie prób biologicznych, wykonanie analiz molekularnych oraz histologicznych, analizę i interpretację uzyskanych wyników, przygotowanie grafik, przygotowanie manuskryptu artykułu, udzielenie odpowiedzi na recenzje oraz naniesienie zasadnych poprawek do zrecenzowanej pracy. Swój udział określam na 60%.

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Plasma biochemistry, gene expression and liver histomorphology in common carp (*Cyprinus carpio*) fed with different dietary fat sources

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ABSTRACT

Demand for omega-3 long chain polyunsaturated fatty acids has become global challenge for aquaculture and different components have been used to increase nutritional value of fillets. The aim of this study was to evaluate influences of feeds on zootechnical parameters, biochemical plasma parameters, expression of lipid-dependent genes, hepatocyte histomorphologies, and fatty acid profiles in common carp fillets. We compared a control diet (CTRL), mimicking a commercial feed formulation for common carp, with three diets containing blends of vegetable oils and a DHA-rich alga (*Schizochytrium* sp.) included at 3.125% (CB1) or 1.563% (CB2), and 2.1% salmon oil (CB3). The study revealed no differences in final body weight of fish fed CB1-3 diets in comparison with significantly lower CTRL. Concentrations of all biochemical parameters in plasma increased gradually in fish fed CB1-3 diets when compared to CTRL diet, with exception of triacylglycerol levels. Expression of hepatic *fas*, *elovl-5a* and *ppara* genes increased significantly in fish fed CB1 and CB2. Additionally, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) accumulation in muscle tissue was directly proportional to the amounts supplied in the diets. Our study revealed that carp fillet profiles can be manipulated for DHA and EPA-contents using enriched diets, depending on the source of fat.

1. Introduction

The essential role of omega-3 polyunsaturated fatty acids (n-3 PUFAs) in human nutrition has been studied widely in recent years (EFSA, 2014). The n-3 PUFAs functions in neurogenesis, neurotransmission, protection against oxidative stress, and are particularly important during brain development (Innis, 2007). Moreover, they are components of cell walls, determining fluidity, elasticity and permeability, and have beneficial effects against development of various human conditions, such as inflammation and autoimmune, cardiovascular and neurodegenerative diseases (Arts and Kohler, 2009; Liu and Ma, 2014; Zhang et al., 2019). The recommended daily intakes for eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (recommended by national and international authorities – 250mg/day) is achieved by only 26% of Europeans (Sioen et al., 2017).

A good source of n-3 fatty acids in human diets is fish, specifically marine fish. Generally, EFA (essential fatty acid) contents of freshwater fish is lower, and dependent on physico-chemical parameters of their habitat, season and geographical location as well as physiology (Williams et al., 2017). Freshwater fish fillets are more susceptible to changes in dietary fatty acid (FA) profile, i.e. ingredients in new feed blends. For example, fillets from Nile tilapia (*Oreochromis niloticus*) fed increased proportions of fish oil (FO) in an experimental diet had contained more DHA and EPA compared with fish fed a control diet (Özlüer Hunt et al., 2018). A comparable observation has been described for Atlantic salmon (*Salmo salar*) (Sissener, 2018). This supports the rational of utilizing sustainable raw materials rich in PUFAs for feed production. Decreasing availability and growing costs associated with animal ingredients rich in PUFAs has forced producers to consider plant-based ingredients (e.g. rapeseed oil, sunflower oil), insects (e.g. black soldier fly larvae meal), microalgae (e.g. *Schizochytrium* sp. meal) and by-products from fish processing (e.g. salmon meal). However, new implemented ingredients are potentially hazardous, because may include a range of contaminants, like heavy metals, mycotoxins, pesticide

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Abbreviations

18SrRNA	18S ribosomal RNA
40sRNA	40S ribosomal protein S11
acox1	peroxisomal acyl-coenzyme A oxidase 1
actb	β-actin
ALA	α-linolenic acid (18:3n-3)
ARA	arachidonic acid (20:4n-6)
DGLA	dihomo-γ-linoleic acid
DHA	docosahexaenoic acid (22:6n-3)
ef-1a	elongation factor 1-alpha
EFA	essential fatty acids
elovl5	fatty acid elongase 5
EPA	eicosapentaenoic acid (20:5n-3)
FA	fatty acid
FBW	final body weight
fads6	fatty acid desaturase 6
fas	fatty acid synthase and
FCR	feed conversion ratio
FM	fish meal
FO	fish oil
FRS	Fisheries Research Station
gapdh	glyceraldehyde 3-phosphate dehydrogenase
GLA	γ-linoleic acid (GLA)
HDL	high-density lipoprotein cholesterol
HUFA	highly unsaturated fatty acid
LA	linoleic acid (18:2n-6)
LC-PUFA	total long chain polyunsaturated fatty acid
LDL	low-density lipoprotein cholesterol
MUFA	monounsaturated fatty acid
n-3 PUFA	total omega-3 polyunsaturated fatty acid
n-6 PUFA	total omega-6 polyunsaturated fatty acid
NLFA	neutral lipid fatty acid
PER	protein efficiency ratio
PLFA	polar lipid fatty acids
pparα	peroxisome proliferator-activated receptor *α*
PUFA	polyunsaturated fatty acids
rpl8	60S ribosomal protein L8
SG	specific growth rate
TAG	triacylglycerol
TC	total cholesterol
TG	total growth
ZUT	West Pomeranian University of Technology in Szczecin

residues, as well as pathogens (Van der Spiegel et al., 2013). Currently studies assessing toxicological safety of new feed ingredients are conducted. For instance, Fedorova-Dahms et al. (2011) evaluated applicability of *Schizochytrium* sp. and revealed that DHA-rich alga is safe for intended use in human consumption and feed production. In contrast to marine fish, adverse effects, linked to feeds with lower EPA and DHA, on fish performance are rarer in freshwater aquaculture, such as Nile tilapia and common carp (*Cyprinus carpio*), because these fish have active metabolic pathways that convert dietary α-linolenic acid (ALA) and linoleic acid (LA) to DHA, EPA and arachidonic acid (AA).

Common carp is one of the world's most consumed freshwater fish, cultured primarily in Europe and Asia. Globally, Cyprinids production accounts for around 40% but, in freshwater aquaculture, it is about 70% (Xu et al., 2014). Carp are reared predominantly in earthen ponds, and nutrition programmes are based on natural food supplemented with cereal grains, such as wheat, maize and barley, depending on price and local availability. The growing demand for aquaculture products, and the high phenotypic plasticity of carp, have led to intensive production models farmed in closed aquaculture systems. Such systems are based solely on artificial feed, often enriched with ingredients intended to improve the nutritional composition of products. Simply put, feeding carp with EFA-enriched feeds translate into increased FA concentrations in fillets (Csengeri et al., 2013). However, different concentrations and compositions of fat in feeds might affect fish blood biochemistry (cholesterol, triacylglycerol) (Nasir and Al-Sraji, 2013), liver histology (Poleksić et al., 2014), nutrient retention and, subsequently, compositions of their different tissues (Böhm et al., 2014) as well as gene expression in the biosynthesis of n-3 fatty acids (Ren et al., 2012). The effectiveness of FA profiling in fish might also depend on levels of lipid oxidation for internal requirements, such as energy production and storage, or membranes construction and maintenance (Sargent et al., 2002).

In the scientific literature, numerous studies report the effects of utilizing feeds containing different amounts of n-3 PUFAs on the chemical composition of fish. However, there is little information about the impact of FA from different sources (especially DHA and EPA) on the function of the organism, which – ultimately – shapes the chemical compositions and FA profiles of fillets. To determine these interactions, multidisciplinary studies are needed, considering molecular (gene activity analysis), biochemical (lipid profile in blood plasma), histological (hepatocyte morphology), and nutritional (chemical compositions and FA profiles of fillets). Thus, the purpose of this study was to determine the influence of feeds containing different sources of fat (i.e. salmon oil, soybean and rapeseed oils and *Schizochytrium* sp. meal) on carp physiology by assessing: i) zootechnical parameters; ii) blood biochemistry (total cholesterol [TC], low-density lipoprotein cholesterol [LDL], high-density lipoprotein cholesterol [HDL], triacylglycerol [TAG] and non-HDL cholesterol); iii) expression of five lipid metabolism-related genes in liver, i.e. peroxisome proliferator-activated receptor *α* (*pparα*), peroxisomal acyl-coenzyme A oxidase 1 (*acox1*), fatty acid desaturase 6 (*fads6*), fatty acid synthase (*fas*) and fatty acid elongase 5 (*elovl5*); iv) liver histomorphology; and v) total fat contents and fatty acid compositions in carp fillets.

2. Materials and methods

2.1. Experimental diets

Four isolipidic, isonitrogenous and isoenergetic diets, three experimental (CB1, CB2, CB3) and one control (CTRL) (Table 1), were designed to evaluate the impact of lipid sources on fish physiology and fillet composition. CB1, CB2 and CB3 were enriched with selenised yeast, microalgae (*Spirulina* sp., *Chlorella* sp. and *Schizochytrium* sp.) and macroalgae (*Laminaria digitata*) meals. Soybean and rapeseed oils (1:1) were the only lipid sources in the CTRL diet while, in CB3, salmon, soybean and rapeseed oils (1.05:1:1) were used. Lipid sources in CB1 and CB2 diets were rapeseed oil and *Schizochytrium* sp., which is ca. 66% fat (Allen et al., 2019). Diets were manufactured by SPAROS Lda (Olhão, Portugal). All powder ingredients were mixed, accordingly to the target formulations, in a double-helix mixer (500L, TGC Extrusion, Roullet-Saint-Estèphe, France) and ground (less than 200 μm) in a micropulveriser hammer mill (SH1, Hosokawa-Alpine, Germany). Diets (floating pellet size 6.0 mm) were manufactured with a twin-screw extruder (BC45, Clextral, Firminy, France; screw diameter 55.5 mm). Extrusion conditions were feeder rate (77 kg/h), screw speed (247 rpm), water addition in barrel 1 (330 ml/min), temperature barrel 1 (32–34 °C) and temperature barrel 3 (111–115 °C). Extruded pellets were dried in a vibrating fluid bed dryer (DR100, TGC Extrusion, Roullet-Saint-Estèphe, France). Oils were added post-extrusion by vacuum coating (PG-10VCLAB, Dinnissen, Sevenum, Netherlands). After coating, diets were packed in bags and shipped to the West Pomeranian University of Technology in Szczecin (Poland, ZUT).

Table 1
Formulation of experimental diets for common carp trail.

Ingredients, %	CTRL	CB1	CB2	CB3
Fishmeal 60 [a]	5.000	2.500	2.500	2.500
Porcine blood meal [b]	2.000	2.000	2.000	2.000
Algae meal (*Spirulina* sp.) [c]		1.000	1.000	1.000
Algae meal (*Chlorella* sp.) [d]		1.000	1.000	1.000
Algae meal (*Schizochytrium* sp.) [e]		3.125	1.563	
Soy protein concentrate [f]	2.500	2.500	2.500	2.500
Corn gluten meal [g]	4.000	4.000	4.000	4.000
Soybean meal 44 [h]	25.000	25.000	25.000	25.000
Rapeseed meal [i]	7.000	7.000	7.000	7.000
Sunflower meal [j]	12.500	12.500	12.500	12.500
Wheat meal [k]	22.500	21.224	21.786	22.349
Wheat bran [l]	5.000	5.000	5.000	5.000
Corn meal [m]	2.500	2.500	2.500	2.500
Salmon oil [n]				2.100
Soybean oil [o]	3.000			2.000
Rapeseed oil [o]	3.000	4.100	5.100	2.000
Vitamins and minerals premix [p]	1.000	1.000	1.000	1.000
Betaine HCl [q]	0.100	0.100	0.100	0.100
Binders [r]	1.000	1.000	1.000	1.000
Macroalgae meal (*Laminaria digitata*) [s]		0.541	0.541	0.541
Antioxidant [t]	0.200	0.200	0.200	0.200
Sodium propionate [u]	0.100	0.100	0.100	0.100
Sodium phosphate [v]	2.100	2.100	2.100	2.100
Selenised yeast [w]		0.010	0.010	0.010
L-Lysine [x]	0.700	0.700	0.700	0.700
L-Tryptophan [y]	0.200	0.200	0.200	0.200
DL-Methionine [z]	0.600	0.600	0.600	0.600
Proximate composition (%)				
Crude protein	30.20	30.60	30.40	30.30
Crude fat	8.10	8.00	8.00	8.10
Crude ash	3.00	3.00	3.00	3.00
Crude fiber	5.00	5.00	5.00	5.00
Main fatty acids (%)				
20:5n−3 (EPA)	0.05	0.27	0.22	0.05
22:6n−3 (DHA)	0.09	0.62	0.38	0.12
ΣEPA + DHA	0.14	0.89	0.60	0.17

[a] CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain.
[b] Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands.
[c] *Spirulina* meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland.
[d] *Chlorella* meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal.
[e] ALL-G RICH (*Schizochytrium*), Alltech Portugal.
[f] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[g] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.
[h] Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain.
[i] Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal.
[j] Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal.
[k] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[l] Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal.
[m] Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal.
[n] Sopropêche, France.
[o] Lamotte Oils GmbH, Germany.
[p] INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's.
[q] ORFFA, The Netherlands.
[r] CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal.
[s] Dry *Laminaria digitata*: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France.
[t] VERDILOX, Kemin Europe NV, Belgium.
[u] PREMIX LDA., Portugal.
[v] Vadequimica, Spain.
[w] ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France.
[x] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.
[y] TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany.
[z] DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

2.2. Feeding trial

Ethical approval was obtained from the ethical committee of Faculty of Food Sciences and Fisheries (ZUT in Poland). We adhered to the "Guidelines for the treatment of animals in behavioural research and teaching" published in Animal Behaviour (Anon, 2012).

The fish trial was performed at the Fisheries Research Station (FRS), Nowe Czarnowo, Poland (N: 53°120 36″ E: 14° 270 48″), in floating cages (net volume 3 m^3 each) submerged in cooling water discharged from the Dolna Odra Power Plant. One week prior to the start, 1200 carp (296 ± 10 g mean initial body weight) were selected from a pond farm located in Maliniec (NW Poland), transported to the FRS and randomly distributed (n = 100) in 12 cages for acclimatization (100 fish/cage). The 100-day trial (July–October 2018) was performed in triplicate (n = 3/diet). Feeding was by hand three times a day (at 10:00, 14:00 and 18:00 h, in equal portions) until apparent satiation (2% metabolic dose). During the experiment, the temperature of water ranged between 13.3 °C and 34.2 °C.

2.3. Measuring growth indices

In order to evaluate the dietary impact on common carp, the following growth parameters were calculated: total growth (TG), feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER) using the following formulas:

$$TG = \frac{\text{weight gain (g)}}{\text{initial body weight (g)}} \times 100$$

$$FCR = \frac{\text{feed consumed (g)}}{\text{weight gain (g)}}$$

$$SGR = \left[\frac{(\text{Ln final weight} - \text{Ln initial weight})}{\text{rearing days}}\right] \times 100$$

$$PER = \frac{\text{weight gain (g)}}{\text{protein consumed (g)}}$$

2.4. Samples collection

On three occasions (days 30, 60 and 100), five fish from each dietary treatment were anesthetised with 0.2 ml/l of 2-phenoxyethanol (Sigma-Aldrich, St. Louis, USA) and blood drawn from the caudal vein using a sterile 5 ml hypodermic syringe and a 23-gauge needle, transferred to 1.5 ml tube (Eppendorf, Hamburg, Germany) before being stored overnight at 4 °C to clot. Then, fish were sacrificed using a lethal dose of the 2-phenoxyethanol (2 ml/l). Liver samples (n = 5 per treatment and sampling period) were collected immediately, preserved in DNA/RNA Shield™ (Zymo Research, Irvine, USA) and stored at −80 °C until RNA extraction. Additionally, at the end of the feeding trial, liver samples (approx. 125 mm^3) were collected for histomorphological assessment; these were placed in 5 ml glass jars and covered with 10% buffered formalin solution for 5 h at room temperature.

2.5. Plasma lipid profile

After overnight clotting, blood samples were centrifuged (Centrifuge 5415 R, Eppendorf, Hamburg, Germany) at 4000×*g* for 10 min. Plasma was transferred to labelled Eppendorf tubes and frozen (−80 °C) until analysis. Biochemical analyses of the lipid profile indices in plasma were conducted at SPSK- 2 hospital laboratory (Pomeranian

Medical University, Szczecin, Poland). Total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), triacylglycerol (TAG) and non-HDL cholesterol were assessed using dedicated reagent sets for chemiluminescence (CMIA) on an ARCHITECT c8000 clinical chemistry analyser (Abbott Diagnostics, Lake Forest, USA).

2.6. Gene expression analysis

At the Department of Aquatic Bioengineering and Aquaculture (ZUT in Szczecin), samples were homogenized in 750 μl Tri Reagent® (Zymo Research, Irvine, USA) for 60 s with a Minilys ® personal homogenizer (Bertin Corp., Rockville, USA). Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, USA), with 15 min DNase I treatment according to manufacturer instructions. Quantity and quality of RNA was assessed using NanoDrop 2000 (TermoFisher Scientific, Waltham, USA), all samples 260/280 ratio varied around 2.0. Reverse transcription was performed immediately after RNA extraction using a Reverse Transcription System kit (Promega, Walldorf, Germany) according to the manufacturer's instructions. Real-time PCR was performed on a QuantStudio™3 (TermoFisher Scientific, Waltham, USA) using PowerUp™ SYBR™ Green Master Mix 2x (TermoFisher Scientific, USA), 0.3 μM of each primer and 1 μl of cDNA templates in a final volume of 10 μl. The reaction was conducted using 2 min of activation at 50 °C, 2 min at 95 °C, 40 cycles of denaturation at 95 °C for 1 s and annealing/extending at 60 °C for 30 s. Melting curve analysis (60–95 °C) was conducted at the end of each PCR thermal profile and negative samples for each primers set were run to ensure the specificity of amplification. The stability of six reference genes [i.e. 18S ribosomal RNA (*18SrRNA*), 40S ribosomal protein S11 (40sRNA), 60S ribosomal protein L8 (*rpl8*), β-actin (*actb*), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) and elongation factor 1-alpha (*ef-1a*)] was evaluated using geNorm and NormFinder algorithms (Table 2). Activities of five lipid metabolism-related genes [i.e. peroxisome proliferator-activated receptor *α* (*ppara*), peroxisomal acyl-coenzyme A oxidase 1 (*acox1*), fatty acid desaturase 6 (*fads6*), fatty acid synthase (*fas*) and fatty acid elongase 5 (*elovl5*)] against two reference genes (40sRNA and *rpl8*) were assessed. Relative gene expression was calculated using GeneEx (MultiD Analyzes, Sweden) software and the results normalised against expression in livers sampled at the beginning of the trial.

2.7. Liver histomorphology

Livers fragments were fixed in buffered formalin and samples dehydrated through alcohol and saturated in intermediate solutions (benzene, benzene: paraffin) before being embedded in paraffin blocks, trimmed, sectioned (4 ± 1 μm, Rotary Microtome MPS-2, Opta-Tech, Poland), stained with haematoxylin and eosin, and mounted on slides with DPX balsam (Burck, 1975). Twelve glass slides (3 fish × 4 slides) for each diet were randomly selected and examined by two members of the laboratory using an Eclipse E600 microscope (Nikon, Nikon, Japan) with 100x objective and, using NIS-Elements Basic Research software (Nikon Instruments Europe B.V, Japan), screened for hepatocyte (C) and hepatocyte nucleus (N) areas (300 measurements/parameter). Additionally, the nuclear:cytoplasm areas ratio (N/C) of hepatocytes were calculated, and the significance of observed differences was assessed for each parameter.

2.8. Fillets fatty acid composition

Samples of muscle tissue from five fish per diet were prepared as pooled homogenates (n = 5 per treatment) and all following analyses were run in triplicates. Crude lipid content was determined gravimetrically after Soxhlet lipid extraction using a Tecator Soxtec System HT

Table 2
Sequences of qPCR primers for the detection of reference and lipid metabolism related genes in common carp by real-time PCR.

Gene symbol	Sequence 5' – > 3'	Tm	Function	References
18SrRNA [a]	CCTGTCGCCGCTGAATACC	55.4 °C	ribosomal RNA gene	Huang et al. (2015)
	TCGCTTTCGTCCGTCTTGC	53.2 °C		
40sRNA [b]	CCGTGGGTGACATCGTTACA	53.8 °C	ribosomal RNA gene	Gonzalez et al. (2007)
	TCAGGACATTGAACCTCACTGTCT	55.7 °C		
rpl8 [c]	CTCCGTCTTCAAAGCCCATGT	54.4 °C	ribosomal protein coding	Bickley et al. (2009)
	TCCTTCACGATCCCCTTGATG	54.4 °C		
actb [d]	ATCCGTAAAGACCTGTATGCCA	53.0 °C	cell stability	Tang et al. (2012)
	GGGGAGCAATGATCTTGATCTTCA	55.7 °C		
gapdh [e]	AGCTCAATGGCAAGCTTACTGG	54.8 °C	cellular homeostasis	
	GTGGATACCACCTGGTCCTCTG	58.6 °C		
ef-1a [f]	GTCAAGTCCGTTGAGATGCACC	56.7 °C	protein biosynthesis	
	GGATGATGACCTGAGCATTGAAGC	57.4 °C		
ppara [g]	GGGAAAGAGCAGCACGAG	52.6 °C	lipid metabolism	Corcoran et al. (2015)
	GCGTGCTTTGGCTTTGTT	48.0 °C		
acox1 [h]	ACAGCACAGCAAGAGCAATG	51.8 °C	peroxisomal β oxidation	
	ACAGAGTGGACAGCCGTATC	53.8 °C		
fads6-a [i]	ATCGGACACCTGAAGGGAGCG	58.3 °C	fatty acid desaturation	Ren et al. (2012)
	CATGTTGAGCATGTTGACATCCG	55.3 °C		
elovl5-a [j]	GTCCTGACCATGTTCCAGACATCTTG	59.5 °C	elongation of very long-chain fatty acids	
	CTGTAAGCGGACGAGGTGTCGTC	60.3 °C		
fas [k]	TGCTGGATGCTTTGTTTGAG	49.7 °C	fatty acid synthesis	Designed based on sequence KY378913.1
	ACTACACCACCAGCGATTCC	53.8 °C		

[a] 18S ribosomal RNA.
[b] 40S ribosomal protein S11.
[c] Ribosomal protein L8.
[d] Beta actin.
[e] Glyceraldehyde 3-phosphate dehydrogenase.
[f] Eukaryotic translation elongation factor 1 alpha.
[g] Peroxisome proliferator activated receptor alpha.
[h] Acyl-CoA oxidase 1.
[i] Fatty Acid Desaturase 6.
[j] Elongation of very long chain fatty acids protein 5.
[k] Fatty acid synthase.

1043 (FOSS Analytical Co., Ltd., Hillerød, Denmark). Fatty acid profile was assessed by Polcargo (Szczecin), using gas chromatography in accordance with PN-EN ISO 12966–1:2015–01. Based on the profiles obtained, specific indicators, such as total saturated fatty acid (SFA), total monounsaturated fatty acid (MUFA), total polyunsaturated fatty acid (PUFA), total long chain polyunsaturated fatty acid (LC-PUFA), total omega-3 polyunsaturated fatty acid (n-3 PUFA), total omega-6 polyunsaturated fatty acid (n-6 PUFA) and ratio between n-3 and n-6, were calculated.

2.9. Statistical analysis

All data are shown as mean ± standard deviation unless otherwise specified. All statistical analyses were performed using Statistica 13 (StatSoft, Inc., Krakow, Poland). Normal distribution of data was assessed with Shapiro-Wilk test (Significance level $p < 0.05$). Differences between parameters were determined using the Kruskal-Wallis test followed by Tukey HSD post-hoc test. Additionally, two-way ANOVA test was applied to assess influence of blends on blood biochemistry parameters. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Growth indices

At the beginning of experiment no significant differences were found for initial body weight. At the end of the 100-day feeding trial period, there were significant differences in weights between CTRL and CB1-3 treatments (Table 3), i.e. later groups had significantly higher final body weight (FBW). No differences were found in growth response (TG or SGR) and feed utilization indices (FCR or PER), and no differences in mortality or disease were noted within or between groups.

3.2. Blood biochemistry

Two-way ANOVA showed that total and non-HDL cholesterol were affected by diet, sampling time, and there was an interaction between these factors (Table 4). HDL was not affected by any factor, while LDL was affected strongly by sampling time. TAG concentrations were time and interaction between time and diet dependent. Non-HDL concentrations associated with CB1-3 were significantly different ($p \leq 0.05$) from CTRL at the end of trial, but not one another. However, CB1-2 TC was significantly different ($p \leq 0.05$) from CRTL after 60 days and remained so at 100 days. All parameters in plasma from carp fed CB1-3 increased during the trial; the opposite was observed in CTRL fish for TC, HDL, LDL and non-HDL, but TAG increased. The increase in TAG was less in fish receiving the experimental diets (CB1-3, increase 41–72%) compared with CTRL fish (183%, $p \leq 0.05$). In fish receiving CB3, changes in TC and non-HDL were the greatest, while TAG, HDL and LDL were the lowest overall throughout the experiment.

3.3. Gene expression

Among six reference genes examined, expressions of *rpl8* and 40sRNA were the lowest and most stable across all samples and dietary treatments. Therefore, both genes were used as internal controls subsequently (Fig. 1). The expression of genes related to lipid metabolism pathways in the liver of common carp are shown in Fig. 2. Dietary levels of EPA and DHA affected significantly the expression of *fads6*, *fas*, *elovl5-a*, *ppara* and *acox1* during the 100-day feeding trial. Liver expression of *fas*, *elovl5-a* and *ppara* increased significantly in carp fed diets containing more EPA and DHA (i.e. CB1, CB2) with an opposite (i.e. down-regulation with increasing EPA and DHA) observed for *acox1*. Transcripts of *fads6-a* in the liver of carp fed diets containing more EPA and DHA were stable, while decreased in CTRL and CB3-fed fish.

3.4. Liver histomorphology

No pathological (disease-related) indicators were found in any samples. However, there were significant differences ($p \leq 0.05$) in hepatocyte morphologies amongst the treatments (Table 5). More specifically, fish fed CB1 had the smallest hepatocytes and nuclei, while the largest were noticed in livers from fish fed CB2. N/C ratios were largest for CB2 (0.204 ± 0.004) and smallest for CB3 (0.164 ± 0.004).

3.5. Fatty acid profile

Significant differences ($p \leq 0.05$) in total lipid content of fillets were found amongst the dietary treatments (Table 6). Total n-3 PUFA, total n-3 LC PUFA, total PUFA, ALA and EPA plus DHA were significantly different in fillets from fish fed CB1-3 ($p \leq 0.05$) compared with CTRL fillets. The highest ($p \leq 0.05$) concentrations of EPA and DHA, n-3/n-6 ratio (0.38 ± 0.01) were in fillets from fish fed CB1. Simultaneously, significantly ($p \leq 0.05$) lower concentrations of palmitic acid (C16:0), total MUFA, oleic acid (C18:1n9c), γ-linoleic acid (GLA), dihomo-γ-linoleic acid (DGLA) and arachidonic acid (ARA) were noted in fillets from all experimental groups (i.e. fish fed CB1-3).

4. Discussion

Demand for essential omega-3 long chain polyunsaturated fatty acids has led to utilization of various plant and animal ingredients in aquafeed production (Tocher et al., 2019). In the present study, we demonstrated a range of impacts on common carp physiology

Table 3
Growth and feed utilization indices of common carp fed diet containing different sources of fat.

	CTRL	CB1	CB2	CB3
IBW[a] (g)	300.67 ± 29.01	294.67 ± 15.37	294.67 ± 14.57	291.67 ± 20.26
FBW[b] (g)	1085.22 ± 16.42[a]	1192.64 ± 75.59[b]	1188.73 ± 49.94[b]	1217.55 ± 36.18[b]
TG[c] (%)	263.14 ± 35.05	305.81 ± 38.40	303.63 ± 12.09	319.28 ± 40.11
FCR[d]	1.52 ± 0.12	1.45 ± 0.11	1.45 ± 0.09	1.39 ± 0.11
SGR[e] (%/d)	1.29 ± 0.10	1.40 ± 0.10	1.40 ± 0.03	1.43 ± 0.10
PER[f] (g/g)	2.18 ± 0.06	2.29 ± 0.06	2.28 ± 0.14	2.38 ± 0.03

Explanations: Results represent mean ± standard deviation (n = 3). Values with different superscripts in rows indicate significant differences ($p \leq 0.05$).
[a] Initial body weight.
[b] Final body weight.
[c] Total growth.
[d] Feed conversion ratio.
[e] Specific growth rate.
[f] Protein efficiency ratio.

Table 4
Dietary influence on blood biochemical parameters of common carp.

		Total cholesterol (mg/dl)	Cholesterol HDL (mg/dl)	Cholesterol LDL (mg/dl)	Triacylglycerol (mg/dl)	Cholesterol non-HDL (mg/dl)
CTRL	30 d	144.75 ± 5.85$^{A\ a}$	7.40 ± 1.34$^{A\ a}$	8.60 ± 1.52$^{A\ a}$	262.60 ± 39.90$^{A\ a}$	142.40 ± 12.74$^{A\ a}$
	60 d	137.67 ± 9.87$^{A\ a}$	6.75 ± 0.96$^{A\ a}$	6.20 ± 2.95$^{A\ ab}$	379.25 ± 30.58$^{A\ b}$	126.40 ± 9.40$^{A\ a}$
	100 d	135.67 ± 27.54$^{A\ a}$	5.75 ± 1.26$^{A\ a}$	6.00 ± 1.00$^{A\ b}$	444.75 ± 27.24$^{A\ c}$	114.50 ± 19.09$^{A\ a}$
CB1	30 d	173.80 ± 26.99$^{A\ a}$	6.50 ± 1.29$^{A\ a}$	9.67 ± 2.52$^{A\ a}$	239.80 ± 29.10$^{A\ a}$	167.00 ± 26.63$^{A\ a}$
	60 d	189.00 ± 1.41$^{B\ a}$	6.50 ± 0.71$^{A\ a}$	10.00 ± 1.41$^{A\ a}$	398.00 ± 45.25$^{A\ b}$	183.00 ± 1.41$^{B\ a}$
	100 d	212.20 ± 28.33$^{B\ a}$	7.20 ± 2.17$^{A\ a}$	15.80 ± 7.26$^{B\ a}$	412.60 ± 76.81$^{A\ b}$	205.00 ± 27.47$^{B\ a}$
CB2	30 d	157.60 ± 13.43$^{A\ a}$	6.67 ± 2.08$^{A\ a}$	9.75 ± 1.71$^{A\ a}$	218.50 ± 47.85$^{A\ a}$	149.40 ± 14.45$^{A\ a}$
	60 d	171.33 ± 19.55$^{B\ ab}$	7.67 ± 0.58$^{A\ a}$	10.33 ± 2.08$^{A\ a}$	358.00 ± 82.31$^{A\ b}$	163.67 ± 19.14$^{AB\ ab}$
	100 d	201.60 ± 21.81$^{B\ b}$	7.80 ± 1.79$^{A\ a}$	17.60 ± 5.94$^{B\ a}$	369.33 ± 53.61$^{A\ b}$	193.80 ± 21.72$^{B\ b}$
CB3	30 d	141.67 ± 26.71$^{A\ a}$	7.33 ± 0.58$^{A\ a}$	9.00 ± 2.07$^{A\ a}$	252.60 ± 37.69$^{A\ a}$	134.33 ± 16.65$^{A\ a}$
	60 d	157.00 ± 7.07$^{AB\ ab}$	7.50 ± 1.91$^{A\ a}$	9.40 ± 5.03$^{A\ a}$	330.50 ± 62.34^{Aab}	141.00 ± 18.03$^{A\ ab}$
	100 d	194.60 ± 28.26$^{AB\ b}$	7.80 ± 1.30$^{A\ a}$	17.40 ± 6.35$^{B\ a}$	357.00 ± 60.32$^{A\ b}$	187.00 ± 28.38$^{B\ b}$
Significance	Diet	**	n.s.	n.s.	n.s.	**
	Time	**	n.s.	**	**	**
	DxT	**	n.s.	n.s.	*	**

Explanations: Results represent mean ± standard deviation (n = 5). Values with different superscripts in columns indicate significant differences ($p \leq 0.05$). Uppercase superscripts indicate differences between diets, lowercase superscripts indicate differences between sampling points for each diet. * – $p \leq 0.05$; ** – $p \leq 0.01$; n.s. – differences not significant; D – diet; T – time (sampling point).

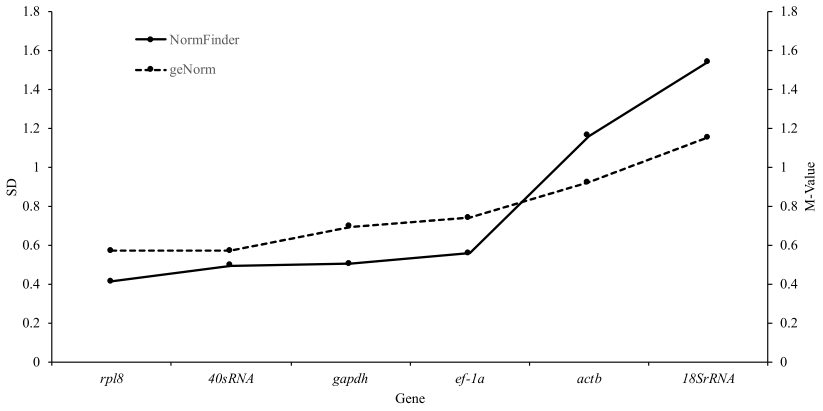

Fig. 1. Stability assessment of six reference genes across all liver samples of common carp under different dietary treatments, using two different algorithms: NormFinder (SD) and geNorm (M-Value) provided in GeneEx software.

associated with feed blends containing various sustainable sources of fat. Zootechnical parameters revealed that carp fed CB1-3 were heavier (FBW) compared with CTRL fish. This difference in FBW was presumably the result of including DHA-enrich algae and salmon oil in test diets, as formulated diets were isoenergetic. Comparable results have been found for rainbow trout (*Oncorhynchus mykiss*), salmon (*Salmo salar*) and whiteleg shrimp (*Litopenaeus vannamei*), the growth of which were higher when fed DHA-rich diets (Bell et al., 2001, 2003, Araújo et al., 2019). However, the differences in FBW for CB1-3 might also arise from addition of selenized yeast in the blends, since organic selenium is more bioavailable than the inorganic form and might affect positively fish health and performance (Berntssen et al., 2018).

Biochemical parameters revealed significantly higher TC concentrations in plasma from fish fed CB1 (containing 3.125% *Schizochytrium* sp.), which contained the most EPA and DHA. Our results are consistent with those obtained for European seabass (*Dicentrarchus labrax*); TC was elevated in fish fed more FAs (Richard et al., 2006a). Some studies have suggested there were no effects of EPA/DHA on Atlantic salmon TC (Jin et al., 2017) whilst others demonstrated TC decreased in blunt snout bream (*Megalobrama amblycephala*, Lu et al., 2013) and rainbow trout (Richard et al., 2006b) fed diets with higher concentrations of n-3 LC-PUFA. In our study, high plasma TC could have influenced CB1-3 FBW. In some fish species, such as rainbow trout and Japanese flounder (*Paralichthys olivaceus*), higher TC improved significantly feed intakes and weight gain (Twibell and Wilson, 2004; Deng et al., 2010). However, in most aquaculture species, TC depends on feed compositions including the source of dietary protein (Romarheim et al., 2006). In most cases, increased TC (likewise TAG) reflects increased internal lipid transport (Du et al., 2005). During the experiment, we also observed increased HDL, LDL and non-HDL in fish receiving CB1-3 as well as a decrease in these indices in the CTRL fish. Deng et al. (2010) observed changes in blood lipoprotein concentrations (HDL, LDL, VLDL, IDL, intermediate density lipoproteins), revealing that fish protein-based diets increased lipoprotein, while plant protein-based diets decreased them. These responses and interactions are specific to species, dietary treatments, and sex (Deng et al., 2010). For carp in particular, this is the first report demonstrating that non-HDL concentrations depend on feed compositions. Therefore, further studies are needed to identify the sources of variation and the optimal diet for fillet composition.

Blood TAG concentrations showed no significant differences amongst dietary treatments, which might be attributed to the isolipidic (8%) feed blends. TAG in blood or liver is a derivative of fat in feeds (Lu et al., 2013). Feeding blunt snout bream with control (5% fat) and high-fat (15% fat) diets showed that hepatic TAG was higher in fish fed the high-fat diet. In contrast, plasma TAG was higher in the control group (5%), suggesting lipid transport from the liver is impacted by high-fat diets (Lu et al., 2013). TAG metabolism also depends on qualitative fat composition in the feed. Results from nutritional trials showed positive (Lemaire et al., 1991; Kjær et al., 2008) and negative (Jordal et al.,

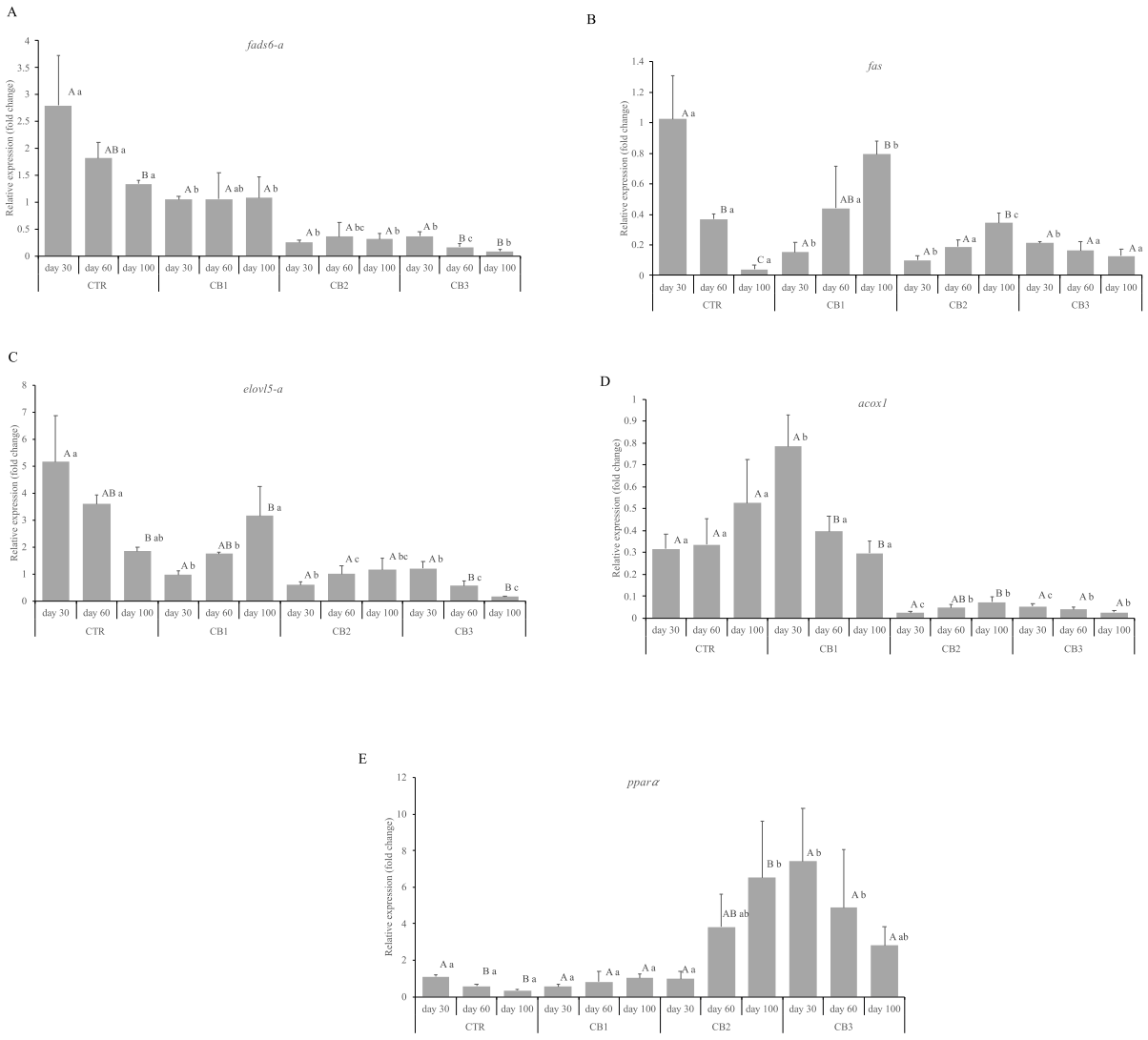

Fig. 2. Relative expression of 5 lipid metabolism related genes (A - fatty acid desaturase 6; B - fatty acid synthase; C - fatty acid elongase 5; D - peroxisomal acyl-coenzyme A oxidase 1; E − peroxisome proliferator-activated receptor α) in liver of common carp under different dietary treatments at 3 sampling points. Values are mean ± SD (n = 5). Different superscripts indicate significant difference ($p \leq 0.05$), small superscripts between sampling points in diet, and large superscripts between different diets at each sampling point.

2007; Leaver et al., 2008) correlations between increased n-3 PUFA feed content and reduced plasma TAG in European seabass and Atlantic salmon. In our study, plasma TAG was similar across all dietary treatments at all time points, despite differences in feed compositions. Similar results were observed in Atlantic salmon fed with a diet where FO was replaced by plant oils with the same fish meal (FM) contents (Jordal et al., 2007). During our trial, plasma TAG increased, probably in relation to increased secretion of TAG-rich VLDL (very low-density lipoprotein) particles from the liver in the post-absorptive phase, which is regulated by dietary FA availability and composition (Torstensen et al., 2011). The greatest increase (183%) was in CTRL fish, which received the diet with the most plant ingredients. Torstensen et al.

Table 5
Liver histological parameters of common carp fed with different sources of fat.

	CTRL	CB1	CB2	CB3
Hepatocyte area (μm^2)	123.37 ± 7.10^{ab}	93.19 ± 30.21^{a}	154.82 ± 13.02^{b}	120.26 ± 8.27^{ab}
Hepatocyte nucleus area (μm^2)	20.68 ± 1.28^{a}	17.08 ± 4.73^{a}	31.00 ± 2.43^{b}	18.81 ± 1.53^{a}
N/C[1]	0.175 ± 0.007^{a}	0.197 ± 0.013^{b}	0.204 ± 0.004^{b}	0.164 ± 0.004^{a}

Explanations: Results represent mean ± standard deviation (n = 3). Values with different superscripts in rows indicate significant differences ($p \leq 0.05$). [1] Hepatocyte nucleus area/hepatocyte area.

Table 6
Muscle fatty acid composition (% of total fatty acids) of common carp fed blends with different sources of fat.

	CTRL	CB1	CB2	CB3
Crude fat (%)	2.98 ± 0.36[a]	3.68 ± 0.52[ab]	3.07 ± 0.13[a]	4.51 ± 0.16[b]
C16:0 (PA)	16.19 ± 0.07[d]	14.90 ± 0.03[b]	14.82 ± 0.06[a]	15.23 ± 0.08[c]
C18:0 (SA)	3.70 ± 0.01[b]	3.45 ± 0.03[a]	3.67 ± 0.04[b]	3.70 ± 0.01[b]
Total SFA [a]	24.55 ± 1.79	24.34 ± 2.14	24.22 ± 2.08	24.07 ± 1.97
C18:1n9c (OA)	43.58 ± 0.02[d]	40.31 ± 0.23[b]	38.96 ± 0.86[a]	42.95 ± 0.07[c]
Total MUFA [b]	53.95 ± 0.02[c]	51.98 ± 0.21[b]	50.17 ± 0.84[a]	52.68 ± 0.05[b]
C18:3n3 (ALA)	1.55 ± 0.02[a]	2.18 ± 0.01[c]	2.05 ± 0.02[b]	2.08 ± 0.01[b]
C20:5n3 (EPA)	0.17 ± 0.01[a]	0.84 ± 0.01[c]	0.81 ± 0.03[c]	0.50 ± 0.00[b]
C22:6n3 (DHA)	0.91 ± 0.02[a]	3.33 ± 0.07[d]	2.97 ± 0.19[c]	1.80 ± 0.03[b]
Total n-3 PUFA [c]	2.68 ± 0.02[a]	6.48 ± 0.07[d]	5.96 ± 0.24[c]	4.49 ± 0.03[b]
Total n-3 LC PUFA [d]	1.12 ± 0.01[a]	4.27 ± 0.07[d]	3.88 ± 0.21[c]	2.38 ± 0.03[b]
C18:2n6c (LA)	15.47 ± 0.04[a]	15.26 ± 0.09[a]	16.97 ± 0.22[c]	16.49 ± 0.03[b]
C18:3n6 (GLA)	0.69 ± 0.00[d]	0.31 ± 0.01[a]	0.39 ± 0.01[b]	0.48 ± 0.00[c]
C20:3n6 (DGLA)	0.73 ± 0.00[c]	0.54 ± 0.03[a]	0.66 ± 0.04[b]	0.62 ± 0.01[b]
C20:4n6 (AA)	1.56 ± 0.02[c]	0.81 ± 0.03[a]	1.08 ± 0.10[b]	0.98 ± 0.01[b]
Total n-6 PUFA [e]	18.45 ± 0.03[b]	16.90 ± 0.14[a]	19.10 ± 0.36[c]	18.57 ± 0.02[b]
Total PUFA [f]	22.10 ± 0.04[a]	24.09 ± 0.22[b]	25.90 ± 0.63[c]	23.81 ± 0.01[b]
EPA + DHA	1.08 ± 0.02[a]	4.17 ± 0.07[d]	3.78 ± 0.22[c]	2.30 ± 0.03[b]
n-3/n-6	0.15 ± 0.01[a]	0.38 ± 0.01[d]	0.31 ± 0.01[c]	0.24 ± 0.01[b]

Explanations: Results represent mean ± standard deviation (n = 3). Values with different superscripts in rows indicate significant differences ($p \leq 0.05$). Some fatty acids in trace amount such as 12:0, 14:0, 16:0, 17:0, 20:0, 22:0, 24:0, 16:1, 18:1n9t, 20:1n − 9, 22:1n − 9, 22:1n-11, 16:2n − 6, 18:4n − 3, 20:3n − 3 and 20:4n − 3 were not listed.

[a] Saturated fatty acids.
[b] Monounsaturated fatty acids.
[c] Omega-3 polyunsaturated fatty acids.
[d] Omega-3 long-chain (C_{20-24}) polyunsaturated fatty acids.
[e] Omega-6 polyunsaturated fatty acids.
[f] Polyunsaturated fatty acids.

(2011) found significantly and consistently increased plasma TAG in fish fed a diet containing high-plant protein and vegetable oils, demonstrating an interaction between dietary lipids and proteins with lipid metabolic consequences. Increased TAG concentrations were also associated with lower water temperatures (Torstensen et al., 2011), in line with the decrease observed in our study (28.9 °C - 23rd July to 14.6 °C - 30th October). Data for the effects of dietary fat intakes and quality on serum indices were inconclusive, meaning further studies are required to better understand the regulation of indices related to lipid metabolism in common carp.

As a freshwater aquaculture species, carp can synthesise HUFA, and nutritional trials with *Cyprinus carpio* var. Jian showed the efficiency of HUFA biosynthesis depended mainly on the quality and quantity of FAs in feeds (Ren et al., 2012). Ren et al. (2012) also demonstrated greater expression of genes involved in HUFA biosynthesis, specifically *fads6-a* and *elovl5-a*, when the fish were fed LA and ALA-fortified diets compared to EPA/DHA rich diet. In our experiment, *fads6-a* expression in fish fed CB1 and CB2 (highest EPA/DHA concentrations) did not differ, which confirms a mechanism inhibiting *fads6-a* activity by dietary HUFA, as described by Tocher (2003) and Zheng et al. (2005). Moreover, constant (CB1 and CB2) or decreasing (CTRL and CB3) *fads6-a* activities were observed during our 100-day trial that might be associated with lower 18:3n-3/18:2n-6 ratios or excess LA or ALA (Izquierdo et al., 2008; Li et al., 2008). It is well known that the *fads6* enzyme in fish has greater affinity with n-3 than n-6 FAs. However, substrates can also compete specifically with one another (Vagner and Santigosa, 2011).

Mechanisms behind *fads6-a* activity regulation require further studies, however, could explain greater expression observed for CTRL and CB1 fed fish in comparison to those receiving CB2 and CB3. Interestingly, expression of *elovl5-a*, except in CB1 fish, was similar to *fads6-a*. Both genes are involved in HUFA biosynthesis, and increased *elovl5-a* expression in CB1 fish is most likely linked to increased proportions of ALA, EPA and DHA in the feed. As shown for Atlantic salmon (Miller et al., 2008) and common carp var. Jian. (Ren et al., 2012), HUFA most likely reduced *elovl5-a* activity through reduced promoter activity (Zheng et al., 2009). Ren et al. (2012) showed that expression of *elovl5-a* and *elovl5-b* were consistent, which is contrary to our findings, which suggest increased *elovl5-a* activity in relation to CB1-3 feed compositions.

In CTRL and CB3 fish, FM and salmon oil HUFA down-regulated *elovl5-a*. In turn, HUFA, derived from the thraustochytrid microalga *Schizochytrium* sp. (CB1 and CB2), together with other algae meal components, stimulated expression of this gene. Differences in *fads6-a* and *elovl5-a* activities might result from the fact that many *elovl5* elongase genes in fish species have been reported to prefer C_{18} and C_{20} PUFAs as substrates, with residual conversion toward C_{22} substrates (Monroig et al., 2012; Ren et al., 2012). Thus, reduced efficiency of elongation, 22:5n-3 to 24:5n-3, which is the substrate for Δ6D, would stabilize hepatic *fads6-a* expression in CB1 carp, as demonstrated in this study. Comparisons of *elovl5-a* and *fas* activities also support these dietary effects on fat metabolism, as described above.

The activity of *fas* gene increased in CB1 and CB2 fed fish (highest EPA/DHA) and increased feed FAs triggered a dose-dependent increase in hepatic *fas* mRNA. However, results for Atlantic salmon (Morais et al., 2011) and mammals (Davidson, 2006) indicated an inhibitory effect of HUFA on hepatic *fas* activity, leading to hypotriglyceridemia resulting from lipogenesis and enhanced fatty acid oxidation. In our study, *fas* expression was dependent on HUFA concentrations, as shown in large yellow croaker (*Larmichthys crocea*), where FM-enriched feed was associated with increased *fas* activity (Qiu et al., 2017). In contrast, Zhu (2005) found that expression of *fas* in grass carp (*Ctenopharyngodon idella*) muscle and liver was inhibited significantly by lard, and soybean and fish oils in the diet. Levels of *fas* activity also correlate with plasma TAG concentrations (Semenkovich, 1997), because encoded FAS enzyme catalyses synthesis of long chain FAs, mainly through acetyl coenzyme A and malonyl coenzyme A. We observed a link between *fas* activity and TAG in relation to the CB1 diet, which contained the most EPA/DHA. Analyses also revealed differences in *fas* expression were quality dependent, as the diets were isolipidic (8% in all variants).

Expression of hepatic *ppar-α* increased over time in CB1 and CB2 fed carp. Lu et al. (2014) observed that feeding blunt snout bream with a

high-fat diet decreased *ppar-α* expression and inhibited fat β-oxidation and steatosis. Higher hepatic *ppar-α* activities in CB1 and CB2 fed fish could be due to increased EPA and DHA concentrations in the feeds and/or additional SFAs and MUFAs, which are utilized preferentially during the β-oxidation (Kjær et al., 2008; Lu et al., 2014). Moreover, EPA induces mitochondrial proliferation and reduces intracellular lipid (Kjær et al., 2008). Hepatic *ppar-α* activity decreased in CTRL and CB3 fish, which might be explained by the increased content of plant-derived components (Ye et al., 2019) that down-regulated lipid metabolism-related gene expression, including carnitine palmitoyltransferase 1 (*cpt1*), apolipoprotein AI (*apo-al*) and lipoprotein lipase (*lpl*). Further research is required to assess the mechanisms underlying *ppar-α* expression amongst the dietary groups in this study.

Activity of *acox1*, which determines β-oxidation capacity, was also dependent on qualitative fat compositions in the feeds. However, the dynamics of this gene's activities were different from those of *ppar-α* across all diets. Decreased *acox1* expression was noted in CB1 fish (the highest EPA and DHA content), while in CTRL fish (the lowest EPA and DHA content) *acox1* activity did not change. Previous studies have shown that feeds with higher proportions of n-3 FAs increased *acox1* activity in rainbow trout (Figueiredo-Silva et al., 2012) but had no effect on Atlantic salmon (Caballero-Solares et al., 2018). Literature for lipid metabolism in common carp is sparse and, thus, further studies are needed to determine the mechanisms of action.

Dietary fat concentrations, as well as composition of the lipids, in feeds influence ectopic lipid accumulation in farmed fish tissues (liver, abdominal adipose tissue), which is often reflected in hepatocyte histomorphology (Caballero et al., 1999; Yan et al., 2015). Isolipidic feeds used in the experiment were not associated with any pathological (disease-related) changes in hepatocytes structure, suggesting the amounts of dietary lipid or energy provided did not exceed the capacity of hepatocytes to oxidise FAs. Differences in hepatocyte and/or nucleus size observed in our study concerned carp fed elevated amounts of EFA (CB1-2). However, a more informative indicator, elaborating on the dynamics of changes occurring in hepatocytes, was the N/C ratio (i.e. hepatocyte nucleus area to hepatocyte area). The highest ratio was found for CB1 and CB2 fed fish, indicating intensive metabolism, as confirmed by increased expression of *fas* and *elovl5-a*. Ye et al. (2019) showed that lower activities of genes involved in hepatic fat metabolism resulted in higher occurrences of hepatocyte vacuolisation and nuclear atrophy, disappearance or reduction, which are signs of nuclear pycnosis. Ostaszewska et al. (2005) showed the highest metabolic activity of rainbow trout hepatocytes in fish fed the control diet without extracted soybean meal or soybean protein concentrate.

Ultimately, the use of CB1 and CB2 feeds was reflected in carp fillet FA profiles. A similar relationship, between feed and fillet composition, has been shown for zebrafish (*Danio rerio*) muscle, where DHA increased as a result of a feed containing 50% of commercial ingredients and 50% freeze-dried marine microalga *Schizochytrium* sp. (Byreddy et al., 2019). Schultz et al. (2018) showed that common carp can almost triple n-3 PUFA contents after consuming a finishing diet containing FO for only 30 days. Schultz et al. (2018) demonstrated that FA profiles in finishing feeds were the most accurate predictors of dorsal fillet composition, specifically total lipid content, regardless of the source (e.g. natural pond zooplankton, terrestrial or marine), although carp fillets contained more lipids when supplied with marine feeds. In our study, the highest fat content (4.51%) was observed in in fillets from CB3 fed fish, the only diet containing oil extracted from wild salmon by-products. Ahlgren et al. (1994) showed that SFA and MUFA (but not PUFA) concentrations were correlated with muscle fat contents, mainly because increasing total lipids in fish muscle is associated with storage lipids (TAG) rather than structural lipids (e.g. phospholipids). Moreover, amounts of phospholipids are influenced primarily by taxa-specific cell membrane requirements (Gause and Trushenski, 2013; Böhm et al., 2014) and farming conditions, e.g. temperature (Vagner and Santigosa, 2011, and references therein). Therefore, results described by Steffens and Wirth (2007) are potentially interesting, as they noted higher n-3 and n-6 FA concentrations in fillets from carp reared in extensive systems and fed natural feeds.

5. Conclusion

The present study demonstrates that different sources of dietary lipids can influence zootechnical parameters, regulate metabolic pathways at biochemical and transcriptional levels, and affect liver histology and muscle FA profiles in common carp. Considering the information available currently, in the near future, carp could contribute more as a source of n-3 PUFA in human nutrition. Carp has huge potential for sustainable aquaculture and, as identified in our study, the high phenotypic plasticity of carp can be exploited for fillets with beneficial nutrient compositions without impacting FBW. Understanding how dietary FA supplies are absorbed, retained and metabolised in common carp, and how FA profiles can be manipulated efficiently, requires further work but could unlock the full potential of an important fish farmed worldwide.

CRediT authorship contribution statement

P. Eljasik: Writing - review & editing, Investigation, Visualization. **R. Panicz:** Conceptualization, Writing - review & editing, Investigation. **M. Sobczak:** Writing - review & editing, Supervision, Formal analysis. **J. Sadowski:** Resources. **V. Barbosa:** Writing - review & editing. **A. Marques:** Writing - review & editing. **J. Dias:** Resources.

Declaration of competing interest

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

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OŚWIADCZENIE DO PRACY P3

W pracy **P3** byłem zaangażowany w przeprowadzenie doświadczenia, pobór i zabezpieczenie prób biologicznych, wykonanie analiz histologicznych, ocenę sensoryczną, tekstury oraz barwy surowca, analizę i interpretację uzyskanych wyników oraz przygotowanie manuskryptu artykułu. Swój udział określam na 40%.

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Quality improvement of common carp (*Cyprinus carpio* L.) meat fortified with n-3 PUFA

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ABSTRACT

The effect of carp feeding with n-3 PUFA-enriched feed (*Schizochytrium* sp. meal or salmon oil) on nutritional quality indicators (proximal composition, fatty acid profile of fat) and culinary quality (color parameters, texture, sensory properties) was evaluated. Highly significant effects of carp nutrition on chemical composition and fat characteristics, L* and a* color parameters, muscle fiber size, endomysium thickness, moisture and taste of fillets were determined. Fillets obtained from carps fed with the experimental feed contained less protein and more crude fat and had larger muscle fibers, but scored more highly in the sensory evaluation of moisture and fishy taste. In the fat of carp fed the enriched feed, a greater share of total PUFA, n-3 PUFA, total EPA and DHA, n-3/n-6 ratio, and a smaller share of total MUFA were observed compared with control fish. However, no effect of nutrition on the texture of carp fillets, assessed either instrumentally or using sensory methods, was found. The use of *Schizchytrium* sp. meal as a source of EPA and DHA gave much better results than salmon oil, as it allowed a higher content of these valuable fatty acids to be achieved, without compromising quality.

1. Introduction

Fish in the human diet are a source of easily digestible proteins, nutritious fats and fat-soluble vitamins (D, A), as well as micro- and macro-elements (e.g. I, Ca, Se). Fish lipids are particularly valuable because of their favorable fatty acid profile, including the high proportion of long-chain fatty acids, especially n-3 and n-6 EFA (essential fatty acid). Fatty acids EPA (20:5, n-3, eicosapentaenoic acid) and DHA (22:6, n-3, docosahexaenoic acid) have a very positive effect on health and are recommended for the reduction of cardiovascular disease (Calder, 2006; Calder and Yaqoob, 2009, 2010). Despite the high nutritional value of fish, consumption is far below levels recommended by nutritionists. The World Health Organization, together with Food and Agriculture Organization (WHO/FAO, 2003), recommend regular consumption of fish (1–2 times a week), including one portion of oily fish providing 200–500 mg of EPA and DHA. Despite these recommendations, most consumers globally eat fish less than once a week (Skibniewska et al., 2009; Pieniak et al., 2010; Dymkowska-Malesa et al., 2014; Maciel et al., 2019). One of the reasons for the lower popularity of fish (compared with meat) are their specific sensory features (i.e. smell, taste). Proteins and lipids (and *post-mortem* transformation of these compounds) are responsible for palatability, and fish lipids are particularly susceptible to oxidative changes due to their high content of unsaturated FA (fatty acids) which contribute strongly to the deterioration of the sensory quality and taste (Fauconneau et al., 1995).

Carp (*Cyprinus carpio* L.) is the main freshwater fish species bred in many European, Asian and Latin American countries. Carp contains 11.8–18% protein and 6.8–12.4% fat, the latter of which consists of 24.3–30.0% SFA (saturated fatty acids) and 70.0–75.7% unsaturated fatty acids, including 48.6% MUFA (monounsaturated fatty acids) and 21.5% PUFA (polyunsaturated fatty acids). Carp lipids are composed of 1.3–6.7% of n-3 FA, including ΣEPA + DHA constituting 0.5–4.3% of FA, and 1.3–14.8% n-6 FA (Grela et al., 2010; Woźniak et al., 2013; Ljubojevic et al., 2013). The variable nutrient content of carp is due to

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Abbreviations

CSA	cross-sectional area
DHA	docosahexaenoic acid (22:6n-3)
EPA	eicosapentaenoic acid (20:5n-3)
FA	fatty acid
HUFA	highly unsaturated fatty acid
MUFA	monounsaturated fatty acid
n-3 PUFA	total omega-3 polyunsaturated fatty acid
n-6 PUFA	total omega-6 polyunsaturated fatty acid
n-9 PUFA	total omega-9 polyunsaturated fatty acid
PUFA	polyunsaturated fatty acids
SFA	saturated fatty acids
WI	whiteness index

different living conditions and food sources. Therefore, these factors can be used by breeders to alter the nutritional make-up of carp. The nutritional quality of this species can be improved for human consumption by selecting the right feed composition and enrichment with nutrients, particularly those present in smaller amounts (e.g., EPA and DHA). Indeed, breeding experiments have shown that feeding common carp with mixed feed affects proximate composition (Manjappa et al., 2002; Aprodu et al., 2012) and fatty acid profile, including the EPA and DHA contents (Buchtová et al., 2007; Aprodu et al., 2012). Increased EPA and DHA in carp can be achieved by the introduction of fats into feed from different natural sources, including microalgae (e.g. *Schizochytium* sp.), fish or vegetables oils. The use of microalgal oils from *Schizochytrium* sp. for fortification is considered safe, as no reports have revealed potential exposure to toxic chemicals or pathogens (Ryan et al., 2010). Microalgal oil is recognized by the Food and Drug Administration of the United States of America (FDA) as permitted for use in infant formulae, and food and dietary supplements (Fedorova-Dahms et al., 2011). However, there is a lack of information about the effects of increased EPA and DHA in carp on culinary quality.

The aim of this study was to assess the quality of carp fed with feed enriched with the EPA + DHA fatty acids from natural sources and to check whether using such blends would increase the nutritional value of carp fillets without compromising their structure, texture and sensory quality parameters.

2. Materials and methods

2.1. Experimental diets

The trial comprised four experimental diets (Table 1). A control diet, mimicking a commercial feed formulation for common carp, with moderate levels of fishmeal (5%) and high levels of plant raw materials and vegetable oils (rapeseed and soybean) as sole fat sources. In comparison, in the three test diets (CB1, CB2 and CB3), half of the fishmeal was replaced with a blend of microalgae (*Spirulina* sp., *Chlorella* sp.), macroalgae (*Laminaria digitata*) and salinized yeast. Additionally, fat fractions were supplied via blends of vegetable oils, and a DHA-rich alga (*Schizochytrium* sp.) at 3.125% (diet CB1) and 1.563% (diet CB2), and salmon oil (diet CB3), respectively. All feeds were produced by extrusion at Sparos Lda facilities (Olhão, Portugal).

2.2. Fish, rearing and housing facilities

This study was conducted at the Division of Aquaculture, Faculty of Food Science and Fisheries, West Pomeranian University of Technology, Szczecin, Poland. Common carp (*Cyprinus carpio* L.) was obtained from the Experimental Fisheries Station in Nowe Czarnowo (53°12′36″N, 14°27′48″E). The fish were housed in floating cages, placed in the cooling water discharge canal of the Dolna Odra power plant, and fed with a commercial diet (Aller Classic, containing 30% protein and 7% fat; Aller Aqua Poland, Czarna Dąbrówka, Poland) prior to the start of the experiment. In total, 12 cuboid cages of 3 m^3 placed in four rows of three were used (three cages per feed). Each cage was stocked with 100 fish with an average starting weight of 296 ± 10 g. The feeding trial was conducted for 100 days during which fish were hand-fed with the feed blends (control and CB1–3, Table 1) in equal portions at 9:00 and 15:00 h. At the end of the last day of the trial, n = 10 fish from each cage were slaughtered, following commercial practices, and filleted. Filleting was performed by one individual. All fish samples were stored at 4 °C until analysis.

2.3. Color measurement

Fillet color was assessed using an NR 20XE Precision Colorimeter (Shenzhen 3NH Technology Co., Ltd., Shenzhen, China) with ϕ = 20 mm extended aperture. L* (lightness), a* (redness), b* (yellowness) were obtained automatically after a light shot was discharged perpendicularly to the inner surface of the fillet. Measurements were done in triplicate. The whiteness index ($WI = 100-[(100-L)^2 + a^2 + b^2]^{0.5}$) and chromaticity ($C = (a^2 + b^2)^{0.5}$) were calculated using the obtained values.

2.4. Chemical analysis

The chemical composition of minced fillets was determined according to AOAC (2012) methods. Moisture was obtained after drying samples in an oven at 105 °C for 24 h, while ash content was determined after incineration at 550 °C for 6 h. Crude protein was measured by determining nitrogen content (N x 6.25), according to the Kjeldahl method, using a Tecator Kjeltec 2100 distillation unit (FOSS Analytical Co., Ltd., Jiangsu, China), and crude lipid was determined gravimetrically, after Soxhlet lipid extraction on a Tecator Soxtec System HT 1043 (FOSS Analytical Co., Ltd., Jiangsu, China). Fatty acid profiles were quantified using gas chromatography (GC) with a flame ionization detector (FID) by Polcargo (Szczecin) in accordance with PN-EN ISO 12966–1:2015–01. Briefly, fatty acids were determined as fatty acid methyl esters (FAME), and individual FAME were identified by comparing their retention times with those of pure standards. Analyses were carried out in triplicate on an Agilent 6890N Network Gas Chromatograph (Agilent Technologies; Palo Alto, CA) equipped with a 7683 automatic liquid sampler and flame ionization detectors.

2.5. Structure

From each fillet, 5 × 5 × 10 mm samples were cut, fixed for 12 h in Sannomiya, dehydrated using alcohol saturated in benzene followed by benzene:paraffin, embedded in paraffin blocks, and sectioned using a Rotary Microtome MPS-2 (Opta-Tech) into slices of 10 ± 1 μm. The sections were mounted on clear glass slides, contrast-stained with hematoxylin and eosin, and sealed with Canada balsam (Burck, 1975). MultiScanBase v.13.01 (Computer Scanning System Ltd., Warszawa, PL). image analysis software was used to record muscle fiber parameters, i.e. cross-sectional area (CSA) and average diameter, as well as thickness of connective tissue surrounding every muscle fiber (*endomysium*). Three slides were prepared from each excised sample and analyzed. Approximately 150 muscle fibers and up to 100 *endomysia* were assessed on each slide.

2.6. Thawing and cooking losses

Right fillets were weighed, wrapped in a plastic wrap, and frozen at −18 ± 1 °C. After 2 months in storage, the fillets were thawed at room

Table 1
Composition of experimental feeds.

Ingredients, %	Control	CB1	CB2	CB3
Fishmeal 60 [a]	5.000	2.500	2.500	2.500
Porcine blood meal [b]	2.000	2.000	2.000	2.000
Algae meal (*Spirulina* sp.) [c]		1.000	1.000	1.000
Algae meal (*Chlorella* sp.) [d]		1.000	1.000	1.000
Algae meal (*Schizochytrium* sp.) [e]		3.125	1.563	
Soy protein concentrate [f]	2.500	2.500	2.500	2.500
Corn gluten meal [g]	4.000	4.000	4.000	4.000
Soybean meal 44 [h]	25.000	25.000	25.000	25.000
Rapeseed meal [i]	7.000	7.000	7.000	7.000
Sunflower meal [j]	12.500	12.500	12.500	12.500
Wheat meal [k]	22.500	21.224	21.786	22.349
Wheat bran [l]	5.000	5.000	5.000	5.000
Corn meal [m]	2.500	2.500	2.500	2.500
Salmon oil [n]				2.100
Soybean oil [o]	3.000			2.000
Rapeseed oil [o]	3.000	4.100	5.100	2.000
Vitamins and minerals premix [p]	1.000	1.000	1.000	1.000
Betaine HCl [q]	0.100	0.100	0.100	0.100
Binder [r]	1.000	1.000	1.000	1.000
Macroalgae meal (*Laminaria digitata*) [s]		0.541	0.541	0.541
Antioxidant [t]	0.200	0.200	0.200	0.200
Sodium propionate [u]	0.100	0.100	0.100	0.100
Sodium phosphate [v]	2.100	2.100	2.100	2.100
Selenised yeast [w]		0.010	0.010	0.010
L-Lysine [x]	0.700	0.700	0.700	0.700
L-Tryptophan [y]	0.200	0.200	0.200	0.200
DL-Methionine [z]	0.600	0.600	0.600	0.600

[a] CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain.
[b] Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands.
[c] *Spirulina* meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland.
[d] *Chlorella* meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal.
[e] ALL-G RICH (Schizochytrium), Alltech Portugal.
[f] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[g] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.
[h] Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain.
[i] Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal.
[j] Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal.
[k] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[l] Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal.
[m] Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal.
[n] Sopropêche, France.
[o] Lamotte Oils GmbH, Germany.
[p] INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's.
[q] ORFFA, The Netherlands.
[r] CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal.
[s] Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France.
[t] VERDILOX, Kemin Europe NV, Belgium.
[u] PREMIX LDA., Portugal.
[v] Vadequimica, Spain.
[w] ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France.
[x] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.
[y] TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany.
[z] DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

temperature and weighed again to calculate thawing losses. Subsequently, the fillets were steamed until their internal temperature reached 68 ± 1 °C in the thickest portion of the fillet, before being cooled to 5 ± 1 °C. After chilling, the samples were weighed to calculate cooking losses.

2.7. Texture profile analysis

The texture of the steamed fillets was measured with a TA.XT Plus Texture Analyser (Stable Micro Systems, Godalming, UK) in a double compression TPA test (Bourne, 1982). Briefly, in the TPA test, a 9.6-mm shaft was driven into the sample twice to a depth of 80% of the original height, and parameters such as hardness (N), cohesiveness (-), springiness (cm) and chewiness (N × cm) were measured. Crosshead speed was 50 mm/min. TPA was run a minimum of five times for each fillet.

2.8. Sensory analysis

Sensory evaluation of the steamed fillets was conducted by a trained team composed of four members (PN–ISO 11036:1999). Color evenness, texture characteristics (tenderness, chewiness, moisture, juiciness, fattiness), as well as intensity of odor and taste descriptors were evaluated. The intensity of these features was rated using a 5-point scale, where 1 point corresponded to the lowest and 5 points the highest intensity.

2.9. Statistical analysis

Data were analyzed using STATISTICA for Windows (version 13.1) and subjected to two-way analysis of variance (ANOVA) and Tukey's test to compare sample means. Differences were considered as statistically significant at $P \leq 0.05$.

3. Results

3.1. Proximate composition and fat characteristics of fillets

A highly significant ($P \leq 0.01$) effect of carp feeding on the chemical composition (Table 2) and fat characteristics (Table 3) of fillets was achieved. Fillets from fish fed fortified feeds were characterized by a lower protein content and greater amounts of dry matter and fat, compared with the controls. No significant differences in ash content were found. The percentage of total MUFA was significantly ($P \leq 0.01$) lower and total PUFA significantly ($P \leq 0.01$) higher in fillets from fish fed fortified diets compared with fillets from fish on the control diet. The largest differences were between the control and the CB3-fed carp (7 and 17%, respectively). Total n-3 PUFA contents in carp fed fortified feeds were 1.7–2.5 times higher than in fat from the control fish. Differences in the relative proportions of EPA + DHA between the fortified-feed and the control carp fillets were even greater; fat from the fish fed CB1–CB3 contained 2.1–3.9 times more of these valuable fatty acids. Fat from the CB3 group contained similar amounts of total n-6 and total n-9 PUFA as the control samples. In contrast, fat from the CB1 and CB2 groups contained significantly ($P \leq 0.01$) less total n-9 PUFAs. The lowest total n-6 PUFA content was recorded in the CB1-fed fish, whereas the highest was in the fish from the CB2 groups. The n-3/n-6 indices for fortified samples were significantly ($P \leq 0.01$) higher compared with the controls. The largest differences were for CB1 (153%), and the smallest for CB3 (60%). However, no significant ($P > 0.05$) differences were found in the contents of SFA or the PUFA:SFA ratio, although the fat from the fish fed with the fortified feeds (CB1–CB3) showed a trend for less SFA and had higher PUFA:SFA ratios.

3.2. Color, thawing and cooking loss of fillets

Data presented in Table 4 show that the types of feed used had a significant ($P \leq 0.05$) effect on the L*, a* and WI parameters, while

Table 2
Effect of fish feeding on proximate composition of carp fillets. Values are expressed as mean ± standard deviation (SD).

Component (% of wet weight)	Control	CB1	CB2	CB3	Significance of influence
Crude protein	16.37a ± 0.020	16.19b ± 0.040	15.99c ± 0.060	15.98cd ± 0.030	**
Dry matter	20.74a ± 0.355	21.58b ± 0.240	21.06 ab ± 0.175	22.28c ± 0.145	**
Crude fat	2.98a ± 0.355	3.68 ab ± 0.515	3.07a ± 0.125	4.51b ± 0.160	**
Ash	1.04 ± 0.025	1.08 ± 0.031	1.01 ± 0.042	1.05 ± 0.018	n.s.

[ab] – values in rows with different index differ significantly ($p \leq 0.05$).
Significance of influence: n.s. – non significant, *$p \leq 0.05$; **$p \leq 0.01$.

dietary composition did not affect ($P > 0.05$) the b* and c color parameters. Fillets from the CB3-fed fish were characterized by a significantly lighter color (L*) than the control carp, which was not significantly different from the CB1 and CB2 groups. The reddest shade was found in fillets from the fish in the CB3 and CB1 groups; the parameter a* for these fish was significantly higher (56.8–58.5%) than in the control samples which were the least red in color. Fillets from the CB2-fed fish did not differ significantly for a* from the controls or the CB3 groups samples. Fish nutrition also had a significant ($P \leq 0.01$) effect on weight losses during thawing: the largest thawing loss was observed in the CB3 group and was 82–117% greater than in other the groups which did not differ significantly from one another. There was no effect of carp nutrition on weight losses after heat treatment.

3.3. Muscle structure

Carp feeding had a significant effect on the size of muscle fiber cross-sectional area (CSA, $P \leq 0.01$) and diameter ($P \leq 0.05$), as well as thickness of the connective tissue surrounding muscle fibers—*endomysium* ($P \leq 0.01$) (Table 5). Similarly sized fibers were found in fillet muscles from CB1, which had the thinnest *endomysium*. CB2 muscles had the largest muscle fibers (approx. 25.6% larger CSA than the controls) and the thickest *endomysium* (approx. 11.5% thicker than CB1).

3.4. Texture of fillets

No significant ($P > 0.05$) effects of fish nutrition on the TPA test parameters were found in this study (Table 6). However, it should be noted that carp fillets receiving fortified feeds (CB1, CB2, CB3) demonstrated trends suggesting greater hardness and chewiness and lower springiness than the controls.

3.5. Sensory assessment of fillets

Feeds with different nutritional compositions significantly ($P \leq 0.05$) affected moisture and taste (Table 7). Fillets from the CB3-fed fish had the least moisture perception, whereas the control fillets had the lowest intensity of fishy taste and odor, compared with the other groups. There was no significant ($P > 0.05$) effect of fish nutrition on other sensory characteristics. However, the control fillets were rated as having the least fatty taste, lower fishy odor and taste, and higher intensity for boiled meat odor and taste compared to fillets from the fish fed with fortified feeds. The least tender, chewy and juicy were fillets (from the CB3-fed fish) characterized by a less intense boiled meat odor and taste. Fillets from carp fed with the fortified feeds received higher scores for fishy odor and taste compared with the control.

4. Discussion

Feeding carp with fortified feeds significantly affected the proximate chemical compositions and fat profiles of their fillets. The highest protein content in the control fillets was probably due to the feed containing twice as much fishmeal as the fortified feeds (CB1–CB3). Mazurkiewicz et al. (2011) did not show significant differences in the crude protein content of carp fed with different proportions of meal (fish, soybean, wheat) and rapeseed oil. Yet, together with an increase in meals and a decrease in rapeseed oil, these authors observed an increase in crude fat content. In our study, the highest fat contents were observed in fillets from the CB3-fed fish. CB3 contained the most fat and was the only one to contain salmon oil and not *Schizochytrium* sp. meal. Fillets from the CB1- and CB2-fed fish were characterized by high fat contents, greater than controls, despite the feed containing lower amounts of fat. In this case, the additional source of fat in the feed was algal meal from *Schizochytrium* sp., which contains 45.3 g/100 g of crude fat (Hadley et al., 2017).

The nutritional value of fish, in addition to their chemical composition, is determined by the quality of fats (Ljubojević et al., 2012). Feeding with *Schizochytrum* sp. algae and salmon oil significantly altered the fatty acid profile not only of the diet but also of the carp muscle fat. According to Fauconneau et al. (1995), supply of high PUFA diets leads directly to a higher PUFA incorporation in fish muscle. Indeed, feed containing fish oil favors accumulation of HUFA (highly unsaturated fatty acids, with > 4 double bonds), especially EPA and

Table 3
Effect of fish feeding on selected fat characteristics of carp fillets. Values are expressed as mean ± standard deviation (SD).

	Control	CB1	CB2	CB3	Significance of influence
Total SFA (% of FA)	22.5 ± 1.79	21.9 ± 2.14	21.8 ± 2.08	21.8 ± 1.97	n.s.
Total MUFA (% of FA)	54.0a ± 0.020	52.0b ± 0.215	50.2c ± 0.845	52.7bd ± 0.055	**
Total PUFA (% of FA)	22.1a ± 0.040	24.1b ± 0.220	25.9c ± 0.630	23.8bd ± 0.010	**
PUFA:SFA	0.99 ± 0.075	1.11 ± 0.103	1.19 ± 0.110	1.10 ± 0.094	n.s.
Total n-3 PUFA (% of FA)	2.68a ± 0.020	6.48b ± 0.065	5.96c ± 0.235	4.49d ± 0.025	**
C20:5n3 (EPA), (% of FA)	0.17a ± 0.001	0.84b ± 0.001	0.81b ± 0.025	0.50c ± 0.001	**
C22:6n3 (DHA), (% of FA)	0.91a ± 0.015	3.33b ± 0.070	2.97b ± 0.190	1.80c ± 0.030	**
EPA + DHA (% of FA)	1.08a ± 0.001	4.17b ± 0.070	3.78c ± 0.215	2.30d ± 0,030	**
Total n-6 PUFA (% of FA)	18.45a ± 0.025	16.90b ± 0.140	19.10c ± 0.360	18.57a ± 0.015	**
Total n-9 PUFA (% of FA)	43.81a ± 0.020	40.53b ± 0.230	39.20c ± 0.850	43.16a ± 0.070	**
n-3/n-6	0.15a ± 0.001	0.38b ± 0.001	0.31c ± 0.006	0.24d ± 0.002	**

[ab] – values in rows with different index differ significantly ($p \leq 0.05$).
Significance of influence: n.s. – non significant, *$p \leq 0.05$; **$p \leq 0.01$.
Explanations: SFA-saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, FA – total fatty acids.

Table 4
Effect of fish feeding on colour parameters, thawing and cooking loss of carp fillets. Values are expressed as mean ± standard deviation (SD).

	Control	CB1	CB2	CB3	Significance of influence
L*	55.8a ± 1.42	53.7 ab ± 0.51	55.1 ab ± 1.34	52.2b ± 1.08	*
a*	2.29a ± 0.198	3.59b ± 0.294	2.75ac ± 0.503	3.63bc ± 0.84	*
b*	11.56 ± 0.610	12.22 ± 0.396	12.03 ± 0.394	12.08 ± 0.298	n.s.
WI	54.2a ± 1.55	52.0 ab ± 0,57	53.4 ab ± 1.41	50.6b ± 0.96	*
c	11.78 ± 0.598	12.74 ± 0.365	12.34 ± 0.470	12.64 ± 0.324	n.s.
Thawing loss (%)	2.02a ± 0.087	2.31a ± 0.071	2.41a ± 0.241	4.39b ± 1.117	**
Cooking loss (%)	9.94 ± 0.800	9.89 ± 1.412	8.49 ± 1.854	9.65 ± 0.886	n.s.

[ab] – values in rows with different index differ significantly ($p \leq 0.05$).
Significance of influence: n.s. – non significant, *$p \leq 0.05$; **$p \leq 0.01$.

Table 5
Effect of fish feeding on structural elements of carp fillets. Values are expressed as mean ± standard deviation (SD).

	Control	CB1	CB2	CB3	Significance of influence
Muscle fibre:					
CSA (μm^2)	3005a ± 382.7	3065a ± 133.7	3774b ± 18.9	3528 ab ± 130.1	**
Diameter (μm)	59.6a ± 4.47	60.2 ab ± 2.16	66.9b ± 0.14	64.9 ab ± 1.26	*
Connective tissue (myocommata)					
Endomysium thickness (μm)	2.27 ab ± 0.079	2.18a ± 0.069	2.43b ± 0.106	2.31 ab ± 0.082	*

[ab] – values in rows with different index differ significantly ($p \leq 0.05$).
Significance of influence: n.s. – non significant, *$p \leq 0.05$; **$p \leq 0.01$.
Explanations: CSA - cross section area.

Table 6
Effect of fish feeding on TPA test parameters of carp fillets. Values are expressed as mean ± standard deviation (SD).

Parameter	Control	CB1	CB2	CB3	Significance of influence
Hardness (N)	4.06 ± 0.775	4.76 ± 0.820	4.79 ± 1.956	4.58 ± 1.046	n.s.
Cohesiveness (-)	0.40 ± 0.024	0.45 ± 0.054	0.38 ± 0.077	0.44 ± 0.025	n.s.
Springiness (cm)	0.77 ± 0.154	0.67 ± 0.075	0.67 ± 0.143	0.63 ± 0.015	n.s.
Chewiness (N × cm)	1.26 ± 0.145	1.61 ± 0.502	1.16 ± 0.139	1.31 ± 0.346	n.s.

[ab] – values in rows with different index differ significantly ($p \leq 0.05$).
Significance of influence: n.s. – non significant, *$p \leq 0.05$; **$p \leq 0.01$.

Table 7
Effect of fish feeding on sensory assessment (points) of carp fillets. Values are expressed as mean ± standard deviation (SD).

Traits (pt.)	Control	CB1	CB2	CB3	Significance of influence
Color evenness	3.3 ± 0.58	3.3 ± 0.58	3.3 ± 0.29	2.8 ± 0.76	n.s.
Texture:					
tenderness	2.5 ± 0.50	2.8 ± 0.29	2.5 ± 0.50	2.3 ± 0.58	n.s.
chewiness	2.3 ± 0.29	2.3 ± 0.29	2.3 ± 0.29	2.2 ± 0.76	n.s.
moisture	2.0 ab ± 0.00	2.5a ± 0.50	2.0 ab ± 0.00	1.7b ± 0.29	*
juiciness	2.3 ± 0.29	2.5 ± 0.50	2.3 ± 0.29	2.0 ± 0.50	n.s.
fattiness	2.0 ± 0.58	2.7 ± 0.58	2.3 ± 0.58	2.3 ± 0.50	n.s.
Odor:					
boiled meat	2.3 ± 0.58	2.2 ± 0.29	1.8 ± 0.29	1.7 ± 0.58	n.s.
fishy	1.3 ± 0.29	1.5 ± 0.00	2.2 ± 1.04	1.8 ± 0.29	n.s.
muddy	1.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00	n.s.
Taste:					
boiled meat	2.3 ± 0.58	2.0 ± 0.00	2.3 ± 0.58	2.0 ± 0.00	n.s.
fishy	1.8a ± 0.29	2.7b ± 0.00	2.8b ± 0.58	2.8b ± 0.00	**
muddy	1.7 ± 0.29	1.5 ± 0.50	1.7 ± 0.29	1.3 ± 0.58	n.s.

[ab] – values in rows with different index differ significantly ($p \leq 0.05$).
Significance of influence: n.s. – non significant, *$p \leq 0.05$; **$p \leq 0.01$.

DHA (Aprodu et al., 2012). The most pronounced effect of fish nutrition has been reported to be on total n-3 PUFA contents (1.7–2.5 times), especially EPA and DHA, and the n-3/n-6 ratio, but these effects are dependent on the source of DHA in the feed. In our research, greater amounts of n-3 PUFA, EPA and DHA, and a higher n-3/n-6 ratio were determined in the muscle fat of carp fed diets in which the source of DHA were the algae *Schizochytrium* sp. (CB1, CB2), and not salmon oil (CB3).

The differences in the EPA and DHA contents observed in fillets were a result of the amounts of these fatty acids in raw materials used for feed production. Oil from *Schizochytrium* sp. contains 40–45% of DHA and up to 10% of EPA (Fedorova-Dahms et al., 2011), whereas the EPA concentrations in salmon oil vary between 4.4% and 8.8% of total FAs, and DHA varies from 5.8% to 7.5% (Horn et al., 2019). These results contrasted with those by Aprodu et al. (2012), in which more EPA, DHA and HUFA were found in fish fed with fish oil supplements compared with fish fed with vegetables oils. In our study, in the fat from the CB1- and CB2-fed carp, PUFA:SFA (> 0.4) and n-6/n-3 (< 4, or n-

3/n-6 > 0.25) ratios were within levels recommended by WHO/FAO.

The effect of nutrition, including the use of feed enriched with PUFAs, on fillet compositions and fatty acid profiles is well recognized in the literature. However, information about the relationship between the amounts of PUFAs in the feed and the parameters of nutritional quality is limited. It is known that fat can have an important role in the visual appearance of fillets, processing yield and taste (Fauconneau et al., 1995). The quality assessment carried out showed significant differences in color brightness (L*, WI) and color redness (a*). In contrast, Mazurkiewicz et al. (2011) did not report differences in the color components of fillets from carp fed varying levels of rapeseed oil and meals. In our study, the brightest color was observed in fillets from the control and CB2-fed groups, in which the largest muscle fibers and lowest muscle fat contents were also noted. Conversely, Kiessling et al. (2006) associated the darker color of Atlantic salmon with smaller muscle fibers. The reddest component was observed in fillets from the CB1- and CB3-fed fish, in which crude fat contents were also the highest. The more intense color of fish fed with salmon oil might be due to the presence of astaxanthin from the salmon, which is the typically intense yellow-orange color of carotenoids (Deepika et al., 2014). Adekoya et al. (2018) also showed that the higher content of fish oil contributed to a reduction in L * and b * in carp. Balev et al. (2017) also found that carp fed solely in a natural way had lower L * and b * values as well as a greater proportion of red (a *) compared to carp fed with wheat.

No significant differences in texture and most sensory quality parameters were detected. Literature data show that texture depends on chemical composition and the size of structural elements (Kiessling et al., 2006). Generally, firmness and hardness of cooked fish increase with the number of smaller fibers, fiber density (Johnston et al., 2000; Kiessling et al., 2006), as well as higher amounts of connective tissue (Periago et al., 2005) and insoluble collagen (Moreno et al., 2012) and lower amounts of fat (Saavedra et al., 2017). However, these relationships are not always clear. For the most part, texture and sensory quality is affected by the size of muscle fibers and connective tissue. Mørkøre et al. (2009) showed that the background for salmon fillet texture was multifactorial, and fiber cross-sectional area explained only 10–12% of the variation in texture properties.

Based on our research, the hardest fillets were those from the CB2 group, the muscle of which were characterized by the largest fibers (CSA, diameter) and the thickest *endomysium*, and contained little crude fat. The control fillets had the smallest fibers and lowest crude fat content, resulting in the lowest hardness values. Periago et al. (2005) noticed a positive correlation between collagen content and textural parameters, such as hardness, springiness, cohesiveness and chewiness, in sea bass fillets, but muscle fiber diameter was correlated negatively with these parameters. Also, Fauconneau et al. (1995) and Mørkøre et al. (2009) showed that salmon fillets, characterized by small fibers, were firmer than fillets with medium-sized or large muscle fibers. Dong et al. (2017) revealed that hardness and chewiness were linked to higher lipid contents in carp, and it has been suggested that the increasing amounts of lipid enable flesh softening (Fauconneau et al., 1995). The higher contents of unsaturated fatty acids in fillets from fish receiving CB1–CB3 did not significantly affect their sensory evaluation. However, interpretation of sensory assessment data is often very difficult, in cases in which there are no differences in organoleptic criteria, even with large differences in composition (Fauconneau et al., 1995).

Lower moisture was only observed in fillets from the CB3-fed fish, which were also the most susceptible to weight loss caused by freezing and thawing. Yet, no differences were observed between the study groups in weight losses after heat treatment. Mazurkiewicz et al. (2011), initially, also did not find differences in the thermal drip of fillets. However, further studies revealed that lower proportions of rapeseed oil and increased content of meals resulted in a decreased weight loss of fillets after heat treatment.

Fillets from the CB1-fed fish, which had the highest total n-3 PUFAs, including EPA + DHA, were rated as the 'fattiest' fillets, and Fauconneau et al. (1995) associated higher contents of unsaturated fatty acids with fat fluidity that was detectable on consumption. Fillets from carp fed with the CB1–CB3 feeds received higher scores for fishy odor and taste than the control fish. Balev et al. (2017) did not report differences in the sensory properties of color or consistency in grilled fish, but grilled carp fed only in a natural way compared to carp fed with wheat had a significantly more pronounced taste and smell. The more intense fish flavor and aroma might be due to the higher contents of unsaturated fatty acids, which contributes to an increased susceptibility to oxidation (Fauconneau et al., 1995). Lazo et al. (2017) linked the specific seafood flavor with lipid-derived aroma components produced by the enzymatic oxidation of PUFA, especially arachidonic acid, EPA and DHA. According to Fauconneau et al. (1995), lipid oxidation affects specific detectable tastes of fish.

5. Conclusion

Feeding carp with feeds enriched with algae and fish fats improved significantly the nutritional value of fillets without compromising quality. Compared with the control fillets, the fillets of fish fed with fortified feeds had lower protein and higher fat contents and better fatty acid profiles, especially the most valuable total n-3 PUFA, EPA and DHA, as well as lighter color, greater redness and low to medium intensity of fishy odor and taste. Yet, none differed in instrumental or sensory texture. At the same time, the use of *Schizchytrium* sp. meal as a source of EPA and DHA in the feed yielded much better results in terms of EPA and DHA fortification than salmon oil, again without compromising quality and weight losses during thawing, which are associated with economic losses. Therefore, the appropriate supplementation of feed for carp could be an excellent tool for aquaculture, significantly contributing to an improved nutritional value of raw materials without adversely affecting culinary quality.

CRediT authorship contribution statement

M. Sobczak: Conceptualization, Writing - review & editing, Investigation, Supervision. **R. Panicz:** Conceptualization, Writing - review & editing, Validation. **P. Eljasik:** Writing - original draft, Investigation, Visualization. **J. Sadowski:** Resources. **A. Tórz:** Formal analysis. **J. Żochowska-Kujawska:** Writing - review & editing. **V. Barbosa:** Writing - review & editing. **V. Domingues:** Investigation. **A. Marques:** Writing - review & editing. **J. Dias:** Resources.

Declaration of competing interest

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

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OŚWIADCZENIE DO PRACY P4

W pracy **P4** byłem zaangażowany w przeprowadzenie doświadczenia, pobór i zabezpieczenie prób biologicznych, wykonanie analiz molekularnych oraz histologicznych, analizę i interpretację uzyskanych wyników, przygotowanie grafik, przygotowanie manuskryptu artykułu, udzielenie odpowiedzi na recenzje oraz naniesienie zasadnych poprawek do zrecenzowanej pracy. Swój udział określam na 60%.

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Structural and molecular indices in common carp (*Cyprinus carpio* L.) fed n-3 PUFA enriched diet

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ABSTRACT

Sustainable freshwater aquaculture has been recently gaining attention owing to the potential of nourishing the world. The study aimed to evaluate the influence of finishing diets on the activity of 21 genes involved in hepatic lipid metabolism and intestinal homeostasis, liver and intestine histology, and the level of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in common carp fillets. We compared two experimental diets: control diet mimicking a commercial feed (CTRL) and a test diet (CB) fortified with EPA and DHA retrieved from salmon by-products. An additional control (eCTRL) from extensively cultured carps was investigated. The study revealed that the expression of seven hepatic genes, e.g., *lipoprotein lipase* and *fatty acid synthase*, and six intestinal genes e.g., *claudin-3c* and *γ-glutamyl transpeptidase*, was influenced specifically by the experimental diets and farming type. Fish from the eCTRL group had the smallest hepatocytes and the largest nuclei compared with CTRL and CB. No pathological signs were found in intestine samples. Additionally, the levels of EPA and DHA in fillets were significantly higher in fish receiving CB compared with CTRL and eCTRL. The use of fortified diets is a promising solution to produce freshwater species with enhanced nutritional value without compromising the safety of fillets.

1. Introduction

Inland aquaculture, mainly freshwater, produced most of the farmed fish in 2018, accounting 51.3 Mt (62.5%), out of which 47 Mt (91.5%) consisted of finfish production (FAO, 2020). The report State of World Fisheries and Aquaculture (SOFIA) forecast that by 2030, freshwater production of finfish will reach 60% of global aquaculture production. Out of the top five freshwater species produced in 2018, four were carp: the herbivorous grass carp, *Ctenopharyngodon idella* (5.7 Mt, 10.5%); the omnivorous common carp, *Cyprinus carpio* (4.2 Mt, 7.7%); and two planktivorous Asian carps, i.e. silver carp, *Hypophthalmichthys molitrix* (4.8 Mt, 8.8%), and bighead carp, *H. nobilis* (3.1 Mt, 5.8%). The fifth species was Nile tilapia, *Oreochromis niloticus* (4.5 Mt, 8.3%), which alongside Pangas catfish (including *Pangasius* spp.), e.g., striped catfish, *Pangasianodon hypophthalmus* (2.3 Mt, 4.3%), is easily accessible to consumers in home countries with high production levels compared with expensive marine aquaculture species (Belton et al., 2018; 2020; FAO, 2020). Moreover, Belton et al. (2020) emphasized that rapid developments in freshwater aquaculture were observed over the last three decades, obtained through intensification rather than horizontal expansion, suggesting further continuity of this trend. Farmed freshwater fish make important contributions to food and nutrition security and, to some extent, try to address global concerns stated in the Sustainable Development Goals (SDGs). In particular, SDGs 1 and 8 are related to poverty and economic growth, respectively, while SDGs 2, 3 and 12 refer to zero hunger, good health and responsible consumption and production, respectively (United Nations, 2015).

Intensification of land-based freshwater production is mainly realised by increasing the internal waste-removal capacity of ponds (Boyd

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Abbreviations

40sRNA	40S ribosomal protein S11
acox1	acyl-CoA oxidase
akp	alkaline phosphatase
cyp7a	cholesterol 7 alpha-hydroxylase
DHA	docosahexaenoic acid (22:6n-3)
elovl2	elongase of very long-chain fatty acids 2
elovl5a	elongase of very long-chain fatty acids 5a
elovl5b	elongase of very long-chain fatty acids 5b
EPA	eicosapentaenoic acid (20:5n-3)
FA	fatty acid
fads6a	fatty acid desaturase 6a
fads6b	fatty acid desaturase 6b
fas	fatty acid synthase
FBW	final body weight
FCR	feed conversion ratio
ggt	γ-glutamyl transpeptidase
HUFA	highly unsaturated fatty acid
IBW	initial body weight
LC-PUFA	total long chain polyunsaturated fatty acid
lpl	lipoprotein lipase
MUFA	monounsaturated fatty acid
n-3 PUFA	total omega-3 polyunsaturated fatty acid
n-6 PUFA	total omega-6 polyunsaturated fatty acid
PER	protein efficiency ratio
pparα	peroxisome proliferator-activated receptor alpha
PUFA	polyunsaturated fatty acids
rpl8 60S	ribosomal protein L8
SDGs	Sustainable Development Goals
SFA	saturated fatty acids
SGR	specific growth rate
SR	survival rate
srebp-1	sterol regulatory element-binding protein 1
TG	total growth
TJ	tight junction proteins
zo-1	zonula occludens-1
ZUT	West Pomeranian University of Technology in Szczecin

et al., 2020), farming fish polycultures (Zhang et al., 2016; Dong et al., 2018) and feed development (Barbosa et al., 2020). As in the case of pond management and multispecies cultures, progress is made mainly by improving water circulation (in partitioned and biofloc ponds) and testing various combinations of fish–fish or fish–invertebrates multispecies polyculture (e.g., *C. carpio* with *H. molitrix*, *H. molitrix* with giant freshwater prawn, *Macrobrachium rosenbergii*). The design and composition of fish feeds is under substantial development globally (Zhang et al., 2016; Dong et al., 2018). The progress and scope of feed production for carp species depend on regional aspects related to the availability of ingredients and farming mode. New blends are based on a variety of additives to supplement primary nutrients for multiple reasons, e.g., to reduce the impact of antinutrients, strengthen the immune system and influence fish metabolism and physiology either directly (e.g., nucleotides) or indirectly (e.g., prebiotics) (Boyd et al., 2020). However, in the case of intensification of common carp culture in Europe, preliminary attempts are being made to fortify fish with increased levels of nutrients that are beneficial to consumers' health. Additives mainly include eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Sobczak et al., 2020), iodine and selenium (Barbosa et al., 2020), and niacin (Choopani et al., 2020). Nevertheless, while the omnivorous common carp (but also herbivorous species) are not high-fat fish and can only provide a limited 'dose' of EPA and DHA to consumers (Sprague et al., 2017a), they are also produced in very high tonnages, being important components of diets in low- and middle-income consumers from Asia, Africa and Europe (Stark et al., 2016).

Since feeding is one of the main budget-demanding drivers affecting fish farming, the idea behind the fortification concept is to apply finishing diets that, in principle, are fed to fish during the last months before harvesting (Schultz et al., 2015). The main drawback is the high cost of the desired feed ingredients, such as fish meal and/or fish oil of marine origin, single-cell proteins (e.g., microalgae, yeasts, bacteria) (Naylor et al., 2009), genetically engineered oils (Sprague et al., 2017b) and, more recently, insect meals (Belghit et al., 2018). The aquafeed sector is also seeking opportunity in retrieving specific ingredients (e.g. DHA, EPA) from fisheries and aquaculture by-products (Shepherd et al., 2017) as an alternative to high-cost compounds. Introducing new or improved feeds, in addition to their cost, requires various studies to assess fortification efficiency, as well as to determine any possible disturbance in fish homeostasis. Therefore, the overarching aim of this study was to evaluate the influence of finishing diet (CB) enriched with EPA and DHA, retrieved from salmon by-products, compared to control diet (CTRL), on the set of biological and molecular indices of common carp. The specific objectives of our study included assessment of: i) zootechnical performance of *C. carpio* fed enriched feeds; ii) activity of genes involved in hepatic lipid metabolism i.e., fatty acid oxidation, cholesterol metabolism, lipogenesis and pathways of omega-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA) biosynthesis, and intestinal homeostasis, i.e. tight junction proteins, digestive enzymes and absorptive enzymes; iii) liver and intestine histology; and iv) levels of EPA and DHA acids in fillets of common carp. Moreover, to assess the true level of fortification with EPA and DHA, the study results were compared with the fillet content of EPA and DHA and the corresponding bio-indices measured in common carps cultured in typical, extensive earthen pond conditions (eCTRL).

2. Materials and methods

2.1. Experimental diets, feeding trial and extensive control group

Approval to conduct the fish trial was obtained from the ethics committee of the Faculty of Food Sciences and Fisheries (ZUT in Poland). We adhered to the "Guidelines for the treatment of animals in behavioural research and teaching" published in Animal Behaviour (Anon, 2012).

Two floating, isolipidic, isonitrogenous and isoenergetic diets, experimental (CB) and control (CTRL), were manufactured by SPAROS Lda (Portugal) as described by Eljasik et al. (2020). The control diet mimicked a commercial feed formulation for common carp, with moderate levels of fishmeal (5%) and high levels of plant raw materials and vegetable oils (rapeseed and soybean) as sole fat sources. The CB diet differed from the CTRL as vegetable oils were replaced by oil from farmed salmon by-products. Additionally, half of the fishmeal in CB was replaced with a blend of microalgae (*Spirulina* sp., *Chlorella* sp.), macroalgae (*Laminaria digitata*) and selenised yeast (Table 1).

The fish trial was performed at a carp farm in Maliniec (Fig. 1, Video S1) located in Northwest Poland (53° 42′ 5.99″ N 15° 21′ 22.19″ E). One week prior to the start, 600 fish (mean weight 250.20 ± 13.54 g) were obtained directly from carp ponds and randomly distributed for acclimation (n = 100 per cage) in two sets of three floating cages (n = 6) with a net volume of 3 m^3 each, and placed in the ongrowing pond, where typical production of market size carp is performed. The 16-week trial (May–September 2019) was performed in triplicate (n = 3 per diet). Feeding was done by hand, two times a day (at 09:00 and 15:00 h, in equal portions) until apparent satiation (2% metabolic dose). During the trial, the water temperature in the pond ranged between 15 °C and

Table 1
Formulation of control and experimental diets for the common carp trial.

Ingredients, %	CTRL	CB
Fishmeal 60 [a]	5.000	2.500
Porcine blood meal [b]	2.000	2.000
Algae meal (*Spirulina* sp.) [c]		1.000
Algae meal (*Chlorella* sp.) [d]		1.000
Soy protein concentrate [e]	2.500	2.500
Corn gluten meal [f]	4.000	4.000
Soybean meal 44 [g]	25.000	25.000
Rapeseed meal [h]	7.000	7.000
Sunflower meal [i]	12.500	12.500
Wheat meal [j]	22.500	22.329
Wheat bran [k]	5.000	5.000
Corn meal [l]	2.500	2.500
Salmon oil [m]		6.100
Soybean oil [n]	3.000	
Rapeseed oil [n]	3.000	
Vitamins and minerals premix [o]	1.000	1.000
Betaine HCl [p]	0.100	0.100
Binder [q]	1.000	1.000
Macroalgae meal (*Laminaria digitata*) [r]		0.541
Antioxidant [s]	0.200	0.200
Sodium propionate [t]	0.100	0.100
Sodium phosphate [u]	2.100	2.100
Selenised yeast [v]		0.030
L-Lysine [w]	0.700	0.700
L-Tryptophan [x]	0.200	0.200
DL-Methionine [y]	0.600	0.600
Proximate composition, %		
Crude protein	30.20	30.30
Crude fat	8.10	8.10
Crude ash	3.00	3.00
Crude fiber	5.00	5.00
Main fatty acids, %		
∑SFA	0.90	1.35
∑MUFA	3.50	3.75
∑n-3 PUFA	0.10	0.80
∑n-6 PUFA	3.20	1.30
20:5n−3 (EPA)	0.008	0.225
22:6n−3 (DHA)	0.016	0.267
∑EPA + DHA	0.024	0.492

[a] CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain.
[b] Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands.
[c] Spirulina meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland.
[d] Chlorella meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal.
[e] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[f] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.
[g] Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain.
[h] Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal.
[i] Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal.
[j] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[k] Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal.
[l] Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal.
[m] Sopropêche, France.
[n] H Lamotte Oils GmbH, Germany.
[o] INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's.
[p] ORFFA, The Netherlands.
[q] CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal.
[r] Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France.
[s] VERDILOX, Kemin Europe NV, Belgium.
[t] PREMIX LDA., Portugal.
[u] Vadequimica, Spain.
[v] ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France.
[w] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.
[x] TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany.
[y] DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

28 °C, and dissolved oxygen between 2 mg O_2 L^{-1} and 17 mg O_2 L^{-1}. Additionally, from the ongrowing pond where the trial was held, seven carps (W = 1024 ± 118.9 g) were collected (eCTRL).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.fct.2021.112146

2.2. Growth indices

In order to evaluate the dietary impact on common carp, the following growth parameters were calculated: total growth (TG), feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER) and survival rate (SR), using the following Equations (1)–(5):

$$TG = WG \times IBW^{-1} \times 100 \quad \text{(Eq1)}$$

$$FCR = FC \times WG^{-1} \quad \text{(Eq2)}$$

$$SGR = (Ln\ FBW - Ln\ IBW) \times D^{-1} \times 100 \quad \text{(Eq3)}$$

$$PER = WG \times PC^{-1} \quad \text{(Eq4)}$$

$$SR = FN \times IN^{-1} \times 100 \quad \text{(Eq5)}$$

where:

WG – weight gain (g)
IBW – initial body weight (g)
FC – feed consumed (g)
FBW – final body weight (g)
D – trial length (in days)
PC – protein consumed (g)
FN – final number of individuals in cage
IN – initial number of individuals in cage

2.3. Samples collection

At the end of the trial, fish (n = 9) from each dietary treatment (n = 3 per cage) and eCTRL (n = 7) were sacrificed using a lethal dose of 2-phenoxyethanol (2 mL L^{-1}) (Sigma-Aldrich, St. Louis, USA). Briefly, fish intestine and liver samples were collected immediately and preserved in DNA/RNA Shield™ (Zymo Research, Irvine, USA) and stored at −80 °C until RNA extraction. Additionally, a piece of intestine bulb (approx. 5 mm) and liver (approx. 125 mm^3) samples were collected and purged with deionised water for histomorphological assessment, subsequently placed in 5 mL glass jars and covered with 10% buffered formalin solution for 5 h at room temperature (Burck, 1975).

2.4. Total RNA extraction and cDNA synthesis

At the laboratories of the Department of Meat Science (West Pomeranian University of Technology, Szczecin, Poland), liver and intestine samples were homogenised in 750 μL Tri Reagent® (Zymo Research, Irvine, USA) for 60 s with a Minilys® personal homogenizer (Bertin Corp., Rockville, USA). Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, USA). An additional step of DNase I treatment was used to avoid contamination with genomic DNA, according to manufacturer's instructions. The quantity and quality of RNA was assessed using NanoDrop 2000 (ThermoFisher Scientific, Waltham, USA) and electrophoresis on 2% agarose gel. The 260/280 ratio of all RNA extracts was approx. 1.8–2.1, and no signs of

Fig. 1. Experimental setup of cages submerged in the ongrowing pond in Maliniec farm located in Northwest Poland.

RNA degradation were observed. Reverse transcription reaction was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) and 1 μg of RNA, according to the manufacturer's instructions, with anchored oligo $(dT)_{18}$ primers.

2.5. Evaluation of gene expression in liver and intestine

Real-time PCR reaction was performed on LightCycler® 480 II (Roche, Switzerland) using LightCycler® 480 SYBR Green I Master (Roche, Switzerland), 0.1 μM of each primer and 5 μL of 10 × diluted cDNA templates in the final volume of 20 μL. All reactions were performed using initial activation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and extending at 72 °C for 15 s. Melting curve analysis (65–95 °C) was conducted at the end of each PCR thermal profile to ensure the specificity of amplification, and a random RNA sample was tested to ensure the absence of genomic DNA contamination. Relative expression of the common carp genes in the liver [*peroxisome proliferator-activated receptor alpha* (*pparα*), *acyl-CoA oxidase* (*acox1*), *cholesterol 7 alpha-hydroxylase* (*cyp7a1*), *sterol regulatory element-binding protein 1* (*srebp-1*), *lipoprotein lipase* (*lpl*), *fatty acid synthase* (*fas*), *elongase of very long-chain fatty acids 2* (*elovl2*), *elongase of very long-chain fatty acids 5a* (*elovl5a*), *elongase of very long-chain fatty acids 5b* (*elovl5b*), *fatty acid desaturase 6a* (*fads6a*) and *fatty acid desaturase 6b* (*fads6b*)] and in the intestine [*zonula occludens-1* (*zo-1*), *occludin*, *claudin-3c*, *claudin-11*, *lipase*, *amylase*, *trypsin*, *γ-glutamyl transpeptidase* (*ggt*), *alkaline phosphatase* (*akp*), Na^+/K^+ *ATPase*] were assessed against two reference genes: *60S ribosomal protein L8* (*rpl8*) and *40S ribosomal protein S11* (40sRNA), (Table S1). Additionally, a series of decimal dilutions was performed to assess and correct the efficiency of qPCR reactions, which ranged from 0.91 to 1.10. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) in the GeneEx (MultiD Analyzes, Göteborg, Sweden) software.

2.6. Liver and intestine histomorphology

Liver and intestine fragments were immersed in fixing solution of 10% buffered formalin until processed. Fixed samples were dehydrated using serial concentrations of alcohol and saturated in intermediate solutions (benzene, benzene: paraffin), before being embedded in paraffin blocks, trimmed and serially sectioned (4 ± 1 μm, Rotary Microtome MPS-2, Opta-Tech, Poland). The slides from both liver and intestine were stained with haematoxylin and eosin, and mounted on slides with DPX balsam (Burck, 1975). Twelve randomly selected glass slides (3 fish × 4 slides) for each diet and tissue type were randomly selected and examined using an Eclipse E600 microscope (Nikon, Nikon, Japan) with 100 × objective and the NIS-Elements Basic Research software (Nikon Instruments Europe B.V, Japan). For liver samples, hepatocyte (C) and hepatocyte nucleus (N) areas and perimeters (300 measurements/parameter) were analysed, and the significance of observed differences was assessed for each parameter. Additionally, the nucleus:cytoplasm area ratio (N:C) of hepatocytes was calculated. Intestine samples were examined to identify pathological changes, i.e., necrosis, pyknosis and karyolysis.

2.7. Chemical analysis

Fatty acid profiles in fillets (CTRL, CB and eCTRL) were quantified using gas chromatography (GC) with a flame ionization detector (FID)

by the Central Diagnostics Laboratory of the University Life Sciences in Lublin in accordance with PN-EN ISO 12966–1:2015–01. The analyses were carried out in triplicate on an Agilent 6890N Network Gas Chromatograph (Agilent Technologies; Palo Alto, CA).

2.8. Statistical analysis and data visualisation

All data are shown as mean ± standard deviation unless otherwise specified. Normal distribution of data was assessed with the Shapiro–Wilk test (significance level $P < 0.05$). The significance of differences was assessed with the paired *t*-test or the Kruskal–Wallis test and the Tukey HSD post-hoc test, depending on the normality of distribution using Statistica 13.3 (TIBCO Software Inc.). Data were visualised using ggplot2 (Wickham, 2016) and biorender.com.

3. Results and discussion

3.1. Growth performance and PUFA fortification possibility

As an omnivorous fish species, common carp has been shown to be highly adaptive to the diet composition received (Zhao et al., 2020a). In our 16-week trial, fish fed CTRL and CB reached a market size (1.2–1.5 kg) with no significant ($P > 0.05$) difference between them, although FBW of fish fed CB was higher (Table 2). No differences were found in growth indices (TG or SGR), and only natural mortality was noted during the trial, with no significant difference between both diets. The obtained results were in line with previously reported data for carp, despite different farming conditions were used (Eljasik et al., 2020). Only one parameter of feed utilisation (i.e., PER) was significantly higher ($P = 0.0347$) in fish fed CB compared with CTRL, while FCR showed no difference between both groups. The better protein utilisation by fish fed CB could be explained by higher concentrations of EPA and DHA in the CB feed. The improved protein utilisation by fish fed increased levels of EPA and DHA was previously reported for Nile tilapia (Sarker et al., 2016), gilthead sea bream (*Sparus aurata*) (Magalhães et al., 2020), and olive flounder (*Paralichthys olivaceus*) (Kim and Lee, 2004). However, in the later species, cumulative levels of EPA and DHA exceeded dietary needs and adversely influenced PER and FCR (Kim and Lee, 2004). From the zootechnical point of view, oil from salmon industry by-products can be successfully implemented in carp farming based on formulated feeds.

The formulated finishing diets show a great potential in various fish species, including Atlantic salmon (*Salmo salar*) (Mørkøre et al., 2020), gilthead sea bream (Barbosa et al., 2020) and common carp (Eljasik et al., 2020). Enrichment with EPA and DHA from salmon by-product oil and microalgae/algae meals tested in this study showed limited success. In particular, the formulated feeds shifted fatty acid (FA) profile towards saturated fatty acids (SFA), at the expense of PUFA, mainly omega-6 (Table S2). However, the inclusion of salmon oil in CB diet improved the levels of EPA and DHA in fillets compared with the CTRL. The FA profile of eCTRL fish also proved that carp is a highly diet-adaptive species (Zhao et al., 2020a), and with natural feed supplemented with grains, it can synthesise EPA and DHA using the n-3 LC-PUFA biosynthesis pathway endogenously (Monroig et al., 2018). Nevertheless, the highest level of combined EPA and DHA noted in fish fed the CB diet may be seen as a determinant of limited success, since the results of a previous experiment with *Schizochytrium* sp. meal enrichment were more promising (Eljasik et al., 2020).

Table 2
Growth and feed utilisation indices of common carp fed control and experimental diets after the 16-week trial in the natural environment.

	CTRL	CB	P
IBW[a] [g]	250.07 ± 21.17	250.33 ± 3.21	n.s.
FBW[b] [g]	1295.14 ± 89.91	1359.44 ± 37.20	n.s.
TG[c] [%]	418.48 ± 14.46	442.99 ± 8.31	n.s.
FCR[d]	1.52 ± 0.04	1.46 ± 0.04	n.s.
SGR[e] [%/d]	1.08 ± 0.03	1.08 ± 0.02	n.s.
PER[f] [g/g]	2.29[a] ± 0.07	2.42[b] ± 0.03	0.0347
SR[g] [%]	96.00 ± 0.00	95.00 ± 1.00	n.s.

Explanations: Results represent mean ± standard deviation (n = 3). Values with different superscripts in rows indicate significant differences, *P* value indicated in last column.
[a] Initial body weight.
[b] Final body weight.
[c] Total growth.
[d] Feed conversion ratio.
[e] Specific growth rate.
[f] Protein efficiency ratio.
[g] Survival rate.

3.2. Lipids metabolism

In an extensive culture, carp feeds on a mixture of zooplankton, benthic organisms and supplemented grains, e.g., wheat, barley, triticale or lupin, depending on seasonal availability (Varga et al., 2020). Our study showed the possibility to fortify common carp with EPA and DHA, utilising finishing diets and their impact on pathways of lipid metabolism. Gene expression analysis showed that the diet specifically affected the activity of three genes involved in lipid metabolism i.e., *srebp-1*, *lpl* and *elovl2*. Subsequently, comparisons between eCTRL (extensive culture) and the trial variants revealed six differentially expressed genes, i.e. *pparα*, *cyp7a1*, *srebp-1*, *lpl*, *fas* and *elovl5a* (Fig. 2 A, B). Common carp, as many other teleost fish, is capable of biosynthesising DHA (Oboh et al., 2017). However, here only two genes involved in n-3 LC-PUFA biosynthesis were expressed differently (*elovl2* and *elovl5a*). The expression of hepatic *elovl2*, which is responsible for the elongation of EPA to a precursor of DHA synthesis i.e., docosapentaenoic acid (Morais et al., 2009), was higher in CB-fed carps compared with CTRL. Interestingly, the upregulation of *elovl2* was commonly noted in fish receiving an insufficient amount of essential fatty acids, therefore, our results are in contrast to previous reports (Betancor et al., 2014; Bou et al., 2017; Janaranjani and Shu-Chien, 2020). In view of the above, the regulatory mechanism behind the *elovl2* activity in carp requires further studies to fully elucidate regulation of this gene activity (Minghetti et al., 2011). The downregulation of *elovl5a* and also no difference in the activity of *elovl5b*, which are mediated by *srebp-1*, in CB and CTRL compared with eCTRL, were probably influenced by *srebp 1 and 2* suppression, since both have different transactivation responses to these transcription factors (Nakamura et al., 2004; Carmona-Antoñanzas et al., 2016). Ren et al. (2012) reported higher expression of *elovl5a* comparing to the *elovl5b* activity in common carp var. Jian exposed to different n-3 LC-PUFA supplementation in the diet, which is in contrast to our findings. Moreover, insignificant differences in expression of hepatic *elovl5b*, *fads6a* and *fads6b* may suggest similar FA metabolism at early stage of LC-PUFA biosynthesis pathway (Betancor et al., 2014).

The up-regulation of hepatic *srebp-1* in fish fed the CB diet compared with CTRL could indicate optimal level of n-3 LC-PUFA for carp in the diet, since *srebp-1*, a major lipogenesis regulator, may both promote or decrease lipids biosynthesis depending on n-3 LC-PUFA intake and the ratio between EPA and DHA in feed (Minghetti et al., 2011; Jin et al., 2017; Wang et al., 2020). Furthermore, down-regulation of *srebp-1* and its target gene *fas* in CTRL fed carp compared with eCTRL may suggest weakened lipogenesis related with inadequate DHA level in the feed mimicking a commercial blend. This was also reflected in levels of DHA deposited in carp meat, which were 0.56%, 2.02% and 0.76% for CTRL, CB and eCTRL, respectively (Table S2). Overexpression of hepatic *srebp-1* was previously shown for gilthead sea bream fed increased levels of EPA and DHA from different sources (Betancor et al., 2016; Houston et al., 2017). In contrast, the expression of *srebp-1* in Manchurian trout (*Brachymystax lenok*), large yellow croaker (*Larimichthys crocea*) or turbot (*Scophthalmus maximus*) decreased with an increasing level of n-3

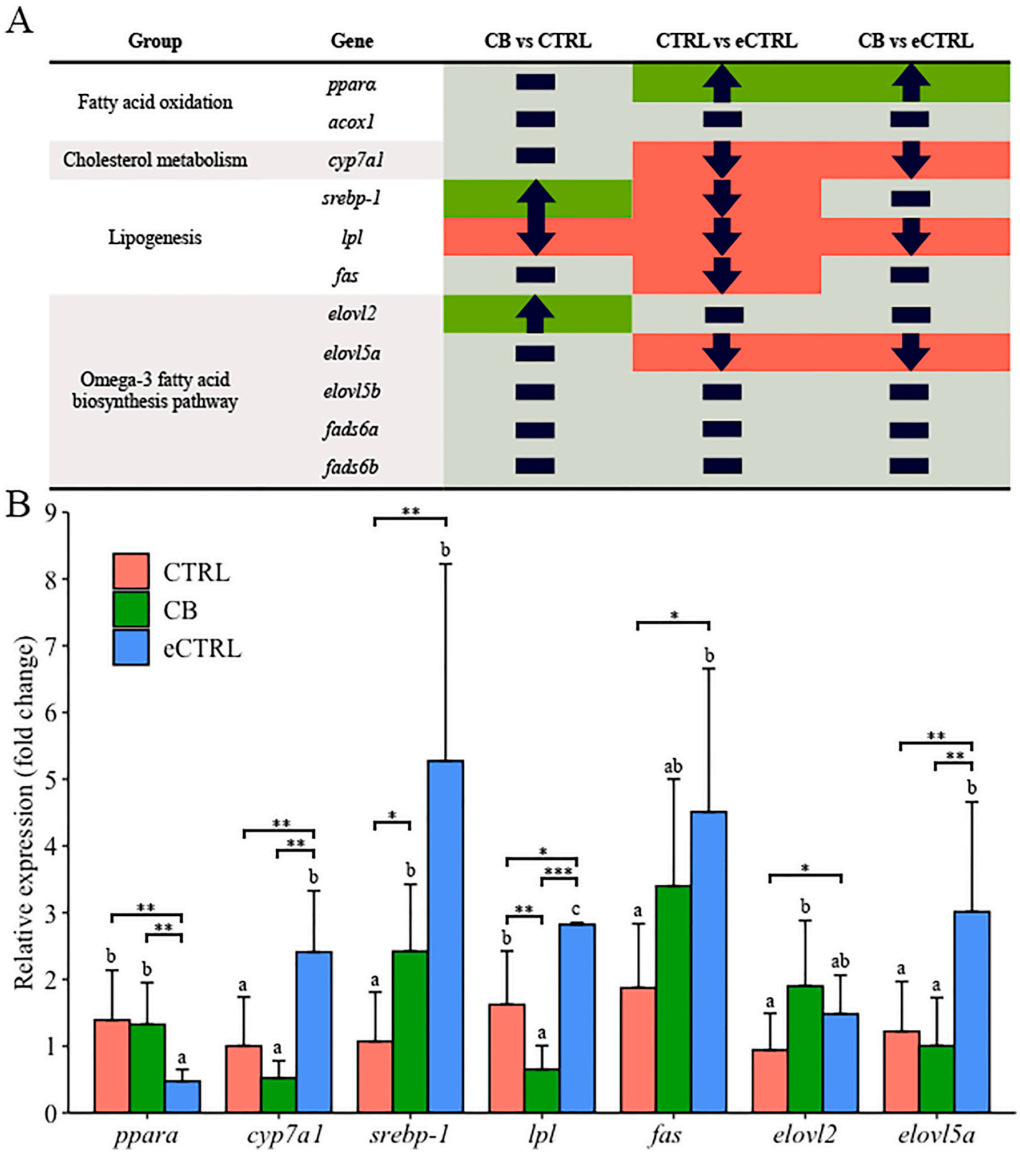

Fig. 2. Relative expression of lipid metabolism-related genes in the liver of common carp. (A) Upregulated (green) and downregulated (red) genes at a significant level, (B) fold change presented as mean ± SD. Asterisks indicate the significance of differences at: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

LC-PUFA (Yu et al., 2019; Peng et al., 2014). However, the regulation of *srebp-1* expression is very complex, and the potential influence of different components should not be ignored (Hagen et al., 2010; Minghetti et al., 2011). For instance, increased selenium intake in Yorkshire–Landrace–Hampshire crossbred pig up-regulated the *srebp-1* activity (Zhao et al., 2016).

The upregulation of hepatic *pparα* in CB and CTRL fed fish identified in our study could be related to a more balanced fatty acid profile of feeds compared with the natural diet (eCTRL). More precisely, the composition of SFA, MUFA and particularly the higher content of EPA and DHA in feed could upturn the expression of hepatic *pparα*, since they are favourably utilized in the β-oxidation process (Kjær et al., 2008; Lu et al., 2014). However, the functioning of *pparα* is multifaceted, and insignificant changes in its target gene *acox1*, an indicator of peroxisome proliferator exposure, may indicate the influence of different environmental factors (Reddy, 2001).

In farmed fish, hepatocyte histomorphology is often used as a measure of ectopic lipid accumulation influenced by dietary lipids (Figueiredo-Silva et al., 2005; Zhou et al., 2019). The microscopy analysis of carp liver in our study revealed no pathological indicators (e.g., vacuolisation, nuclei displacement) in CB and CTRL fed fish, as well as in eCTRL. However, we found significant differences ($P \leq 0.05$) in hepatocyte morphologies between fish fed the experimental diets and eCTRL (Table 3). More specifically, fish fed CB and CTRL had significantly smaller nucleus area and N:C ratio compared with eCTRL, and a significantly larger hepatocyte area. The difference in histomorphology, particularly the N:C ratio, indicates that formulated feeding with an increased level of available fat and its specific fractions, compared with natural plant-based diet, may result in the accumulation of fat in the liver. For instance, Caballero et al. (1999) found that high-fat diet results

Table 3
Liver histological parameters of common carp fed formulated feeds and from extensive pond culture.

	CTRL	CB	eCTRL
Hepatocyte area (N) [μm^2]	231.51^b ± 14.52	206.48^b ± 26.80	164.98^a ± 4.39
Nucleus area (C) [μm^2]	21.45^a ± 0.57	18.44^a ± 0.50	29.92^b ± 2.58
Ratio (N:C)[1]	0.0976^a ± 0.0042	0.0940^a ± 0.0100	0.1959^b ± 0.0180
Hepatocyte perimeter [μm]	57.99^b ± 2.05	54.14^b ± 3.04	48.49^a ± 0.81
Nucleus perimeter [μm]	17.21^b ± 0.25	15.66^a ± 0.34	20.37^c ± 0.96

Explanations: Results represent mean ± standard deviation. Values with different superscripts in rows indicate significant differences ($P \leq 0.05$). [1] Hepatocyte nucleus area/hepatocyte area.

in fat deposition in the liver of gilthead seabream. These findings were also confirmed in blunt snout bream, *Megalobrama amblycephala* (Zhou et al., 2019). The deposition of fat in the liver of fish fed CB and CTRL could result from the lower expression of hepatic *lpl*, which regulates gluconeogenesis from liver fat to maintain stable blood glucose levels, and thus regulates fat deposits in the liver (Tian et al., 2013). Additionally, accumulation of fat in the liver of carp may be associated with plant oils and proteins used in this study, as was previously shown for the rainbow trout, *Oncorhynchus mykiss* (Caballero et al., 2002). Therefore, in our study, the inclusion of oil from salmon by-products in the CB diet reduced the size of hepatocytes and suppressed the expression of hepatic *cyp7a.* The plant protein- and oil-dependent activity of *cyp7a1* was previously reported for several fish species and, plausibly, *cyp7a1* overexpression in fish fed plant-based diet results from: (i) binding of various plant components with bile salts, and thus reduced reabsorption; or (ii) lower amounts of cholesterol in plant-based diet

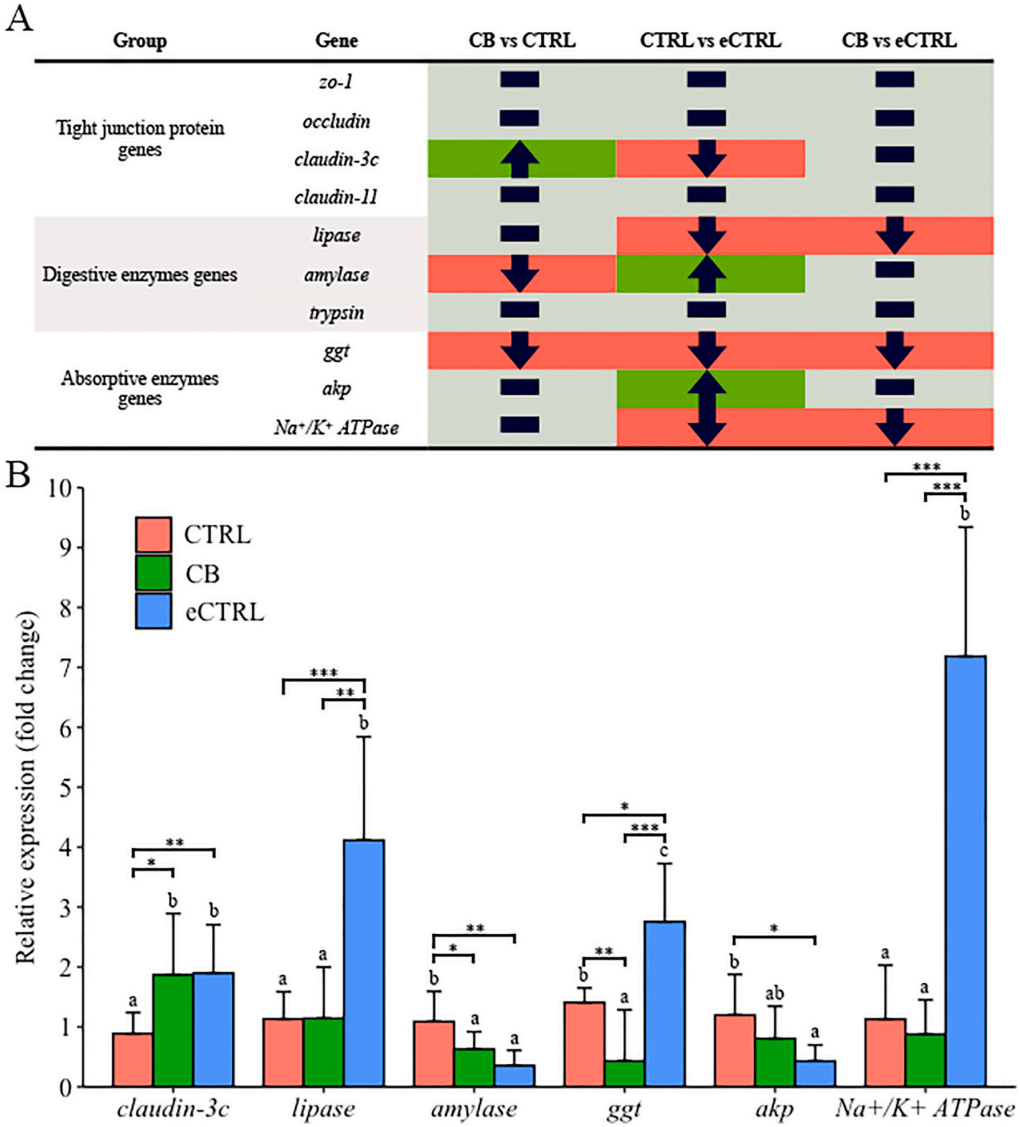

Fig. 3. Relative expression of homeostasis-related genes in the intestine of common carp. (A) \upregulated (green) and downregulated (red) genes at a significant level, (B) fold change presented as mean ± SD. Asterisks indicate the significance differences at: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Romano et al., 2020).

3.3. Intestinal health and nutrients absorption

The intestine plays an important role in maintaining fish health status, since epithelial cells create mechanical, microbiological and immunological barriers against infection and inflammation (Ringø et al., 2010; Dawood, 2021). Epithelial cells are bound together with junctional complex, which includes tight junction proteins (TJ) that are essential in the regulation of paracellular permeability to ions and other components (Milatz et al., 2010; Cao et al., 2019). In our study, we found downregulation of *claudin-3c* in fish fed CTRL compared with CB and eCTRL (Fig. 3 A, B). No other differences in the activities of TJ genes were noted, and no pathological changes (e.g., necrosis, pyknosis and karyolysis) were identified in the intestine histological samples in all groups. The difference in the *claudin-3c* expression may be due to multiple factors. For instance, soybean proteins can disrupt the intestinal integrity and downregulate *claudin-3c* expression (Zhang et al., 2021). The higher concentrations of EPA and DHA in the CB feed, compared with CTRL, possibly inverted the effect. A positive influence of dietary EPA and DHA on TJ was previously described in a laboratory trial on mice (Cao et al., 2019). The other possible explanation was the inclusion of selenium (selenised yeast) in the CB feed, which showed a great fortification potential for carp and gilthead seabream (Barbosa et al., 2020). Dietary selenium is known to enhance TJ functioning (Martin et al., 2007), and adverse effects of selenium deficiency on TJ were previously reported for grass carp (Zheng et al., 2018a, 2018b). Therefore, we can suppose that down-regulation of *claudin-3c* in fish fed CTRL was influenced by soybean proteins, and EPA, DHA and selenium supplementation in the CB diet restored the expression to a level of eCTRL. Additionally, similar expression levels of other TJ genes (*zo-1*, *occludin*, *claudin-11*), absence of pathological changes observed in intestine and liver histology, but improved growth (even not significant) may suggest that selenium (from selenised yeast) does not have the toxic effect on adult common carp (Lemly, 2002).

Absorptive enzymes are responsible for maintaining homeostasis of the intestine by limiting inflammation and reducing the adverse metabolic effects of consumed nutrients, and are useful indicators of nutrient uptake (Lallès, 2020; Yuan et al., 2020). In our study, we observed differences in the expression of absorptive enzymes i.e., *ggt*, *akp* and Na^{+}/K^{+} *ATPase*. The intestinal activity of the latter differed significantly between fish fed formulated diets (CB and CTRL) and eCTRL. The upregulation of intestinal Na^{+}/K^{+} *ATPase* in eCTRL fed fish could be explained by the deficiency of cholesterol in natural diet (Crockett and Hazel, 1997), which probably also altered the activity of hepatic *cyp7a1*. Moreover, the mechanism behind the Na^{+}/K^{+} *ATPase* activity is very complex, since many transport systems use the sodium gradient created by this enzyme to move components (e.g., amino acids, glucose) into the cells (Geering, 1990). Therefore, many factors, for example, salinity, heavy metal exposure, allergens or mechanical injury can influence the expression of Na^{+}/K^{+} *ATPase* (Zhang et al., 2013; Castaldo et al., 2020; Yuan et al., 2020). However, in our study, fish were reared in the same pond, thus the influence of environment conditions should be equal for all investigated variants.

We observed downregulation of intestinal *ggt* in fish fed the CB diet compared with both CTRL and eCTRL, and downregulation in fish fed CTRL compared with eCTRL. The activity of intestinal *ggt* is related to the passage of amino acids across cell membranes (Griffith and Meister, 1980). Chen et al. (2012) showed that the activity of this enzyme depends on dietary arginine level, and studies in different fish species indicated a dependency of dietary protein level and amino acid composition (Hakim et al., 2006; Tibaldi et al., 2006; Messina et al., 2019). However, in our study, formulated feeds were isonitrogenous, thus we suggest an influence of dietary EPA and DHA on the expression of *ggt*, since fish fed CB showed better dietary protein utilisation. The direct influence of EPA and DHA level on the activity of absorptive enzymes was previously shown for carp (Behar et al., 1989). Similarly, Camuesco et al. (2006), revealed higher activity of alkaline phosphatase in rats supplemented with n-3 LC-PUFA. Nonetheless, we observed an opposite effect, since the expression of intestinal *akp* was similar for fish fed CB and CTRL diets. The relation between n-3 LC-PUFA and the activity of *akp* remains unclear, and further research in this field is still needed (Lallès, 2020).

We also observed downregulation of *amylase*, involved in the digestion of carbohydrates, in fish fed CB compared with CTRL. The downregulation in fish fed CB could be related to minor differences in the component formulation of both diets, since the enzyme is carbohydrate sensitive (Zhao et al., 2020b). However, the difference may be also associated with dietary selenium supplementation. Iqbal et al. (2020) showed that dietary selenium reduces amylase activity in a dose-dependent manner in Nile tilapia. Therefore, we can assume that the expression of *amylase* could be influenced by both factors. In this study, we also found differences in the intestinal *lipase* and *amylase* expression in fish fed formulated diets compared with eCTRL and in fish fed CTRL compared with eCTRL, respectively. The activity of digestive enzymes in fish is correlated with the feed composition, consumption and starvation (Furné et al., 2008), and it is difficult to indicate when exactly carps nourished in extensive pond farming. However, Hofer and Sturmbauer (1985) reported inhibition of carp amylase by wheat, which was supplemented to eCTRL fish in this study.

4. Conclusion

Our study showed potential of utilising oil from salmon industry by-products in feeds for common carp farming with affordable increase in costs. The formulated feed provides both enrichment with EPA and DHA and contributes to the circular economy in the aquaculture sector. Noteworthily, we revealed no adverse effects of finishing diets on the intestine integrity, which is an important barrier against inflammation and infection, thus regulating the health status of fish. Furthermore, we reported changes in the activity of hepatic genes (*elovl2*, *elovl5a*) involved in lipid metabolism driven by n-3 LC-PUFA supplementation.

CRediT authorship contribution statement

Piotr Eljasik: Writing - review & editing, Investigation, Visualization. **Remigiusz Panicz:** Conceptualization, Writing - review & editing, Supervision. **Małgorzata Sobczak:** Writing - original draft, Validation, Investigation. **Jacek Sadowski:** Resources. **Agnieszka Tórz:** Formal analysis. **Vera Barbosa:** Writing - review & editing. **António Marques:** Writing - review & editing. **Jorge Dias:** Resources.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.112146.

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Supplementary Table S1. Sequences of qPCR primers for the detection of reference, lipid metabolism- and intestine health-related genes in common carp by real-time PCR.

Gene		Primer sequence 5' -> 3'	Tm (°C)	Function	Reaction efficiency	Reference
zo-1 [1]	F	GCGAAATGACACGGGCTAT	51.1	Tight junction protein	1.07	Yuan et al., 2020
	R	CTCTGTTGTGGTTGAGTGTAGGC	57.1			
occludin [2]	F	ATCGGTTCAGTACAATCAGG	49.7	Tight junction protein	0.97	
	R	GACAATGAAGCCCATAACAA	47.7			
claudin-3c [3]	F	TCACGGCACAAGTCATCTGG	53.8	Tight junction protein	0.96	Syakuri et al., 2013
	R	CGGTGGACAGTAACCGGGTTG	58.3			
claudin-11 [4]	F	GTGCTGGGAGGACTTCTTATAC	54.8	Tight junction protein	0.96	Zhao et al., 2019
	R	GAGAGAATCCGAACGACATCAG	54.8			
amylase [5]	F	GGCTGGATTCAGAGTAGA	48.0	Digestive enzyme	1.06	Yuan et al., 2020
	R	CAAGTGGTATTGAGGGTC	48.0			
lipase [6]	F	GCGAAATGACACGGGCTAT	51.1	Digestive enzyme	1.00	
	R	CTCTGTTGTGGTTGAGTGTAGGC	57.1			
trypsin [7]	F	TCGTTGGTGGATTTGAG	44.6	Digestive enzyme	0.98	Zhao et al., 2012
	R	GTAACAGTGAGCAGCAGACA	51.8			

ggt [8]	F	GTGGCTCAGCGGTAGATG	52.6	Absorptive enzyme	0.99	Yuan et al., 2020
	R	CCACTTTGTTCCCGTATTG	48.9			
akp [9]	F	ACCAATGCTCAGGTCCCA	50.3	Absorptive enzyme	0.98	
	R	CGCTCACTCCAACCGTAC	52.6			
Na^+/K^+ *ATPase* [10]	F	TGCCAGAACTTCTCCACA	48.0	Absorptive enzyme	0.94	
	R	AGCGATACCCATAGCCAC	50.3			
pparα [11]	F	GGGAAAGAGCAGCACGAG	52.6	Lipid metabolism	0.98	Corcoran et al., 2015
	R	GCGTGCTTTGGCTTTGTT	48.0			
acox1 [12]	F	ACAGCACAGCAAGAGCAATG	51.8	Peroxisomal β oxidation	1.00	
	R	ACAGAGTGGACAGCCGTATC	53.8			
cyp7a [13]	F	AAGTCTTGCACCGCTTTCTG	51.8	Cholesterol metabolism	0.93	Zhao et al., 2020b
	R	GCAGCTAACAGAGACCATGC	53.8			
srebp-1 [14]	F	CGCCTGCTTCACTTCACTACTC	56.7	Induction of lipogenesis	1.10	Yan et al., 2020
	R	GGACCAGTCCTCATCCACAAA	54.4			
lpl [15]	F	CGCTCCATTCACCTGTTCAT	51.8	Lipoprotein metabolism	0.91	
	R	GCTGAGACACATGCCCTTATT	52.4			
fas [16]	F	TGCTGGATGCTTTGTTTGAG	49.7	Fatty acid synthesis	1.00	Eljasik et al., 2020
	R	ACTACACCACCAGCGATTCC	53.8			

elovl2 [17]	F	TGGGCCAATGCTCAACAG	50.3	Elongation of very long-chain fatty acids	0.98	Zhang et al., 2019
	R	TGAAGCCACCAGGAACGA	50.3			
elovl5a [18]	F	GTCCTGACCATGTTCCAGACATCTTG	59.5	Elongation of very long-chain fatty acids	1.00	Ren et al., 2012
	R	CTGTAAGCGGACGAGGTGTCGTC	60.3			
elovl5b [19]	F	GTCCTGACCATGTTCCAGACATCTTG	59.5	Elongation of very long-chain fatty acids	0.99	
	R	CATGAAGCTCCTCTACTGCGCTG	58.8			
fads6a [20]	F	ATCGGACACCTGAAGGGAGCG	58.3	Fatty acid desaturation	0.93	
	R	CATGTTGAGCATGTTGACATCCG	55.3			
fads6b [21]	F	GTACCAATGGGAGGTTCGGCAC	58.6	Fatty acid desaturation	1.07	
	R	GAGTTGAAGGTTTGGATGAAATGCATG	56.7			
rpl8 [22]	F	CTCCGTCTTCAAAGCCCATGT	54.4	Ribosomal protein coding	0.97	Bickley et al., 2009
	R	TCCTTCACGATCCCCTTGATG	54.4			
40sRNA [23]	F	CCGTGGGTGACATCGTTACA	53.8	Ribosomal RNA gene	0.92	Gonzalez et al., 2007
	R	TCAGGACATTGAACCTCACTGTCT	55.7			

Supplementary Table S2. Summarised fatty acid profile of carp fillets fed CTRL and CB diets and eCTRL.

	CTRL	**CB**	**eCTRL**
Total SFA	$29.35^{a} \pm 0.33$	$31.07^{b} \pm 0.01$	$24.12^{c} \pm 0.68$
Total MUFA	$53.48^{a} \pm 0.88$	$50.64^{b} \pm 0.03$	$52.23^{ab} \pm 0.96$
EPA	$0.23^{a} \pm 0.03$	$0.55^{b} \pm 0.01$	$1.19^{b} \pm 0.45$
DHA	$0.56^{a} \pm 0.01$	$2.02^{b} \pm 0.01$	$0.76^{c} \pm 0.21$
EPA + DHA	$0.79^{a} \pm 0.03$	$2.58^{b} \pm 0.01$	$1.94^{b} \pm 0.65$
Total n-3 PUFA	$1.38^{a} \pm 0.04$	$3.95^{b} \pm 0.02$	$6.53^{c} \pm 0.84$
Total n-6 PUFA	$9.22^{a} \pm 0.07$	$9.31^{a} \pm 0.08$	$17.10^{b} \pm 0.54$
Total PUFA	$10.59^{a} \pm 0.11$	$13.26^{b} \pm 0.07$	$23.63^{c} \pm 0.30$

Supplementary Video S1. Video showing experimental setup of cages submerged in the ongrowing pond in Maliniec farm located in Northwest Poland.

OŚWIADCZENIE DO PRACY P5

W pracy **P5** byłem zaangażowany w przeprowadzenie doświadczenia, pobór i zabezpieczenie prób biologicznych, wykonanie analiz histologicznych, ocenę sensoryczną, tekstury oraz barwy surowca, analizę i interpretację uzyskanych wyników oraz przygotowanie manuskryptu artykułu. Swój udział określam na 40%.

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Nutritional value and sensory properties of common carp (*Cyprinus carpio* L.) fillets enriched with sustainable and natural feed ingredients

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ABSTRACT

Declines across global fishery stocks forced aquaculture feed manufacturers to search for new and sustainable components. Therefore, the aim of study was assessing nutritional value and sensory properties of meat of common carp (*Cyprinus carpio* L.) fed for 116 days with two blends. The control feed contained 5% of fishmeal and vegetable oils (rapeseed and soybean) as sole fat sources. While in the experimental diet half of the fishmeal was replaced with a blend of microalgae (*Spirulina* sp., *Chlorella* sp.), macroalgae (*Laminaria digitata*) and vegetable oil was replaced with salmon oil. Proximate composition, energy value, fatty acid profile of meat, nutritional characteristics of fat and protein as well as culinary properties of fillets were assessed. Fillets of carp fed experimental diet had a higher level of protein, lower level of fat and energy value. Intramuscular fat of fish fed with the experimental diet had a better parameters of quality. Protein in the meat of fish from both groups was characterized by a high quality comparing to the protein standard. Our study showed that meat of carp fed with experimental feed enriched with sustainable and natural feed ingredients can be a sensorily attractive source of nutritious ingredients in the human diet.

1. Introduction

Common carp is the most common fish species consumed in Central and Eastern Europe. The history of carp breeding in this area dates back to the Middle Ages. Currently, carp is among the top four cultivated fish species in the world (Ćirković et al., 2012), which accounted for 8% of fish species produced in aquaculture in 2016 (FAO, 2018). Freshwater species, such as carp, catfish (including *Pangasius* spp.) and tilapia, are expected to represent about 62% of total aquaculture production in 2030, as compared with 58% in 2016 (FAO, 2018). Carp meat is a valuable raw material due to the presence of highly nutritious protein, fat, minerals and vitamins. The average protein content of carp meat is 15.9–18.5% (Yeganeh et al., 2012; Ćirković et al., 2012; Trbović et al., 2013; Kurćubić et al., 2017; Skibniewska et al., 2013). With a fat content of 1.5–6.8% (Kmínková et al., 2001; Yeganeh et al., 2012; Ćirković et al., 2012; Trbović et al., 2013; Kurćubić et al., 2017), carp meat can be considered lean or moderately fatty (Ackman, 1989). Despite the low fat content in carp meat, it has good nutritional properties (Kmínková et al., 2001; Yeganeh et al., 2012; Linhartová et al., 2018; Kłobukowski et al., 2018). Carp fat contains 15.5–31.7% of saturated fatty acids (SFAs), 20.7–63.5% of monounsaturated fatty acids (MUFAs) and 8.9–65.5% of polyunsaturated fatty acids (PUFAs), with eicosapentaenoic acid (EPA) constituting 0.4–4.8% of fatty acids (FAs) and docosahexaenoic acid (DHA) constituting 0.8–11.6% of FA (Yeganeh et al., 2012; Csengeri et al., 2013; Trbović et al., 2011). The chemical composition and, consequently, the nutritional value of carp meat depend on several factors, i.e. lines, crossbreds, age, environmental conditions, season and feed composition (Kmínková et al., 2001; Trbović et al., 2013; Mráz and Pickova, 2011), as well as the type of fertilization (Kour et al., 2016). Previous results showed differences in the proximate composition and FA profile of meat in various genetic groups of common carp (Buchtová et al., 2010), as well as between wild and farmed fish and among seasons (Yeganeh et al., 2012). Freshwater fish, including carp, have much less essential unsaturated fatty acids (UFAs), (DHA, EPA) but are richer in amino acids compared with marine fish (Borowiec et al., 2010;

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Abbreviations

AA(s)	amino acid(s)
AAA(s)	aromatic amino acid(s)
AI	index of atherogenicity
BMI	body mass index
CS	chemical score
DHA	docosahexaenoic acid (22:6n-3)
EAA	essential amino acids
EAA:NEAA	essential amino acids/nonessential amino acids ratio
EAAI	essential amino acids index
EPA	eicosapentaenoic acid (20:5n-3)
EUFA(s)	essential unsaturated fatty acid(s)
FA(s)	fatty acid(s)
h:H	hypocholesterolemic/hypercholesterolemic ratio
HUFA(s)	highly unsaturated fatty acid(s)
MUFA(s)	monounsaturated fatty acid(s)
NEAA	nonessential amino acids
n-3 PUFA(s)	total omega-3 polyunsaturated fatty acid(s)
n-6 PUFA(s)	total omega-6 polyunsaturated fatty acid(s)
n-9 PUFA(s)	total omega-9 polyunsaturated fatty acid(s)
PUFA(s)	polyunsaturated fatty acid(s)
SAA(s)	sulfur amino acid(s)
SFA(s)	saturated fatty acid(s)
TI	index of thrombogenicity
UFA(s)	unsaturated fatty acid(s)

Łuczyńska et al., 2014). Yet, it is the long-chain UFA of the total omega-3 polyunsaturated fatty acids (n-3 PUFA) and total omega-6 polyunsaturated fatty acids (n-6 PUFA) groups that are particularly valuable owing to the positive effects on human health (Mráz et al., 2012b; Trbović et al., 2013; Simopoulos, 2016). DHA is one of the most abundant components of brain structural lipids, as well as the cerebral cortex, retina, testis and sperm (Simopoulos, 2016). DHA is also necessary for the functioning of the central nervous system, infant brain development and visual activity (Vahmani et al., 2020). High intake of omega-6 FAs during the perinatal period is associated with increased adiposity in the offspring. In human studies, the level of archaidonic acid in adipose tissue is associated with the body mass index (BMI) and overweight status of children (Simopoulos, 2016). They are also recommended by nutritionists in the prevention of many diseases, e.g. reduction of coronary heart disease risk (especially myocardial infarction, arteriosclerosis, hypertension and other cardiovascular diseases) and prevention of inflammatory and autoimmune diseases, cancer, diabetes and obesity (Simopoulos, 2016; Trbović et al., 2013; Mráz et al., 2012b). For the general population, 2–3 servings of fish per week (Simopoulos, 2016) or the consumption of 250 mg of EPA and DHA per day are recommended (EFSA, 2009).

In the case of farmed fish, including carp, FA composition can vary considerably, being strongly depending on rearing methods and ingested feed. The possibility of modulating diet quality according to fish species and human requirements can be used to produce sustainable and nutritionally high-quality fish (Steffens and Wirth, 2007). Owing to this, farmed carp and other freshwater species may be as nutritionally valuable as wild fish. Previous results confirmed the effect of feed enriched with n-3 PUFA (fish oil, vegetable oil, olive oil and algae) on fat content and FA profile of carp meat (Borowiec et al., 2010; Mráz et al., 2012a; Csengeri et al., 2013). Moreover, the replacement of animal protein (fish meal) in fish feed by plant protein (bean, soybean, sunflower, grain, legume seeds) may strongly affect the proximate composition of the produced meat, FA and AA profile, as well as nutritional quality of fat (Steffens and Wirth, 2007; Steffens, 2016; Kłobukowski et al., 2018), without any deterioration in fish growth, organoleptical properties and meat nutritional quality (Mazurkiewicz, 2009; Adekoya et al., 2018). However, in case of common carp information on influence of feeds manufactured using aquaculture and/or fisheries ingredients (seaweeds, by-products) on fillet properties are still scarce. Therefore, the aim of this study was to assess the nutritional (proximate composition, energy value, profile of FAs and amino acids, nutritional value of fat and protein) and sensory quality of carp fed with diets enriched with sustainable and natural ingredients (algae meal and salmon oil).

2. Materials and methods

2.1. Experimental diets

The trial comprised two diets: control and experimental feed (Table 1). The control diet, mimicking a commercial feed formulation for common carp, contained moderate levels of fishmeal (5%) and high levels of plant raw materials and vegetable oils (rapeseed and soybean) as sole fat sources. In comparison, in the experimental diet, half of the fishmeal was replaced with a blend of microalgae (*Spirulina* sp., *Chlorella* sp.), macroalgae (*Laminaria digitata*) and selenized yeast. Additionally, vegetable oil was replaced with salmon oil extracted from by-products of farmed Atlantic salmon. Both feeds were produced by extrusion at Sparos Lda facilities (Olhão, Portugal).

2.2. Fish, rearing and housing facilities

The study was carried out at the carp farm in Maliniec located in north-east Poland (53° 42′ 5.99″ N 15° 21′ 22.19″ E). Common carp (*Cyprinus carpio* L.) were obtained from an earthen pond, where fish were fed according to typical extensive model, i.e. natural food and grains (triticale, wheat, rye), and transferred to floating cages placed in the same earthen pond. In total, 6 cuboid cages of 3 m^3 were used (3 cages per feed). Each cage was stocked with 100 fish (average starting weight of 250 ± 10 g). The feeding trial was conducted for 116 days (representing a finishing diet) during which fish were hand-fed with the feed blends (control and experimental, Table 1) in equal portions at 9:00 and 15:00 h. At the end of the last day of the trial, n = 10 fish from each cage were slaughtered, following commercial practices, and filleted. Filleting was performed by one individual. All fish samples were stored at 4 °C until analysis.

2.3. Color and pH measurement

Fillet color was assessed using a NR 20XE Precision Colorimeter (Shenzhen 3NH Technology Co., Ltd., Shenzhen, China) with ϕ20mm extended aperture. L* (lightness), a* (redness), b* (yellowness) were obtained automatically after a light shot was discharged perpendicularly to the inside surface of fillet. Measurements were done in triplicate.

pH was measured in quadruplicates for each raw meat sample (n = 20). The measurements were done using a portable pH meter (CP-411, Elmetron, Zabrze, Poland) with a glass penetrating electrode. Before the analysis, the pH meter was calibrated using standard phosphate buffers (pH 4.00 and 7.00). Between measurements, the electrode was rinsed thoroughly with distilled water.

2.4. Chemical analysis

The chemical composition of minced fillets was determined

Table 1
Characterization of fish feeds.

Component (% of wet weight)	CONTROL FEED	EXPERIMENTAL FEED
Fishmeal 60 [a]	5.000	2.500
Porcine blood meal [b]	2.000	2.000
Algae meal (*Spirulina* sp.) [c]	–	1.000
Algae meal (*Chlorella* sp.) [d]	–	1.000
Soy protein concentrate [e]	2.500	2.500
Corn gluten meal [f]	4.000	4.000
Soybean meal 44 [g]	25.000	25.000
Rapeseed meal [h]	7.000	7.000
Sunflower meal [i]	12.500	12.500
Wheat meal [j]	22.500	22.329
Wheat bran [k]	5.000	5.000
Corn meal [l]	2.500	2.500
Salmon oil [m]	–	6.100
Soybean oil [n]	3.000	–
Rapeseed oil [n]	3.000	–
Vitamins and minerals premix [o]	1.000	1.000
Betaine HCl [p]	0.100	0.100
Binder [q]	1.000	1.000
Macroalgae meal (*Laminaria digitata*) [r]	–	0.541
Antioxidant [s]	0.200	0.200
Sodium propionate [t]	0.100	0.100
Sodium phosphate [u]	2.100	2.100
Selenized yeast [v]	–	0.030
L-Lysine [w]	0.700	0.700
L-Tryptophan [x]	0.200	0.200
DL-Methionine [y]	0.600	0.600
Proximate composition		
Crude protein (%)	30.20	30.30
Crude fat (%)	8.10	8.10
Crude ash (%)	3.00	3.00
Crude fiber (%)	5.00	5.00
Main fatty acids		
∑SFA (mg $100g^{-1}$)	900	1350
∑MUFA (mg $100g^{-1}$)	3500	3750
∑n-3 PUFA (mg $100g^{-1}$)	100	800
∑n-6 PUFA (mg $100g^{-1}$)	3200	1300
EPA (mg $100g^{-1}$)	8.390	224.500
DHA (mg $100g^{-1}$)	16.060	266.760

[a] CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain.
[b] Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands.
[c] Spirulina meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland.
[d] Chlorella meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal.
[e] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[f] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.
[g] Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain.
[h] Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal.
[i] Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal.
[j] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[k] Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal.
[l] Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal.
[m] Sopropêche, France.
[n] Lamotte Oils GmbH, Germany.
[o] INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulfate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's.
[p] ORFFA, The Netherlands.
[q] CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal.
[r] Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France.
[s] VERDILOX, Kemin Europe NV, Belgium.
[t] PREMIX LDA., Portugal.
[u] Vadequimica, Spain.
[v] ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France.
[w] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.
[x] TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany.
[y] DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

according to Association of Official Analytical Chemists procedures (Latimer, 2019). Moisture was obtained after drying samples in an oven at 105 °C for 24 h, while ash content was determined after incineration at 550 °C for 6 h. Crude protein was measured by determining nitrogen content (N x 6.25), according to the Kjeldahl method, using a Tecator Kjeltec 2100 distillation unit (FOSS Analytical Co., Ltd., Jiangsu, China), whereas crude lipid was determined gravimetrically, after Soxhlet lipid extraction on a Tecator Soxtec System HT 1043 (FOSS Analytical Co., Ltd., Jiangsu, China). Fatty acid profiles were quantified by gas chromatography (GC) with a flame ionization detector (FID in accordance with PN-EN ISO 12966–1:2015-01 by the Central Diagnostics Laboratory of the University Life Sciences in Lublin. Analyses were carried out in triplicate on an Agilent 6890N Network Gas Chromatograph (Agilent Technologies; Palo Alto, CA) equipped with a 7683 automatic liquid sampler and flame ionization detectors. The amino acid profile of proteins in meat samples was determined by High Performance Liquid Chromatography (HPLC) using an AAA 400 amino acid analyser (Ingos, Prague, Czech Republic). The chromatograms were analyzed using the CHROMuLAN V 0.88 program (PiKRON, Prague, Czech Republic) by comparison with the standard chromatogram, taking into account dilution and weight.

2.5. Nutritional value

Energetic value was calculated using the relative percentage of each nutrient (protein and fat), which was multiplied by the correction factors, 4 kcal g^{-1} (17 kJ g^{-1}) and 9 kcal g^{-1} (37 kJ g^{-1}) for protein and fat, respectively, as described in the Regulation (EU) No 1169/2011.

Protein quality was described by the chemical score (CS) of essential amino acids (EAA) and the essential amino acids index (EAAI). The CS was calculated in relation to the reference protein pattern suggested by FAO/WHO/UNU (2007) according to the following equation:

$$CS = \frac{g\ EAA in tested protein}{g\ EAA in pattern protein} \times 100$$

The essential amino acids index (EAAI) was calculated according to the equation described by Shahidi and Synowiecki (1993).

$$EAAI = 100 \times \sqrt[n]{\frac{a}{a_p} \times \frac{b}{b_p} \times \ldots \times \frac{i}{i_p}},$$

where:

a, b, …, i – content of histidine, isoleucine, leucine, lysine, sulfur amino acids (SAAs as sum of methionine and cysteine), aromatic amino acids (AAAs as sum of phenylalanine, tyrosine and tryptophan), threonine and valine in the sample;
a_p, b_p, …, i_p – content of histidine, isoleucine, leucine, lysine, SAAs, AAAs, threonine and valine in the protein standard;
n – number of amino acids (FAO/WHO/UNU, 2007).

Fat quality was described by the following factors: SFA as sum of SFAs, MUFA as sum of MUFAs, PUFA as sum of PUFAs, hypocholesterolemic/hypercholesterolemic ratio (h/H), index of atherogenicity (AI) and index of thrombogenicity (TI). These factors were calculated using the following equations:

SFA = Σ (C6:0, C8:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0),
MUFA = Σ (C14:1n5, C16:1n7, C17:1n7, C18:1n9t, C18:1n9c, C20:1n9, C22:1n9),

PUFA = Σ (C18:2n6t, C18:2n6c, C18:3n6, C18:3n3, C20:2n6, C20:3n3, C20:3n6, C20:4n6, C20:5n3, C22:6n3),
h/H = Σ(C18:1n 9, C18:1n 7, C18:2n 6, C18:3n 6, C18:3n 3, C20:3n 6,C20:4n 6, C20:5n 3, C22:4n 6, C22:5n 3, C22:6n 3)/Σ(C14:0, C16:0), (Fernández et al., 2007),
AI = (C12:0 + 4 × C14:0+C16:0)/((n-6)PUFA+(n-3)PUFA + MUFA), (Ulbricht and Southgate, 1991; Fehily et al., 1994),
TI = (C14:0+C16:0+C18:0)/(0.5 × MUFA+0.5 × (n-6)PUFA+3.0 × (n-3)PUFA+(n-3)PUFA/(n-6)PUFA), (Fehily et al., 1994).

2.6. Cooking loss

Right fillets were weighed and steamed until their internal temperature reached 68 ± 1 °C in the thickest portion of the fillet, followed by cooling to 5 ± 1 °C. The temperature was measured using a portable thermometer DT-34 (Termoprodukt, Bielawa, Poland). After chilling, samples were weighed to calculate cooking losses.

2.7. Texture profile analysis

The texture of fillets was measured with a TA.XT Plus Texture Analyser (Stable Micro Systems, Godalming, UK), by applying the double compression texture profile analysis (TPA) test (Bourne, 1982). Briefly, in the TPA test, a 9.6 mm shaft was driven into the sample twice to the depth of 80% of the original height, and parameters, such as hardness (N), cohesiveness (−), springiness (cm) and chewiness (N × cm), were measured. The crosshead speed was 50 mm/min and TPA test was conducted at least five times for each fillet.

2.8. Sensory analysis

Sensory evaluation was conducted by a trained team composed by four members using individual light and temperature controlled booths, providing privacy and comfort for the participants (PN-ISO 11036:1999). The steamed samples were placed in petri dishes, coded and served at room temperature to the panelists. Mineral water was used to cleanse the panelists' palate to ensure the correct sensory evaluations between samples. All tests were performed on the same day. Texture characteristics (tenderness, springiness, cohesiveness, chewiness, moisture, juiciness, fattiness), odor and taste descriptors intensity were evaluated. Intensity of these features was rated using a 5-point scale, where 1 point corresponded to the lowest and 5 points the highest intensity. The sensory evaluation was repeated 3 times on each sample.

2.9. Statistical analysis

Data were analyzed using STATISTICA for Windows (version 13.1) and subjected to analysis of one-way variance (ANOVA) and *t*-test to compare sample means. The significance level in *t*-test was 0.05.

3. Results

3.1. Nutritional value of fillets

Significant differences ($P \leq 0.05$) were found in the proximate chemical composition of fish meat fed with different feeds (Table 2). The meat of carp fed with the experimental feed was characterized by a higher content of crude protein (6%) and moisture (5%), as well as a lower content of crude fat (37%), dry matter (14%) and energy value (2%) compared to the control group.

The analysis of amino acid composition (Table 3) did not reveal significant difference ($P > 0.05$) in the content of most amino acids between the two fish groups tested. The only exceptions were found for proline that revealed 3% higher levels in fillets of fish fed with the experimental diet, whereas histidine, methionine, tryptophan, glycine and the EAA:NEAA ratio presented reduced values in fillets of fish fed

Table 2
Proximate composition and energy value of carp fillets fed with the control and experimental diet.

Component (% of wet weight)		Fish group		Influenceof fish group
		CONTROL	EXP DIET	
Crude protein (%)		$17.16^{a} \pm 0.32$	$18.19^{b} \pm 0.10$	**
Crude fat (%)		$1.88^{a} \pm 0.30$	$1.18^{b} \pm 0.03$	**
Moisture (%)		$74.16^{a} \pm 0.05$	$77.86^{b} \pm 0.38$	**
Dry matter (%)		$25.84^{a} \pm 0.05$	$22.14^{b} \pm 0.38$	**
Energy value	(kcal 100 g^{-1})	$85.54^{a} \pm 0.99$	$83.42^{b} \pm 0.18$	*
	(kJ 100 g^{-1})	$361.20^{a} \pm 4.25$	$353.08^{b} \pm 0.78$	*

Values are expressed as mean ± standard deviation (SD), ^{ab} – values in rows with different index differ significantly ($P \leq 0.05$), significance of influence: n.s. – non-significant, *$P \leq 0.05$; **$P \leq 0.01$.

Table 3
Content of essential and nonessential amino acids in fish meat fed with the control and experimental diets.

Amino acids(g 100 g^{-1} of protein)	Fish group		Influenceof fish group
	CONTROL	EXP DIET	
Histidine	$4.02^{a} \pm 0.07$	$3.57^{b} \pm 0.02$	**
Arginine	6.30 ± 0.12	6.21 ± 0.03	n.s.
Isoleucine	4.70 ± 0.09	4.64 ± 0.03	n.s.
Leucine	8.51 ± 0.16	8.52 ± 0.05	n.s.
Lysine	11.20 ± 0.20	11.16 ± 0.06	n.s.
Methionine	$3.69^{a} \pm 0.07$	$3.56^{b} \pm 0.02$	*
Tryptophan	$3.18^{a} \pm 0.06$	$2.54^{b} \pm 0.01$	**
Tyrosine	3.56 ± 0.07	3.55 ± 0.02	n.s.
Phenylalanine	4.69 ± 0.09	4.68 ± 0.03	n.s.
Valine	5.57 ± 0.10	5.45 ± 0.03	n.s.
Threonine	5.26 ± 0.10	5.29 ± 0.03	n.s.
Total essential amino acids (EAA)	60.69 ± 1.11	59.19 ± 0.33	n.s.
Alanine	6.76 ± 0.12	6.60 ± 0.04	n.s.
Aspartic acid	11.90 ± 0.22	11.98 ± 0.07	n.s.
Cysteine	1.42 ± 0.03	1.38 ± 0.01	n.s.
Glutamic acid	16.91 ± 0.31	17.32 ± 0.10	n.s.
Glycine	$5.18^{a} \pm 0.10$	$4.96^{b} \pm 0.03$	*
Proline	$3.56^{a} \pm 0.07$	$3.68^{b} \pm 0.20$	*
Serine	4.61 ± 0.08	4.73 ± 0.03	n.s.
Total nonessential amino acids (NEAA)	50.34 ± 0.92	50.66 ± 0.28	n.s.
EAA: NEAA	$1.21^{a} \pm 0.02$	$1.17^{b} \pm 0.02$	**

Values are expressed as mean ± standard deviation (SD), ^{ab} – values in rows with different index differ significantly ($P \leq 0.05$), significance of influence: n.s. – non-significant, *$P \leq 0.05$; **$P \leq 0.01$.

with the experimental diet (by approx. 11%, 12%, 20%, 4% and 3%, respectively).

The study also compared the nutritional quality of protein from the meat of fish fed with the control and experimental feeds (Table 4). The meat of both groups of fish was characterized by a high CS index, indicating that its consumption exceeds the FAO/WHO/UNU (2007) recommended amino acid demand, both for children aged 3–10 years and adults (>18 years old). The CS index for amino acids covered the demand for amino acids in the range 136–489% in children and 141–530% in adults. Significant differences between the groups were found in CS for histidine, tryptophan ($P \leq 0.01$) and SAAs (sulfur amino acids) ($P \leq 0.05$). Differences in the content of these amino acids between groups were 11%, 20% and 3%, respectively. There was no significant difference between the feeds in respect to the essential amino acid index (EAAI).

The nutritional value of fish meat was characterized by the FA profile of its fat. The study analyzed the FA content of muscle fat in fish fed with the different feeds (Table 5). In both groups, MUFAs were the most prevalent FA type in muscle fat (50.64–52.94%), whereas PUFAs were

Table 4
Nutritional quality of proteins from fillets of carp fed with the control and experimental diets.

Amino acids	FAO/WHO/UNU (2007) Scoring pattern (g 100 g^{-1} of protein)	CS (% of scoring pattern)		
		Fish group		Influenceof fish group
		CONTROL	EXP DIET	
For adults (>18 years old)				
Histidine	1.5	268.23^{a} ±4.93	237.87^{b} ± 1.31	**
Isoleucine	3.0	156.66 ± 2.88	154.85 ± 0.85	n.s.
Leucine	5.9	144.30 ± 2.65	144.43 ± 0.79	n.s.
Lysine	4.5	248.80 ± 4.57	248.01 ± 1.36	n.s.
SAA	2.2	232.45^{a} ± 4.27	224.66^{b} ± 1.24	*
Phe + Tyr	3.8	217.29 ± 3.99	216.58 ± 1.19	n.s.
Threonine	2.3	228.68 ± 4.20	230.19 ± 1.27	n.s.
Tryptophan	0.6	529.66^{a} ± 9.73	423.32^{b} ± 2.33	**
Valine	3.9	142.94 ± 2.62	139.84 ± 0.77	n.s.
EAAI		141.52 ± 0.32	141.05 ± 0.10	n.s.
For children 3–10 years old				
Histidine	1.6	251.47^{a} ± 4.62	223.00^{b} ± 1.23	**
Isoleucine	3.1	156.66 ± 2.88	154.85 ± 0.85	n.s.
Leucine	6.1	141.89 ± 2.61	142.02 ± 0.78	n.s.
Lysine	4.8	233.25 ± 4.28	232.51 ± 1.28	n.s.
SAA	2.4	222.34^{a} ± 4.08	214.89^{b} ± 1.18	*
Phe + Tyr	4.1	201.39 ± 3.70	200.73 ± 1.10	n.s.
Threonine	2.5	210.39 ± 3.86	211.77 ± 1.16	n.s.
Tryptophan	0.66	488.92^{a} ± 8.98	390.76^{b} ± 2.15	**
Valine	4.0	139.36 ± 2.56	136.34 ± 0.75	n.s.
EAAI		140.42 ± 0.32	139.95 ± 0.10	n.s.

SAA – sulfur amino acids, EAAI – essential amino acids index.
Values are expressed as mean ± standard deviation (SD), ^{ab} – values in rows with different index differ significantly ($P \leq 0.05$), significance of influence: n.s. – non-significant, *$P \leq 0.05$; **$P \leq 0.01$.

Table 5
Content of fatty acids and nutritional value of fat in fillets of carp fed with the control or experimental diets.

Fatty acids(% of fat)	Fish group		Influenceof fish group
	CONTROL	EXP DIET	
(C6:0) Caproic acid	0.08 ± 0.01	< LOD	–
(C8:0) Caprylic acid	0.06 ± 0.01	< LOD	–
(C12:0) Lauric acid	0.03 ± 0.01	< LOD	–
(C14:0) Myristic acid	0.90^{a} ± 0.08	1.46^{b} ± 0.02	**
(C15:0) Pentadecanoic acid	0.14^{a} ± 0.01	0.21^{b} ± 0.01	**
(C16:0) Palmitic acid	20.85^{a} ± 0.14	23.43^{b} ± 0.09	**
(C17:0) Heptadecanoic acid	0.26 ± 0.01	< LOD	–
(C18:0) Stearic acid	6.86^{a} ± 0.07	5.83^{b} ± 0.05	**
(C20:0) Arachidic acid	0.16^{a} ± 0.01	0.14^{b} ± 0.01	*
Total SFA	**28.80^{a} ± 0.53**	**31.07^{b} ± 0.01**	**
(C14:1n5) cis-9-Tetradecenoic acid	0.36^{a} ± 0.01	0.12^{b} ± 0.01	**
(C16:1n7) Palmitoleic acid	5.66^{a} ± 0.05	7.89^{b} ± 0.10	**
(C17:1n7) cis-10-Heptadecenoic acid	0.15^{a} ± 0.01	0.13^{b} ± 0.01	*
(C18:1n9t + C18:1n9c) Elaidic acid + Oleic acid	42.76^{a} ± 0.75	39.08^{b} ± 0.19	**
(C20:1n9) cis-9-Eicosenoic acid	3.82^{a} ± 0.10	2.95^{b} ± 0.06	**
(C22:1n9) Erucic acid	0.19^{a} ± 0.01	0.46^{b} ± 0.01	**
Total MUFA	**52.94^{a} ± 1.00**	**50.64^{b} ± 0.03**	*
(C18:2n6c + C18:2n6t) Linoleic acid + Linolelaidic acid	8.55^{a} ± 0.06	7.51^{b} ± 0.05	**
(C18:3n6) gamma-Linolenic acid [GLA]	< LOD	0.13 ± 0.01	–
(C18:3n3) alfa- Linolenic acid [ALA]	0.58^{a} ± 0.01	1.25^{b} ± 0.01	**
(C20:2n6) cis-11.14-Eicosadienoic acid	< LOD	0.35 ± 0.02	–
(C20:3n6) cis-8.11.14-Eicosatrienoic acid [DGLA]	0.28^{a} ± 0.01	0.57^{b} ± 0.01	**
(C20:4n6) Arachidonic acid [AA]	0.39^{a} ± 0.01	0.74^{b} ± 0.01	**
(C20:3n3) cis-11.14.17-Eicosatrienoic acid [ETE]	< LOD	0.12 ± 0.01	–
(C20:5n3) cis-5.8.11.14.17-Eicosappentaenoic acid [EPA]	0.23^{a} ± 0.03	0.55^{b} ± 0.01	**
(C22:6n3) cis-4.7.10.13.16.19-Docosahexaenoic acid [DHA]	0.56^{a} ± 0.01	2.02^{b} ± 0.01	**
Total PUFA	**10.59^{a} ± 0.11**	**13.26^{b} ± 0.07**	**
Total n-3 PUFA	1.38^{a} ± 0.04	3.95^{b} ± 0.02	**
EPA + DHA	0.79^{a} ± 0.03	2.58^{b} ± 0.01	**
Total n-6 PUFA	9.22 ± 0.07	9.31 ± 0.08	n.s.
Total n-9 PUFA	46.77^{a} ± 0.84	42.49^{b} ± 0.12	**
PUFA: SFA	0.36^{a} ± 0.00	0.43^{b} ± 0.00	**
MUFA: SFA	1.82^{a} ± 0.01	1.63^{b} ± 0.00	**
UFA: SFA	2.18^{a} ± 0.01	2.06^{b} ± 0.00	**
n-6/n-3	6.70^{a} ± 0.14	2.36^{b} ± 0.03	**
n-3/n-6	0.15^{a} ± 0.00	0.42^{b} ± 0.01	**
AI	0.35^{a} ± 0.03	0.46^{b} ± 0.00	**
TI	0.79^{a} ± 0.01	0.73^{b} ± 0.00	**

(*continued on next page*)

Table 5 (*continued*)

Fatty acids(% of fat)	Fish group		Influenceof fish group
	CONTROL	EXP DIET	
h: H	2.52[a] ± 0.06	2.08[b] ± 0.00	**

Values are expressed as mean ± standard deviation (SD), [ab] – values in rows with different index differ significantly ($P \leq 0.05$), significance of influence: n.s. – non-significant, *$P \leq 0.05$; **$P \leq 0.01$.
LOD – level of detection.

the least prevalent (10.59–13.26%). Palmitic acid (C16:0) was dominant in the SFA group, the sum of elaidic and oleic acids (C18:1n9t c) in the MUFA group, and the sum of linoleic and linolelaidic acids (C18:2n6c t) in the PUFA group. The feed used in carp had a significant effect ($P \leq 0.01$) in fillets FA profile. The fat of carp muscle from the experimental diet contained significantly ($P \leq 0.01$) more PUFAs and SFAs (by approx. 25.2% and 7.9%, respectively) and less MUFAs (by approx. 4.3%). Moreover, carp fed with the experimental diet had 2 times more EPA and 4 times more DHA in their fat than those from the control diet.

Based on quality indexes, both groups differred significantly ($P \leq 0.01$) in the nutritional quality of intramuscular fat (Table 5). The fat of carp muscle from the experimental diet had higher n-3 PUFA, PUFA:SFA ratio, n-3/n-6 ratio, EPA + DHA and AI, while the fat of carp from the control diet had higher n-9 PUFA, MUFA:SFA ratio, UFA:SFA ratio, n-6/n-3 ratio, TI and h:H ratio. The consumption of 100 g of carp meat from the experimental diet provides approximately 30.4 mg EPA + DHA, while 100 g of carp meat from the control diet provides 14.89 mg EPA + DHA.

3.2. Sensory properties of fillets

The sensory evaluation did not reveal significant ($P > 0.05$) differences in the quality of carp meat from both groups (Table 6). Nonetheless, it must be stressed that carp meat from the experimental diet revealed a higher springiness, cohesiveness and chewiness, as well as a higher odor intensity when boiled. In contrast, the carp meat from the control diet was more tender, juicy, fatty, and had a higher intensity of fishy and muddy odor, as well as boiled meat and muddy taste.

In order to provide a comprehensive description of carp quality, the assessment of sensory properties of carp meat was extended (supplemented) with measurement of pH and of losses during heat treatment, as well as instrumental assessment of color and texture (Table 7). No significant ($P > 0.05$) differences in meat pH, lightness (L*), redness (a*), yellowness (b*) and cooking loss was registered between the two diet groups (Table 7). In the instrumental texture assessment, no significant differences were found between the fillets from both dietary groups for most parameters, except springiness that was significantly lower in carp fillets from the experimental diet.

Table 6
Sensory properties of fillets from carp fed with the control and experimental diets.

Sensory traits (pt.)	Fish group		Influenceof fish group
	CONTROL	EXP DIET	
Texture:			n.s.
Springiness	1.88 ± 0.25	2.50 ± 0.82	n.s.
Cohesiveness	2.00 ± 0.41	2.62 ± 1.03	n.s.
Tenderness	2.25 ± 0.96	2.12 ± 0.85	n.s.
Moisture	3.00 ± 0.82	2.88 ± 0.95	n.s.
Juiciness	2.88 ± 0.32	2.75 ± 0.87	n.s.
Chewiness	2.12 ± 0.63	2.88 ± 0.48	n.s.
Fattiness	2.38 ± 0.25	2.12 ± 0.85	n.s.
Odor:			n.s.
Boiled meat	1.50 ± 0.58	1.62 ± 0.75	n.s.
Fishy	2.62 ± 1.25	2.25 ± 0.65	n.s.
Muddy	1.50 ± 0.58	1.12 ± 0.25	n.s.
Taste:			n.s.
Boiled meat	2.12 ± 0.25	2.00 ± 0.41	n.s.
Fishy	2.38 ± 1.11	2.38 ± 1.44	n.s.
Muddy	2.00 ± 0.91	1.38 ± 0.48	n.s.

Values are expressed as mean ± standard deviation (SD), [ab] – values in rows with different index differ significantly ($P \leq 0.05$), significance of influence: n.s. – non-significant, *$P \leq 0.05$; **$P \leq 0.01$.

Table 7
Physicochemical properties and texture profile analysis (TPA) test parameters of fillets from carp fed with the control and experimental diets.

Parameter	Fish group		Influenceof fish group
	CONTROL	EXP DIET	
pH	6.19 ± 0.06	6.20 ± 0.05	n.s.
L* (lightness)	55.08 ± 1.17	55.48 ± 0.63	n.s.
a* (redness)	0.97 ± 0.23	1.17 ± 0.41	n.s.
b* (yellowness)	10.74 ± 0.54	11.87 ± 1.20	n.s.
Cooking loss (%)	9.56 ± 0.99	8.51 ± 0.98	n.s.
Hardness (N)	3.81 ± 0.38	3.40 ± 0.34	n.s.
Cohesiveness (–)	0.385 ± 0.05	0.384 ± 0.30	n.s.
Springiness (cm)	1.25[a] ± 0.09	1.09[b] ± 0.09	*
Chewiness (N × cm)	1.83 ± 0.32	1.52 ± 0.26	n.s.

Values are expressed as mean ± standard deviation (SD), [ab] – values in rows with different index differ significantly ($P \leq 0.05$), significance of influence: n.s. – non-significant, *$P \leq 0.05$; **$P \leq 0.01$.

4. Discussion

Fillets of carp from the experimental diet revealed more proteins, less fat and lower energy value than fillets from the control diet. Trbović et al. (2013) showed that feed influence the proximate composition of carp during rearing. Aprodu et al. (2012) showed that carp fed with a diet with fish oil, compared with those fed with a control feed or a feed with the addition of olive or soybean oils, had less protein, more fat and lower energy value (kcal/100 g) of the meat. In contrast, Borowiec et al. (2010) showed that the mixture of sunflower and rapeseed oils had no effect in the content of basic nutrients of meat between the experimental and control carp groups. The different effects of nutrition on the composition of carp meat observed herein and in previous studies are likely due to the diversity of ingredients used in feed formulation. As demonstrated by Steffens and Wirth (2007), Steffens (2016) and Kłobukowski et al. (2018), the replacement of animal protein (fish meal) in feed with plant protein (bean, soybean, sunflower, grain, legume seeds) affects the proximate composition of fish meat, FA and AA profiles, as well as the fat quality. In our previous study (Sobczak et al., 2020), we showed that using salmon oil to enrich feed given to carp causes e.g. a reduction in protein and increase in fat levels in fish meat.

The present study did not reveal major differences in the AA composition (the only exceptions were His, Met, Trp, Gly and Pro) in carp fillets between the two diets. Nonetheless, the less favorable amino acid composition of carp fillets from the experimental diet due to the increase in nonessential amino acid (NEAA) and reduction of essential amino acids (EAA) contents, may have been caused by the higher proportion of algae (Spirulina, Chlorella) and macroalgae (*L. digitata*) meals. Spirulina contains all essential amino acids, vitamins and minerals, and is a rich source of carotenoids and FAs, especially γ-linolenic acid (GLA), (Holman and Malau-Aduli, 2013). However, according to Øverland et al. (2018), marine macroalgae meals have a much lower content of Lys and His compared with fishmeal. The EAA:NEAA ratio of marine macroalgae meals is 0.82 (Øverland et al., 2018), which is lower than 0.9–1.09 found in fishmeal (Ween et al., 2017; Kim et al., 2018). Aprodu et al. (2012) did not observe major differences in the AA profile between groups of fish fed with different feeds. Compared with wild carp, the experimental carp had more Ser, Tyr, Leu and Lys, and less of the remaining AAs (Aprodu et al., 2012). In contrast, Borowiec et al.

(2010) showed significant differences between the control and experimental groups in the AA composition of meat, indicating that the oil mixture added to the feed caused differences in the AA composition of carp meat protein. The present study showed that muscle protein from carp in both dietary groups had a high nutritional value (CS of EAA and EAAI) and contained essential amino acids in amounts much higher than in the standard protein. The consumption of 100 g of carp meat covers the daily demand for all EAAs in children of 3–10 years of age and in adults (>18 years of age).

The nutritional value of fish meat is also determined by the FA profile of intramuscular fat. Our study demonstrated that muscle fat of carp fed with the experimental diet had a higher degree of saturation (higher SFAs) and higher PUFAs content, including the nutritious EPA and DHA FAs, compared with the fat of fillets from the control diet. Ćirković et al. (2012), assessing the nutritional value of common carp fat, observed a SFA:MUFA:PUFA ratio of 24.2:64.3:11.0, which is similar to values found in this study for carp fillets from the control diet. The differences in EPA and DHA content observed in fillets were caused by the amounts of these FAs in the ingredients used for feed production. The influence of diet on FA profile of carp meat has been shown in a number of studies (Borowiec et al., 2010; Aprodu et al., 2012; Trbović et al., 2013). The additional feeding of carp with extruded feed influenced an increase in the quantities of MUFAs and n-6 PUFAs, and a decrease in the quantities of nutritionally important n-3 PUFAs (Trbović et al., 2013). Also, Aprodu et al. (2012) found more EPA, DHA and highly unsaturated fatty acids (HUFAs) in fish fed with fish oil supplements compared with fish fed with vegetable oils. Borowiec et al. (2010) also found an increase in PUFAs, including essential unsaturated FAs (EUFAs), i.e. C20:5, (EPA) and C22:6 (DHA), as well as a decrease in the sum of SFAs in carp fed with pellets containing a mixture of sunflower and rapeseed oils. This is considered as very favorable from the perspective of consumers of fish meat. EPA and DHA are needed for normal growth and development. They also affect retinal and brain phospholipid composition, intelligence quotient (IQ) and motor development (Simopoulos, 2006). Due of that consumption of meat carp fed experimental blend can have beneficial effects on consumers health.

The present study shows that carp meat from both dietary groups had a favorable fat nutritional value. The UFA:SFA ratio was 2.06–2.18, which is well above the recommended minimum of 0.35 (Kmínková et al., 2001). AI for both groups was estimated at 0.35–0.46, with the recommended ratio of <1.0 (Fernandes et al., 2014). Only TI and the PUFA:SFA ratio were worse than those recommended by nutritionists (Fernandes et al., 2014; Simopoulos, 2002). Low AI and TI values, as well as high h:H index, are considered as beneficial to human health and are desirable for the prevention of cardiovascular disorders (Zhang et al., 2020). However, the fat of carp fillets from the experimental diet reached much better n-3/n-6 and n-6/n-3 ratios than those recommended by nutritionists (Simopoulos, 2002), likely caused by the use of algae and macroalgae in feed formulation. The feed rich in saccharides leads to a decrease in n-3 PUFA percentage in fish body lipids (Fajmonová et al., 2003; Buchtová et al., 2007). According to Simopoulos (2016), omega-6 and omega-3 fatty acid balance in the human diet is important because these FAs often have important physiological effects. A high omega-6/omega-3 ratio is associated with overweight/-obesity, whereas a balanced ratio decreases obesity and weight gain (Simopoulos, 2016) as well as may play a vital role in preserving skeletal integrity of old age (Simopoulos, 2006). The consumption of meat of carp fed experimental diet may have beneficial effects on consumer health and can help prevent numerous diseases including depression, Alzheimer's disease, cardiovascular diseases, cancer as well as inflammatory and autoimmune diseases. The optimal ratio n-6/n-3 may vary with disease under condition (Simopoulos, 2006). Our study showed 2.4:1 and 6.7:1 n-6/n-3 ratio respectively for carp fed control and experimental blends. These results suggest that consumption carp meat can reduce rectal cell proliferation in patients with colorectal cancer and suppress inflammations in patients with rheumatoid arthritis. It can have beneficial effect on patients with asthma. It is essential to decrease omega-6 FAs levels in the diet, while increasing omega-3 FA intake. This can be accomplished by, e.g., increasing fish intake to 2–3 times per week, while decreasing meat intake (Simopoulos, 2016). One way to achieve this may be to consume farmed fish fed with diets that are designed to maximize n-3/n-6 ratio and to improve muscle fat quality. Such strategy can definitely be implemented as shown in results presented in this study.

The proximate chemical composition of fish meat and FA profile can affect the quality of fish fillet, though often no differences are perceptible from the organoleptic point of view, even if large differences in fish meat nutritional composition and other physicochemical and texture characteristics of the flesh could be demonstrated (Fauconneau et al., 1995). The present study did not reveal any significant differences in the assessment of meat organoleptical properties, as well as physicochemical and texture parameters between the two carp dietary groups, despite the significant differences found in protein, fat and FA content. However, it can be pointed out that carp meat from the experimental diet had a lower intensity of fishy and muddy odor and taste, as well as higher lightness and redness. This result suggests that carp fed with the experimental diet can be a source of fish meat for consumers that do not eat fish because of their typical meat taste, smell and color after culinary preparation.

The present study showed influence of feeding common carp with two blends on the selected quality parameters of carp meat. However, next studies are needed in order to perform further evaluation of nutritional, culinary and technological quality of carp meat. For example, nutritional benefits of carp meat consumption include not only beneficial FA profile and protein quality but also presence of other essential components such as vitamins and elements as well as toxicological safety. Moreover, further studies should assess effect of experimental diets on shelf life, lipid and protein oxidation, storage and cooking losses or processing suitability of carp meat.

5. Conclusion

This study concludes that feeding carp with feeds enriched with specific ingredients like microalgae/macroalgae meal and fish oil can significantly improve the nutritional quality of fillets. Compared with the control diet, fortified fillets had more protein, less fat and lower energy value, but revealed better nutritional indexes (i.e. greater share of total PUFAs, especially sum of EPA and DHA, as well as PUFA:SFA and n-3/n-6 ratios and AI) and also better taste and smell. With such characteristics, carp may constitute a nutritionally and sensorily attractive fish source for the human diet, especially for people avoiding consumption of fish exactly because of their typical fish palatability. However, further studies are needed to assess profitability and feasibility of the carp farming using functional feeds that include ingredients (seaweeds, by-products) from aquaculture and fisheries sector. Additionally, feed sector should monitor the content of heavy metal concentrations in seaweeds and upcycled by-products and assess their possible toxic effects during fish trials to include natural ingredients in feeds while reducing use of chemical compounds.

CRediT authorship contribution statement

M. Sobczak: Conceptualization, Writing – review & editing, Investigation, Supervision. **R. Panicz:** Conceptualization, Writing – review & editing, Validation. **P. Eljasik:** Writing – original draft, Investigation, Visualization. **J. Sadowski:** Resources. **A. Tórz:** Formal analysis. **J. Żochowska-Kujawska:** Writing – review & editing. **V. Barbosa:** Writing – review & editing. **J. Dias:** Resources. **A. Marques:** Writing – review & editing.

Declaration of competing interest

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

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