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HABILITAČNÍ PRÁCE

Optimalizace intenzivního chovu a výživy ryb
v recirkulačních akvakulturních systémech

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Prohlášení

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Ve Vodňanech, dne 28. 1. 2022 Ing. Vlastimil Stejskal, Ph.D.

Obsah

1. Úvod

Celosvětovým megatrendem je růst lidské populace, což spolu se zvyšující se oblibou konzumace ryb v důsledku moderních návyků a informací o pozitivním vlivu rybího masa na lidské zdraví neustále zvyšuje světovou spotřebu ryb. Konkrétní odhady hovoří o nárůstu populace o 2 miliardy do roku 2050, což znamená, že celkový počet lidí na planetě Zemi by tak vzrostl na 9,6 miliard. Synergie výše popsaných tendencí vyvíjí enormní tlak na lov ryb a dalších vodních organismů v mořích a oceánech. Nicméně světový rybolov dosáhl v devadesátých letech svého ekologického limitu a další zvyšování výlovu z moří je možné jen za cenu pravděpodobně nevratné devastace populací ryb, které jsou pro potřeby humánního konzumu loveny. Z tohoto důvodu bude v budoucnu nutno poptávku po rybách zajistit produkcí z akvakultury.

Recirkulační akvakulturní systémy jsou ekologicky šetrné a vysoce produktivní systémy intenzivní akvakultury, kde produkce ryb není spojena s nepříznivými dopady na životní prostředí, jako je ničení stanovišť původních druhů živočichů a rostlin, znečištění a eutrofizace okolního recipientu. Při chovu ryb či jiných vodních organismů v těchto systémech jsou značně redukovány až eliminovány negativní ekologické vlivy na biodiverzitu v důsledku úniku ryb (často nepůvodních druhů) či rozšiřování nemocí a parazitů.

Takzvané „blue foods“ neboli potraviny původem z vodních organismů pocházejících z mořských a sladkovodních lovů nebo akvakultury (Naylor et al., 2021), hrají významnou roli v lidské výživě. Odhaduje se, že v roce 2018 bylo celkem 178 milionů tun (tj. přibližně 90 %) těchto potravin využito pro výživu světové populace, zbývajících zhruba 10 % bylo zpracováno pro nepotravinové využití (FAO, 2020). V nadcházející dekádě je v souvislosti s neustále rostoucí populací v kombinaci se zvyšující se oblibou konzumace produktů akvakultury očekáván výrazný nárůst celosvětové poptávky po těchto potravinách (FAO, 2020). Je odhadováno, že se poptávka po produktech akvakultury ze strany hlavních spotřebitelských zemí (např. Čína, Indie) téměř zdvojnásobí z 80,7 milionů tun na 154,6 milionů tun v období od 2015 do 2050 (Naylor et al., 2021b). Vzhledem k tomu, že výlov ve volných vodách i při uplatnění veškerých technologických a institucionálních inovací může do roku 2050 dosáhnout maximální produkce 57,4 milionů tun (16% nárůst oproti současnému stavu) (Costello et al., 2020), bude rostoucí globální poptávka po rybách a mořských plodech pokryta především produkcí z akvakultury (Béné et al., 2015). Akvakultura se stala nejrychleji rostoucím potravinářským odvětvím s průměrným ročním tempem růstu 5,3 %. V období 2001–2018 toto odvětví přispělo přibližně 46 % do celosvětové potřeby ryb a mořských plodů pro humánní konzum. Odhaduje se další růst s dalším zvýšením podílu až na 50 % v období 2018 až 2030 (FAO, 2020).

V rámci akvakultury především chovné systémy, kde jsou aplikovaná kompletní krmiva s obsahem zpracovaných (tj. rybí moučky) nebo nezpracovaných krmných ryb (např. chovy tuňáků obecných *Thunnus thynnus* v klecích), významně přispěly (69,5 %) k celkové roční produkci akvakultury. Produkce z těchto akvakulturních systémů předčila produkci z podmínek, kde nejsou aplikována krmiva (např. rybniční systémy), ta v roce 2018 přispěla 30,5 % k celkové roční produkci akvakultury (FAO, 2020). Kompletní krmiva pro intenzivní akvakulturu byla tradičně založena hlavně na mořských zdrojích, nicméně v současnosti stále více využívá suroviny ze suchozemských plodin (Hua et al., 2019; Naylor et al., 2021a; Turchini et al., 2019). Neustálý růst akvakultury, který bude podněcován nárůstem poptávky po rybách

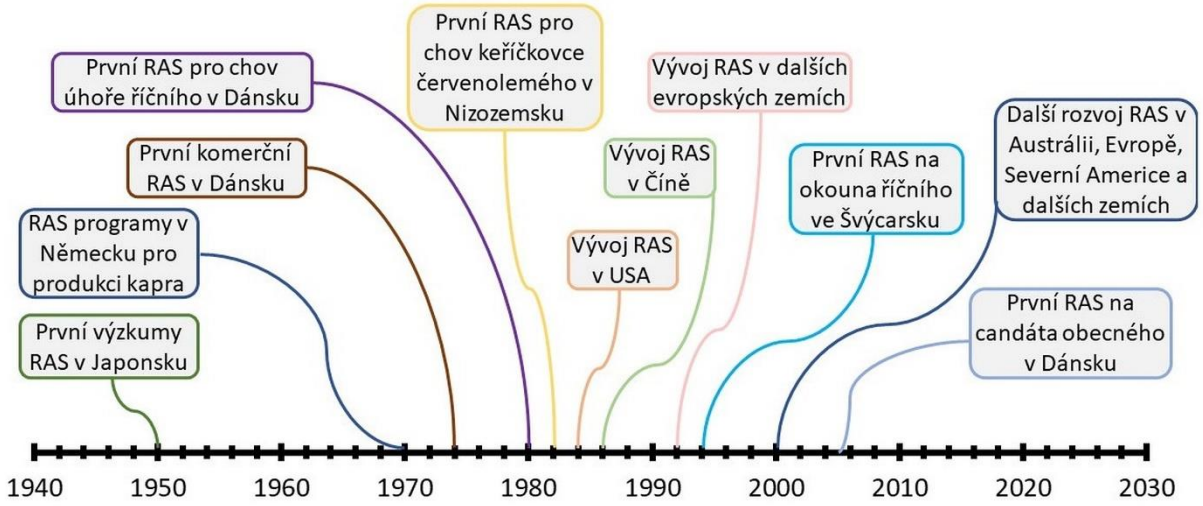
a mořských plodech, v budoucnosti povede ke zvýšení poptávky po kvalitních krmivech pro intenzívní akvakulturu včetně recirkulačních akvakulturních systémů. Tacon (2020) sumarizuje, že celková produkce krmiv pro intenzivní akvakulturu využitá v roce 2017 byla 51,2 milionu tun a odhaduje, že v roce 2025 bude potřeba přibližně 73,2 milionu tun. To znamená, že v následujících letech bude potřeba k pokrytí celosvětového růstu akvakultury vyprodukovat o přibližně 22 milionů tun ročně více. Současně jsou k dispozici prognózy, že ryby lovené pro produkci rybí moučky z mořských zdrojů dosáhnou ekologické hranice udržitelnosti populace do roku 2037 (Cottrell et al., 2020) a další využívání tohoto omezeného zdroje tedy není dlouhodobě udržitelné (Naylor et al., 2009). Současně další intenzifikace produkce suchozemských plodin jako surovin pro krmiva v akvakultuře vyvolává obavy z ekologických zátěží, jako je nadměrné využívání orné půdy, vody a odlesňování (Foley et al., 2011; Fry et al., 2016).

1.1. Historie a význam recirkulačních akvakulturních systémů

Přestože základní technologie recirkulačních akvakulturních systémů (RAS) existuje již více než 65 let, nedoznala zatím výrazněji kosmopolitního rozšíření tak, jako tomu je u průtočných, klecových nebo rybničních systémů (Murray et al., 2014). V některých zemích je stále považována za recentní inovaci. Relativně malému rozšíření do určité míry napomáhá fakt, že v akvakultuře je chováno velké množství druhů živočichů (odhaduje se 360 druhů ryb, 100 druhů měkkýšů a 60 druhů korýšů). Historicky bylo průkopnickou zemí v testování RAS Japonsko, kde první pokusy proběhly v 50. letech 20. století (Saeki, 1958) (Obr. 1). Nicméně většina technických a technologických zdokonalení byla provedena zhruba v období od roku 1970 do současnosti (Espinal a Matulic, 2019). V sedmdesátých letech probíhalo ověřování především v Německu pro účely intenzivního chovu kapra obecného (*Cyprinus carpio*). V těchto letech myšlenku chovu ryb v plně kontrolovaných (zastřešených) RAS velmi intenzivně podporovalo Dánsko, především za účelem intenzivní produkce úhoře říčního (*Anguilla anguilla*). První komerční RAS byl poté postaven v Dánsku v roce 1980 (Warrer-Hansen, 2015). Stejná země byla později podnícena k dalšímu rozvoji venkovních RAS pro chov pstruha duhového (*Oncorhynchus mykiss*) v důsledku vysoké frekvence výskytu patogenních organismů (virového i bakteriálního původu) v povrchových vodách a s tím spojenými častými problémy na průtočných farmách (Goldman, 2016). Rozvoj a prvotní úspěchy s technologií RAS v Německu, a hlavně Dánsku inspirovaly další rozvoj RAS v jiných evropských zemích (Holandsko, Francie, Norsko) na konci 80. a 90. let (Martins et al., 2010). Vývoj v Evropě byl inspirací i pro sladkovodní RAS v Severní Americe a mořské RAS v Číně (Goldman, 2016; Ying et al., 2015).

Zhruba od roku 2000, lze pozorovat akceleraci rozvoje RAS v Evropě, Severní Americe, Austrálii a dalších zemích kde akvakultura figuruje jako významné odvětví živočišné výroby (Espinal a Matulić, 2019; Martins et al., 2010; Summerfelt et al., 2004). V každé geografické oblasti byla technologie RAS rozvíjena z různých důvodů. V Norsku využití kontrolovaného prostředí RAS vede ke stabilní produkci smoltů lososa a následně vysazování větších a odolnějších ryb pro klecové chovy. Recirkulační technologie rovněž umožnují lepší kontrolu procesu smoltifikace, protože parametry jako teplota, salinita a délka dne mohou být jednoduše řízeny. Ve Švýcarsku jsou na vzestupu chovy okouna říčního (*Perca fluviatilis*) a candáta obecného (*Sander lucioperca*) díky oblíbenosti konzumace filet těchto ryb

a nedostupnosti kvalitní lokální produkce (zákazníci preferují místní produkty), přičemž se odhaduje, že v současnosti RAS technologie zde využívá 11–15 farem. Zatímco v Holandsku stojí za využitím RAS systémů neutuchající poptávka po tržních úhořích a očekává se mírný nárůst produkce, jakmile se podaří uzavřít celý produkční cyklus (v současnosti je hlavní překážkou odchov raných stádií úhořů). V USA byla důvodem rozvoje RAS sezónnost produkce tlamouna nilského (*Oreochromis niloticus*) v jižněji položených rybničních systémech. Farmy s RAS technologií se však budují i v pouštních oblastech (např. farma pro výrobu kaviáru v Abu Dhabí s odhadovanou produkcí 35 tun/rok) nebo velmi odlehlých oblastech (farmy na smolty lososa obecného v Chile). V současnosti je z pohledu produkce v RAS nerozvinutější zemí Dánsko, kde technologii využívá cca 130–140 farem (Jokumsen a Svendsen, 2010; EUMOFA, 2020).



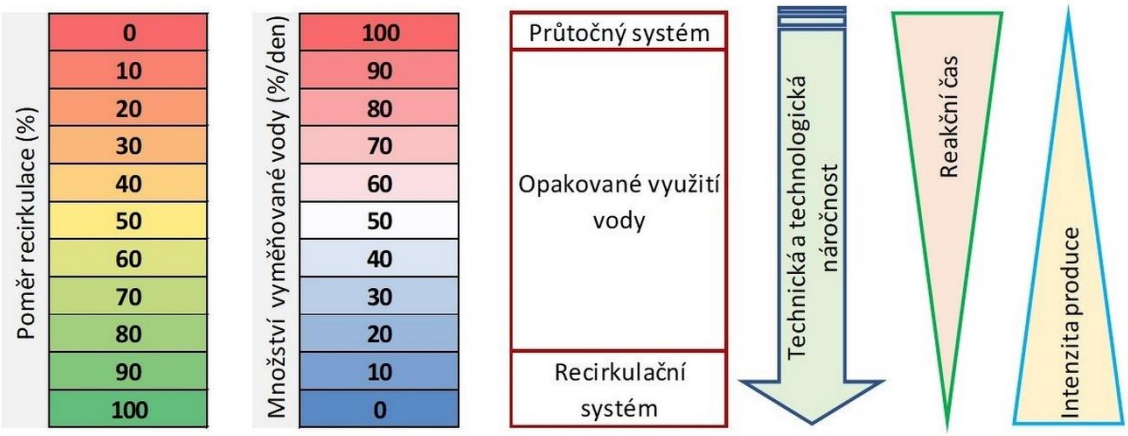
Obr. 1. Historický vývoj chovu ryb v recirkulačních akvakulturních systémech (RAS).

1.2. Podstata a fungovaní RAS

V nejzákladnější podobě se recirkulační systémy sestávají z odchovných nádrží (různé tvary a rozměry mají svoje specifika), mechanické filtrace (většinou automatický mechanický filtr na bázi bubnového nebo diskového mikrosíta) a biologické filtrace (zkrápěný či ponořený biofiltr s pevným nebo pohyblivým ložem). Produkt metabolismu ryb, toxický amoniak, je v těchto systémech odstraňován procesem nitrifikace v biologických filtrech různé konstrukce. Obecným trendem je využívání ponořených biofiltrů s pohyblivým médiem namísto dříve široce rozšířených skrápěných filtrů. Další varianty jako ponořený filtr s fixním filtračním ložem a biokontaktory jsou vyžívány méně. Odchovné nádrže bývají většinou plastové nebo betonové opatřené nátěrem a mají pravoúhlý nebo kruhový půdorys. V některých případech jsou začleněny na vedlejším okruhu také denitrifikační věže (zdrojem uhlíku je obvykle metylalkohol) pro snížení obsahu dusičnanů, čímž je dosaženo snížení spotřeby dopouštěné vody a energie na ohřev této vody. Další úpravy recirkulované vody zpravidla zahrnují odplynění (zejména snížení obsahu ve vodě rozpuštěného oxidu uhličitého, případně dusíku), úpravu pH, zvýšení obsahu ve vodě rozpuštěného kyslíku (pomocí aerace či oxygenace), případně též dezinfekci vody (pomocí ozonizace nebo UV záření) a úpravu teploty vody

(ohřev). Nedílnou součástí RAS jsou systémy měření, regulace a řízení provozu a signalizace mezních hodnot a stavů (včetně využití dálkové kontroly a přenosu dat). Stávající technologie RAS umožňují chov ryb v optimálních podmínkách zajišťujících maximální růst chovaných ryb. Udržováním vhodné teploty, chemismu vody a vyváženou kompletní krmnou směsí lze zkrátit růstový interval pro dosažení tržní hmotnosti ryb, případně požadované kategorie násadových ryb (Obr. 2).

Především investiční náročnost spolu s nedostatkem informací a zkušeností však brání širšímu využití RAS pro produkci hospodářsky významných druhů ryb. Pokud jde o provozní náklady, Losordo et al. (1998) uvádí investiční náklady pro rybniční systémy ve výši 0,41 USD/ kg/rok ve srovnání s náklady v RAS, které činí 0,45–1,81 USD/kg/rok. Ze zmíněného vyplývá, že produkce ryb v RAS je tedy efektivní a rentabilní pouze při použití vysokých hustot obsádek a plném využití odchovné kapacity daného systému. Z provozních sledování RAS a řešení problémů v praxi bylo zjištěno, že RAS musí poskytovat optimální podmínky nejen pro chované ryby, ale i pro bakterie žijící na biologických filtrech a volně rozptýlené v prostředí, proto se nové směry výzkumu orientují na poznání složitých vztahů mezi aplikovaným krmivem, mikrobiomem ryb a mikrobiomem v různých částech RAS.



Obr. 2. Závislost mezi poměrem recirkulace, množstvím vyměňované vody, technologickou náročností, reakčním časem a intenzitou produkce pro průtočné a recirkulační systémy.

1.3. Současný stav a výzvy spojené s fungováním RAS

Prostředí existujících RAS systémů lze charakterizovat jako velmi rozmanité s několika zavedenými technologiemi a principy pro různé druhy ryb. Právě tato diverzifikace podporovaná národními evropskými strategickými plány z pohledu spektra chovaných druhů, používaných krmiv, odchovných postupů a funkčních (technických) nastavení RAS systémů s sebou nese potřebu hlubšího poznání vztahů, dynamiky a vlivů různých faktorů. Systémy RAS většinou využívají kontrolované prostředí z pohledu teploty (Matoušek et al., 2017a), obsahu kyslíku (Matoušek et al., 2017b; Prokešová et al., 2020; Schäfer et al., 2021 – **Příloha 3**), fotoperiody (Prokešová et al., 2017; Malinovskyi et al., 2022) a obsahu metabolitů. Ryby jsou v RAS chovány v optimálních hustotách obsádek (Policar et al., 2013 – **Příloha 1**; Stejskal et al., 2020 – **Příloha 2**), aby byl maximálně využit technologický potenciál daného RAS a biologický potenciál chovaného druhu. Produkce v RAS je tak jen minimálně ovlivněná klimatickými vlivy,

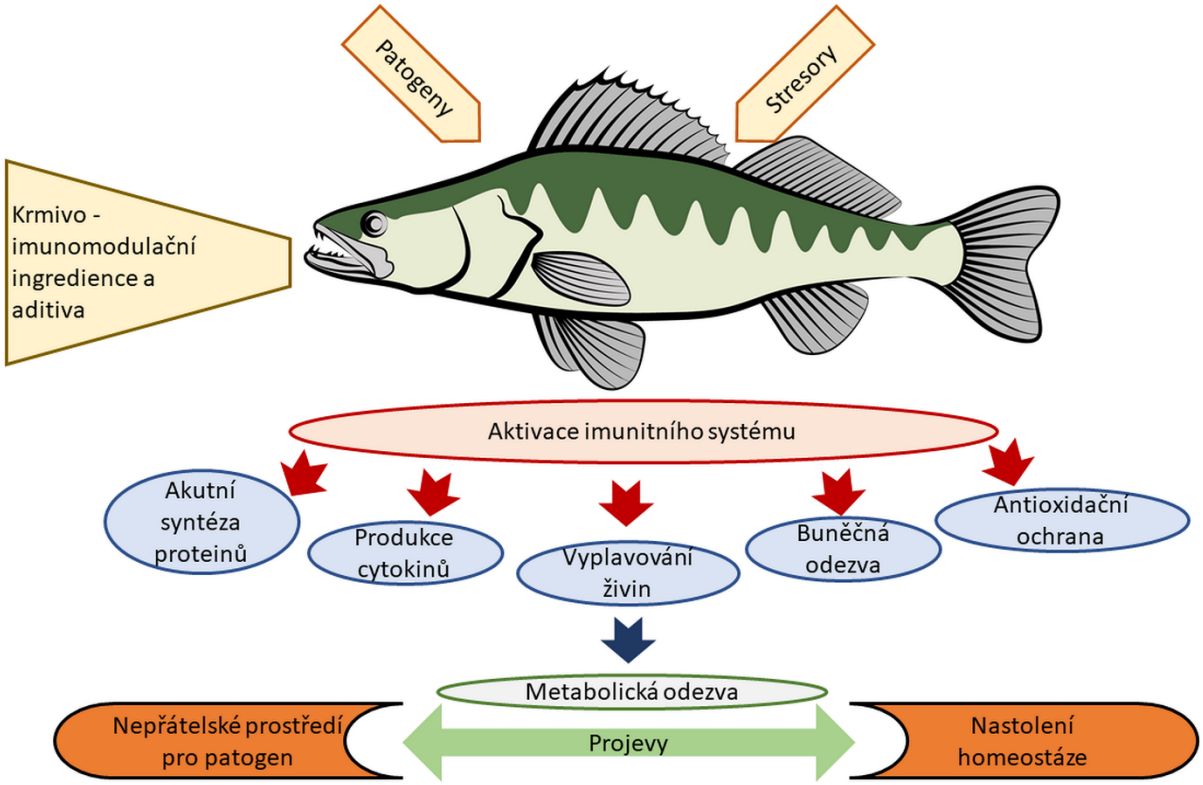
jako je kolísání množství srážek, záplavy, období sucha, globálního oteplování, cyklóny, kolísání salinity, acidifikace oceánů a vzestup hladiny moře. Výše zmíněné vlivy silně ovlivňují stabilitu a celkovou výši produkce v ostatních produkčních systémech jako jsou klecové, průtočné či rybniční systémy.

Z environmentálního hlediska jsou limitujícími faktory pro RAS především spotřeba energie a emise skleníkových plynů. Navzdory potenciálu a pozitivním vlastnostem, které RAS mají, nejsou dosud široce rozšířeny především kvůli investičním nákladům a vysokým nárokům na kvalitní a spolehlivou obsluhu. Další směry výzkumu, vývoje a technologických inovací by měly být zaměřeny na zavedení nízkonákladových (jak investičně, tak provozně) energeticky účinných RAS pro zintenzivnění produkce ryb a mořských plodů a snížení emisí skleníkových plynů. Systémy RAS mohou v budoucnu hrát důležitou roli jako jedna z adaptačních strategií na změnu klimatu.

Z pohledu spotřebitele vyvolává chov ryb v RAS otázky především s ohledem na welfare chovaných organismů (Stejskal et al., 2011). Pojmy blahobyt, zdraví, welfare či well-being jsou stále více v centru zájmu spotřebitelských a obchodních řetězců a vypořádání se s těmito otázkami bude nepochybně jednou z hlavních výzev současných a budoucích udržitelných politik EU v oblasti akvakultury. Na konferenci Evropské komise o dobrých životních podmínkách zvířat (EU Animal Welfare Today and Tomorrow, 2021) panovala shoda na tom, že předpisy na toto téma jsou zastaralé a je třeba je přezkoumat. Za tímto účelem by měla proběhnout kontrola současného stavu systému chovaných druhů ryb (Gebauer et al., 2020 – **Příloha 4**). Vzhledem k tomu, že v tuto chvíli neexistují žádná jasná doporučení v tomto směru, je pro producenty velmi obtížné vyhodnotit a monitorovat stav a pohodu chovaných ryb, případně najít způsoby, jak neuspokojivý stav zlepšit.

1.4. Recentní trendy ve výživě intenzivně chovaných ryb

V RAS stejně jako v ostatních produkčních systémech, kde jsou využívány kompletní krmné směsi, je snahou optimalizovat spotřebu krmiv a maximalizovat využití jejich nutričního potenciálu (Stejskal et al., 2020 – **Příloha 2;** Stejskal et al., 2021). Provozní pozorování a bilance z RAS prokazují, že krmné náklady mohou tvořit až 33–50 % provozních nákladů (Liu et al., 2016; Ahmed a Turchini, 2021). Intenzifikací akvakultury často dochází k ekonomickým ztrátám vlivem stresu a infekčních onemocnění. Dosavadní léčebné metody zahrnující schválená chemoterapeutika a antibiotika nejsou mnohdy ani efektivní, ani šetrné ke spotřebiteli či životnímu prostředí. Například antibiotika mohou podpořit zvýšenou odolnost a změny struktury mikrobiálního společenství v přírodním prostředí (Kiron, 2012) (Obr. 3). Bioaktivní substance či ingredience s preventivním i kurativním účinkem jsou úspěšně testovány na experimentální úrovni v chovech ryb v celosvětovém měřítku (Stratev et al., 2018). Dále s tím, jak je navyšování světového výlovu trvale neudržitelné (dochází k postupné devastaci populací krmných ryb a ohrožení výskytu některých druhů), přichází urgentní potřeba výzkumu a vývoje nových technologií a strategií, jak tento negativní trend redukovat. Proto je současný výzkum orientován na možnosti náhrady rybí moučky a rybího oleje alternativními surovinami a v současnosti jsou již dostupné některé informace o limitech náhrady rybí moučky a oleje včetně efektů na růst, přežití, fyziologický, imunologický stav ryb a enviromentální dopady zkrmování inovovaných krmiv (Tran et al., 2022 – **Příloha 6**).



Obr. 3. Koncept nutriční modulace imunity ryb (upraveno podle Kiron, 2012).

1.4.1. Fytoaditiva jako strategie pro podporu růstu a zdraví chovaných ryb

Zvýšení rychlosti růstu a odolnosti chovaných ryb vůči negativnímu působení stresu a patogenům (Hashemi a Davoodi, 2011) představují důležité výzvy v udržitelném růstu akvakultury. Intenzifikace akvakultury jako odvětví vedla ke zvýšenému používání antibiotik a růstových stimulátorů s cílem zajistit stabilně vysokou produkci zdravých ryb (Romero et al., 2012). Lze konstatovat, že antibiotika jsou v akvakultuře široce užívaná a v nedávné zprávě stojí, že nejméně jedenáct zemí používalo v období 2008 až 2018 celkem 67 různých antibiotik (Lulijwa et al., 2020). Používání antibiotik a dalších chemických látek v akvakultuře vzbuzuje obavy o veřejné zdraví, životní prostředí, bezpečnost a kvalitu potravin, welfare ryb a dobré životní podmínky pro personál (Jennings et al., 2016). Nadměrné užívání antibiotik a léčiv navíc způsobuje zvýšení rezistence patogenů vůči těmto látkám. Recentní strategií k omezení užívání farmaceutických produktů představují rostlinné moučky a extrakty (fytoaditiva), jejichž bioaktivní sloučeniny představují levnou a bezpečnou alternativu (Stratev et al., 2018). Fytoaditiva byly u ryb potvrzeny jako přirozené stimulátory růstu a imunostimulanty (Akrami et al., 2015; Kanani et al., 2014; Xu et al., 2020;). V poslední dekádě bylo provedeno několik studií zaměřených na účinky rostlinných aditiv v krmivech pro hospodářská zvířata včetně ryb. Byly identifikovány zajímavé produkty z pohledu alternativních surovin s obsahem bioaktivních látek (Zare et al., 2021 – **Příloha 5**), které jsou často dostupné jako vedlejší produkty (Doan et al., 2020; Prokešová et al., 2020; Doan et al., 2022), mohou fungovat jako stimulanty imunity či prevence stresu. Zároveň však bylo zjištěno, že navzdory velkému potenciálu bioaktivních látek zlepšit využitelnost krmiva a zdravotní stav ryb je jejich

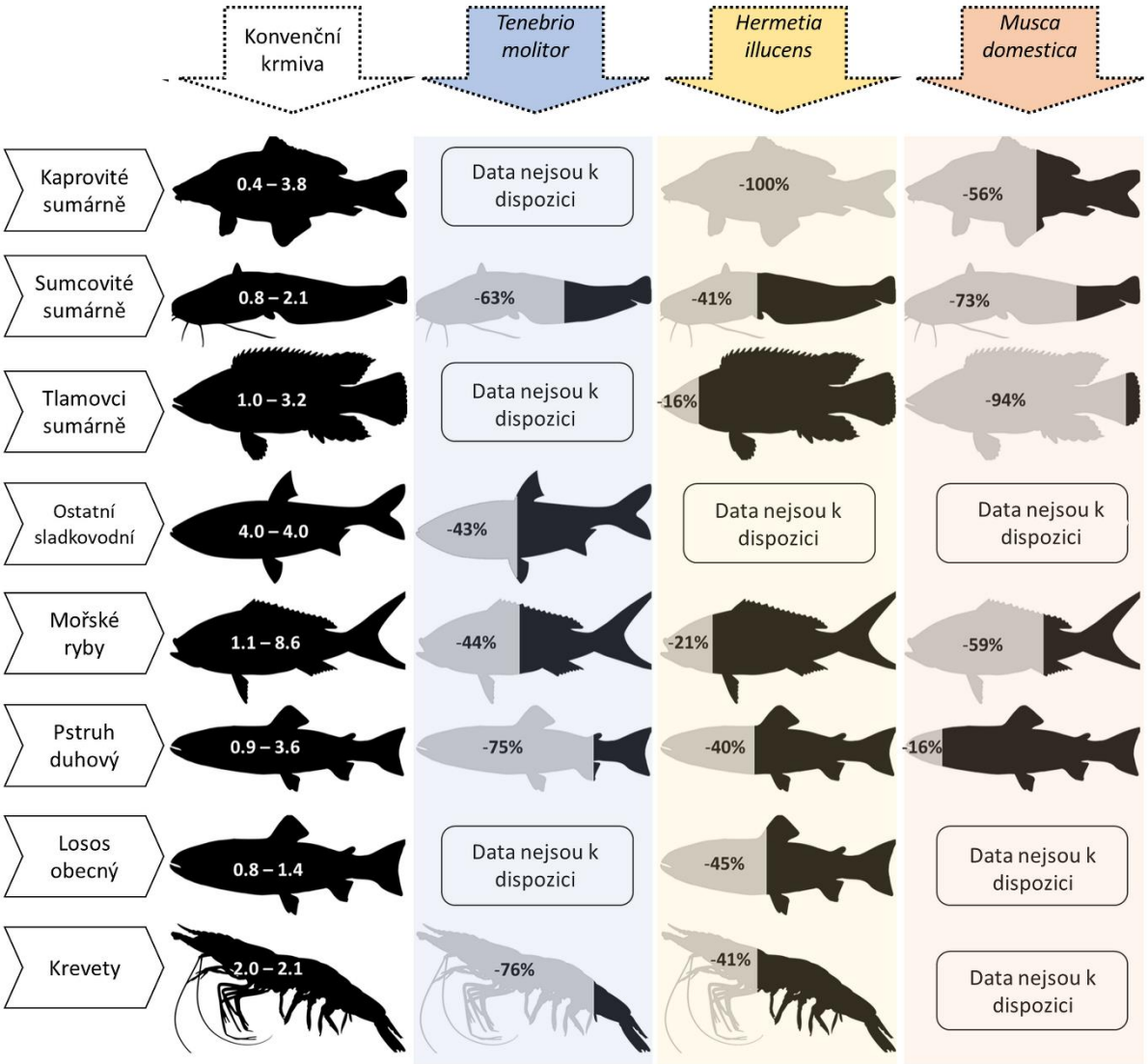
majorita buď špatně vstřebatelná, nebo nemají dlouhodobou účinnost v trávicím traktu, anebo jsou degradovány při výrobě (vysoká teplota a tlak při extruzi, popř. nelze je aplikovat kvůli nerozpustnosti v oleji při následném nástřiku oleje po extruzi), skladování krmiv, nebo průchodem trávicím traktem (kyselé pH, enzymy) (Shah a Mraz, 2020). V oblasti výživy ryb je tedy nutný další vývoj krmiv se specificky účinnými látkami s efektivní metodou podávání pro podporu nejen růstu ryb díky efektivnější konverzi živin, ale také zdravotního stavu a odolnosti ryb vůči nemocem a stresu.

Česnek (*Allium sativum*) z čeledi Amaryllidaceae je jednou z nejstarších léčivých rostlin. Je známo, že již před více než 4000 lety byl široce používán jako potravina a lék (Rivlin, 2001). Byly prokázány antimikrobiální, antitrombotické, hypolipidemické, antiartritické, hypoglykemické a protinádorové vlastnosti česneku (Thomson a Ali, 2003). Účinná látka aminokyselina alliin je komplexní sloučenina bez zápachu, který za pomoci enzymu alliinázy tvoří diallyl-thiosulfinát – allicin. Teprve allicin je organosírová sloučenina, která vytváří typický zápach čerstvého česneku (Lawson a Wang, 1993). Další aktivní látkou je organosírová sloučenina S-allylcystein, která snižuje růst nádorů u různých druhů zvířat (Thomson a Ali, 2003). Další bioaktivní sloučeniny zahrnují fenolické sloučeniny (flavonoidy), vitamíny (A, B-komplex, C, E), kyselinu linolovou, různé proteiny (lektiny jsou nejhojnější bílkovinou), esenciální aminokyseliny a minerály (např. selen) (Lawson a Wang, 1993; Rivlin, 2001; Thomson a Ali, 2003).

1.4.2. Alternativní zdroje proteinu pro krmiva v intenzivní akvakultuře

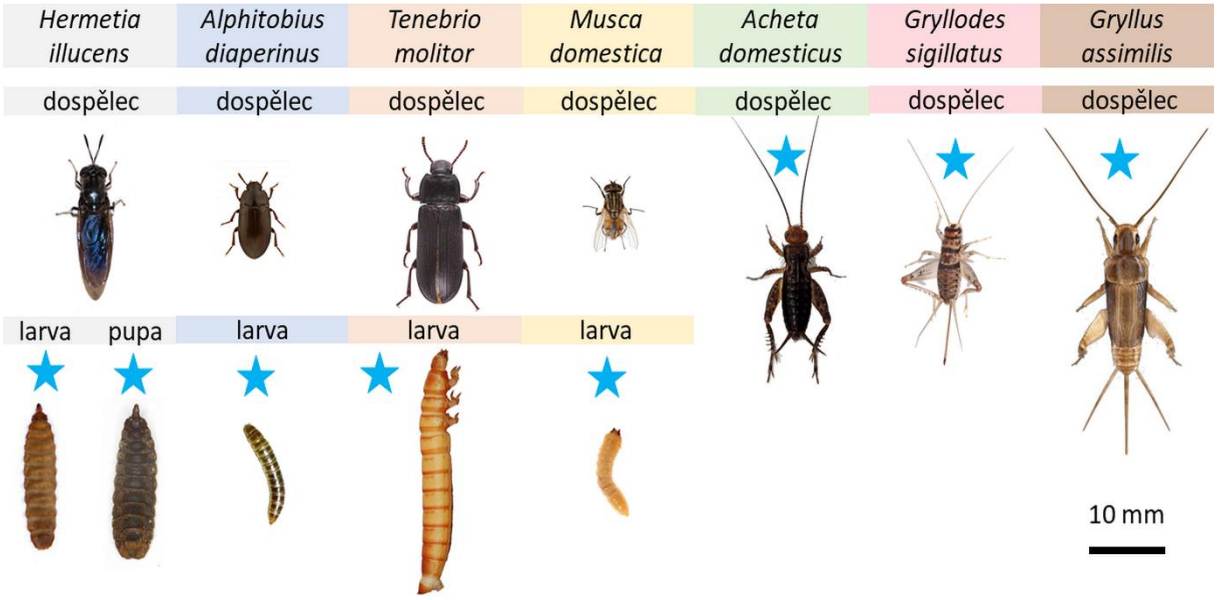
Velkou prioritou je tedy hledání alternativních složek pro zajištění dalšího růstu akvakultury. V současnosti je testována široká škála nových ingrediencí pro jednotlivé v akvakultuře chované druhy ryb, mezi kterými hmyzí moučky vykazují slibné výsledky pro zajištění zdroje proteinů v nadcházející dekádě (Gasco et al., 2020b; Hua et al., 2019; Hua, 2021). Mezi příhodné vlastnosti hmyzích mouček používaných jako alternativního zdroje bílkovin patří příznivé nutričního složení, především vysoký obsah bílkovin a vyvážený profil aminokyselin (Nogales-Mérida et al., 2019) a nadlepšení zdravotního stavu krmených organismů (Gasco et al., 2020a). Dále jsou zde environmentální přínosy produkce krmiv obsahující hmyz spojené se snížením ukazatele FIFO (parametr vyjadřující množství ryb vylovených z moře na produkci 1 kg ryb chovaných v akvakultuře), využíváním půdy a produkcí fosforu ve srovnání s krmivy bez obsahu hmyzu (Tran et al., 2021) (Obr. 4).

Využití hmyzích mouček pro vodní živočichy se v posledních letech těší rostoucímu zájmu ze strany výzkumných institucí, krmivářských firem a producentů hmyzu. Z komerčního hlediska se například v Evropě očekává, že produkce hmyzích proteinů pro krmiva dosáhne do roku 2025 přibližně 60 000 tun s dalším růstem až na 200 000 tun v roce 2030 (IPIFF, 2021a). Podíl složek hmyzí moučky používaných v akvakultuře do roku 2030 dosáhne 40 %, čímž překročí podíl v krmivech pro domácí zvířata na úrovni 30 % (IPIFF, 2021b).

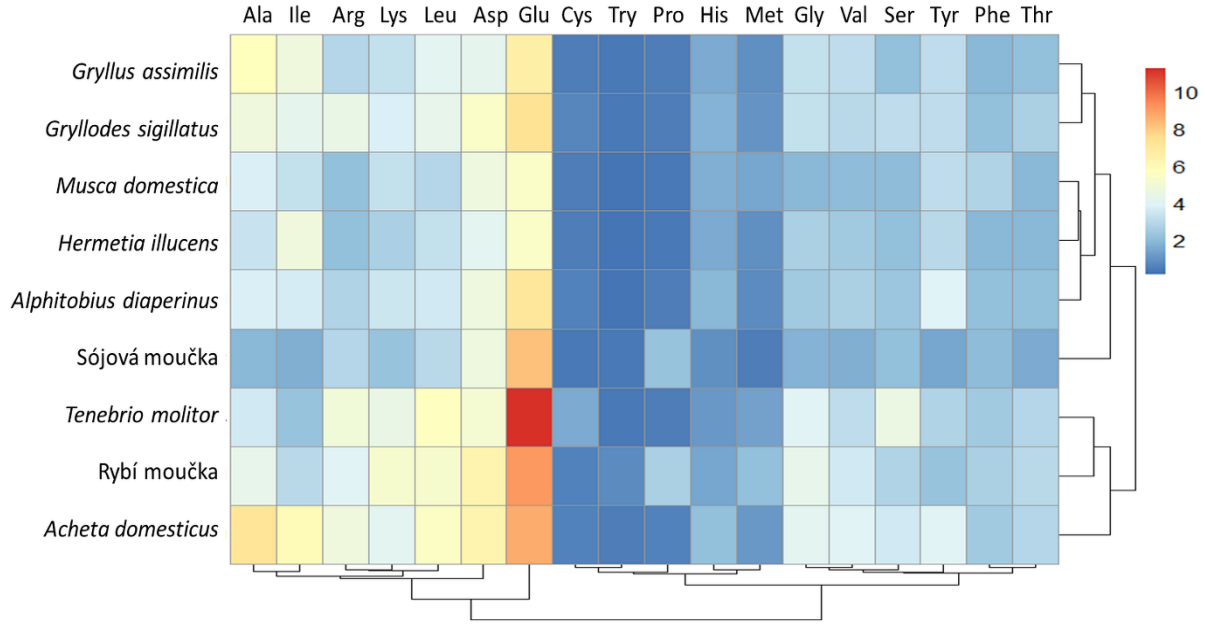


Obr. 4. Redukce ukazatele FIFO při porovnání produkční účinnosti krmných receptur na bázi hmyzí moučky s konvenčními krmivy (kontrolní krmiva). Data extrahovaná ze studií využívajících *Tenebrio molitor*, *Hermetia illucens* a *Musca domestica* jako náhradu rybí moučky pro hlavní chované skupiny ryb (nepublikovaná data).

Současný výzkum v oblasti výživy ryb naznačuje velký potenciál hmyzích mouček, bakteriemi produkovaného proteinu a vedlejších produktů ze zpracování ryb jako surovin pro krmiva v akvakultuře v nadcházejících desetiletích (Gasco et al., 2020b; Hua et al., 2019). Nedávno bylo schváleno sedm druhů hmyzu pro použití v akvakulturních krmivech jmenovitě bráněnka *Hermetia illucens*, moucha domácí *Musca domestica*, potemník domácí *Tenebrio molitor*, potemník stájový *Alphitobius diaperinus*, cvrček domácí *Acheta domesticus*, cvrček krátkokřídlý *Gryllodes sigillatus* a cvrček stepní *Gryllus assimilis* (Obr. 5 a Obr. 6). Zmíněné druhy byly schváleny Evropskou komisí (nařízení 893/2017) a vyvolaly rostoucí zájem o tyto suroviny. V důsledku toho je produkce hmyzích mouček celosvětově na vzestupu.



Obr. 5. Druhy hmyzu schválených Evropskou komisí (No. 2017/893 dne 1. 7. 2017) pro využití v krmivech pro hospodářská zvířata včetně ryb. Modrá hvězdička označuje vývojové stádium využívané pro produkci hmyzích mouček.



Obr. 6. Vizualizace obsahu aminokyselin v hmyzích moučkách sedmi druhů hmyzu schválených současnou legislativou Evropské komise v porovnán s rybí a sójovou moučkou. Data pro alanin (Ala), izoleucin (Ile), arginin (Arg), lysin (Lys), kyselina asparagová (Asp), kyselina glutamová (Glu), cystein (Cys), tryptofan (Try), prolin (Pro), histidin (His), methionin (Met), glycin (Gly), valin (Val), serin (Ser), tyrosin (Tyr), fenylalanin (Phe), threonin (Thr) jsou vyjádřena jako % ze sušiny (vlastní nepublikovaná data).

1.5. Cíle práce

Prvním cílem této práce bylo optimalizovat chov karnivorních ryb v intenzivních RAS z pohledu: i) hustoty obsádky a krmného režimu, ii) posoudit, zda u v současnosti intenzivně chovaných populací okouna existují rozdíly v personalitě, fitness a odolnosti proti stresu a dále iii) ověřit, jakým způsobem mohou hypoxické podmínky ovlivnit imunitní systém.

Druhým cílem práce pak bylo optimalizovat výživu v RAS především zavedením alternativních surovin a zjistit jejich vlivy na: i) růst, stravitelnost a produkční charakteristiky, ii) energetický metabolismus a fitness, iii) výstavbu tkání, iv) kompozici střevní mikroflóry a histomorfologii střeva a v) stresovou odolnost.

2. Výsledky a diskuse

2.1. Optimalizace chovu ryb v RAS z pohledu abiotických faktorů

Aplikovaný výzkum v oblasti technických a technologických inovací intenzivní akvakultury je prioritním zájmem reflektujícím aktuální a budoucí potřeby produkce potravin. Diverzifikace z pohledu spektra chovaných druhů, používaných krmiv, odchovných postupů a funkčních (technických) nastavení RAS systémů s sebou nese potřebu hlubšího poznání vztahů, dynamiky a vlivů v rámci RAS. V první fázi jsme se v rámci týmu doc. Policara zaměřili na možnosti kombinace raného odchovu candáta obecného v rybničních podmínkách s navazující adaptací rychleného plůdku na prostředí RAS. Pro tento druh v současnosti platí, že obliba a poptávka po tomto druhu na trhu výrazně převyšuje produkci v Evropě (Müller-Belecke a Zienert, 2008). Candát obecný je proto považován za perspektivní druh s potenciálem diverzifikovat evropskou intenzivní akvakulturu, a to především v plně kontrolovaných RAS (Policar et al., 2019). V naší práci bylo základní otázkou, jak dlouho lze ryby odchovávat v rybničních podmínkách, respektive jaká velikost ryb je vhodná pro bezproblémový průběh odchovu v rybničních podmínkách, výlov, transport do RAS, třídění, a hlavně kritické období potravní adaptace rychleného plůdku na kompletní krmnou směs. Proto jsme se na tento aspekt zaměřili v rozsáhlé studii Policar et al. (2018), která navíc v metodické části popisuje postup reprodukce, odchov v rybničním prostředí, výlov, transport a postup adaptace na kompletní krmnou směs (**Příloha 1**).

2.1.1. Vliv hustoty obsádky a krmných režimů na welfare a produkční ukazatele

V první části studie jsme se zaměřili na vliv počáteční velikosti ryb, přičemž testovány byly tři počáteční velikosti rychleného plůdku, kdy první skupina byla charakterizována rybami menší velikosti o průměrné celkové délce 40,3 ± 2,3 mm a hmotnosti 0,42 ± 0,15 g (skupina S). Prostřední velikostní skupina zahrnovala ryby o průměrné celkové délce 56,2 ± 2,7 mm a hmotnosti 1,66 ± 0,40 g (skupina M). V experimentu byla zařazena i třetí velikostní skupina s průměrnou celkovou délkou 71,0 ± 3,2 mm a počáteční hmotností 2,95 ± 0,65 g. Z výsledků studie vyplývá, že nasazování velikostně menších ryb (tj. skupina S) při adaptaci plůdku na intenzivní podmínky je výhodnější a projeví se vyšším růstem v porovnání se skupinou M a skupinou L. Tento výsledek je pouze potvrzením obecné závislosti, kdy menší ryby vykazují rychlejší růst. Z chovatelského hlediska je podstatně významnějším výsledkem, že skupina S (ryby nejmenší velikosti) vykazovala nejvyšší hodnoty přežití při potravní a prostorové adaptaci dosahující až 81,7 % v experimentální části a 78,7 % při masovém (poloprovozním)

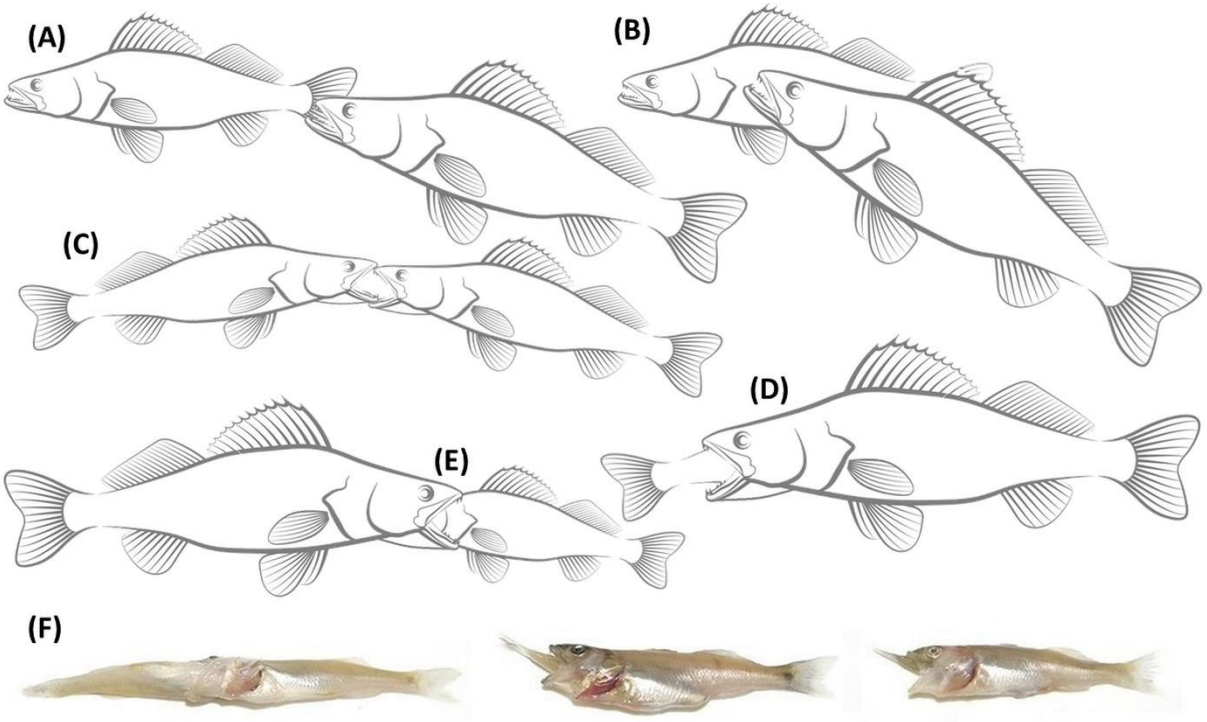
ověření experimentálně získaných dat. Dalším pozitivním aspektem adaptace plůdku candáta o hmotnosti cca 0,5 g (skupina S) byl signifikantně nižší kanibalismus. Nejmenší velikostní skupina vykazovala nejnižší kanibalismus a nejvyšší přežití i přesto, že to byla při nasazení nejvíce heterogenní skupina z testovaných variant (variační koeficient 35,7 %). Tyto výsledky naznačují, že rozvoj kanibalismu je u plůdku candáta obecného věkově a velikostně závislý. V této souvislosti lze dodat, že populace rychleného plůdku odchovaného v rybnících je charakteristická bimodálním rozdělením a náchylností vzniku kanibalismu již v rybničním prostředí. Při odchovu rychleného plůdku v rybničních podmínkách je proto důležité odchov ukončit včas v období, kdy se v rybnících ještě vyskytuje dostatečné množství zooplanktonu a podíl kanibalů k celkovému počtu odchovaných a vylovených ryb je minimální, což má výrazné dopady na další odchov v intenzivních podmínkách. Vliv počáteční velikosti ryb je v intenzivním chovu okounovitých druhů ryb velmi významný aspekt, neboť již malé rozdíly ve velikosti mohou vyvolat agresivní chování, projevy hierarchie a dominance (Magnhagen, 2015). Prevencí výše popsaných negativních vlivů a běžnou praxí je velikostní třídění ryb tak, aby došlo ke snížení heterogenity obsádky (Policar et al., 2015). Na RAS farmách se provádí rutinně (často jednou za 2–3 týdny) při odchovu juvenilních ryb (Fontaine a Teletchea, 2019). Časté velikostní třídění se osvědčilo v raných fázích odchovu u okounovitých a vedlo k vyšším přírůstkům biomasy především díky vyššímu přežití (Król et al., 2019). Nicméně v dalších fázích odchovu se výhodnost velikostního třídění částečně kompenzuje výskytem rychle rostoucích ryb v každé tříděné skupině, což může vést k dalšímu zvýšení hmotnostní heterogenity, ale nezvyšuje to celkovou produktivitu chovu (Mélard et al., 1996). V této souvislosti lze dodat, že třídění může působit jako další stresor (Policar et al., 2015) a opakované stresové události mohou snižovat příjem krmiva a zvyšovat výdej energie (Strand et al., 2007).

Postup, který je v práci popsán racionálně využívá rybniční fond, jenž je k dispozici a zároveň šetří náklady v raných fázích odchovu často spojenými především s potřebou kvalifikované lidské práce, nutností kultivovat krmné organismy (*Artemia* sp). V praxi tak tento postup poskytuje částečnou alternativu plně kontrolovanému procesu, kde jsou po vylíhnutí larvy odchovávány v plně kontrolovaném prostředí pomocí živé potravy a startérových krmiv (Kestemont et al., 2007; Lund a Steenfeldt, 2011). V této souvislosti je třeba dodat, že v praxi intenzivní chovy candáta obecného v plně kontrolovaných RAS řeší problémy spojené s nižší frekvencí zralých a ovulujících jikernaček, sníženou pohyblivostí a životaschopností spermií, problémy s oplozeností jiker, poruchy líhnivosti larev, vyšší podíl deformit a obecně nižší vitalitu larev (Wang et al., 2009; Kestemont et al., 2007).

Intenzivní chov candáta obecného jakožto karnivorního druhu má svoje specifika. V další části studie Policar et al. (2018), jsme se zaměřili na vliv hustoty obsádky při adaptaci plůdku candáta obecného na podmínky intenzivního chovu (**Příloha 1**). Ze studií na jiných druzích ryb a v jiných odchovných systémech je známo, že nepřiměřeně vysoká hustota obsádky může být příčinou stresu. Stresové efekty je třeba v rámci intenzivního odchovu candáta kombinací rybničního a intenzivního chovu redukovat na co nejnižší míru i vzhledem k tomu, že vlastní postup již zahrnuje stresové situace jako výlov, transport a třídění, a mohlo by tak dojít ke kumulativnímu účinku na stresovou odezvu ryb.

Organismus ryb, candáta obecného nevyjímaje, reaguje na nadměrnou hustotu obsádky zvýšením hladiny kortizolu v plazmě, což následně ovlivňuje (blokuje) produkci tyroidního hormonu (Herrera et al., 2016) a v konečném důsledku redukuje rychlost růstu

(Żarski et al., 2008), případně se může v dlouhodobějším horizontu projevit i snížením přežití ryb (Molnár et al., 2004). U intenzivně chovaných druhů lososovitých ryb bylo prokázáno, že nepřiměřeně vysoká hustota obsádky se může projevit snížením pohody chovaných ryb a zvýšenými projevy kanibalismu (Ellis et al., 2002; Liao a Chang, 2002). V naší studii bylo zjištěno, že jako optimální se jeví vyšší hustoty hustota obsádek na úrovni 4–8 ryb/l. Při porovnání adaptace plůdku candát obecný v jednotlivých testovaných hustotách obsádek (1, 2, 4 a 8 ryb/l) nebyly zaznamenány žádné rozdíly v konečné hmotnosti a délce ryb. Ve skupinách s nižšími hustotami obsádek (1–2 ryby/l) bylo zaznamenáno nižší přežití. Překvapivě nebyly zaznamenány žádné rozdíly v odhadu kanibalismu napříč spektrem testovaných hustot obsádek. Vzhledem k tomu, že jsme v této práci založili počtem nasazených ryb velmi rozsáhlý experiment, nebylo logisticky možné se detailně věnovat monitorování jednotlivých typů kanibalismu.

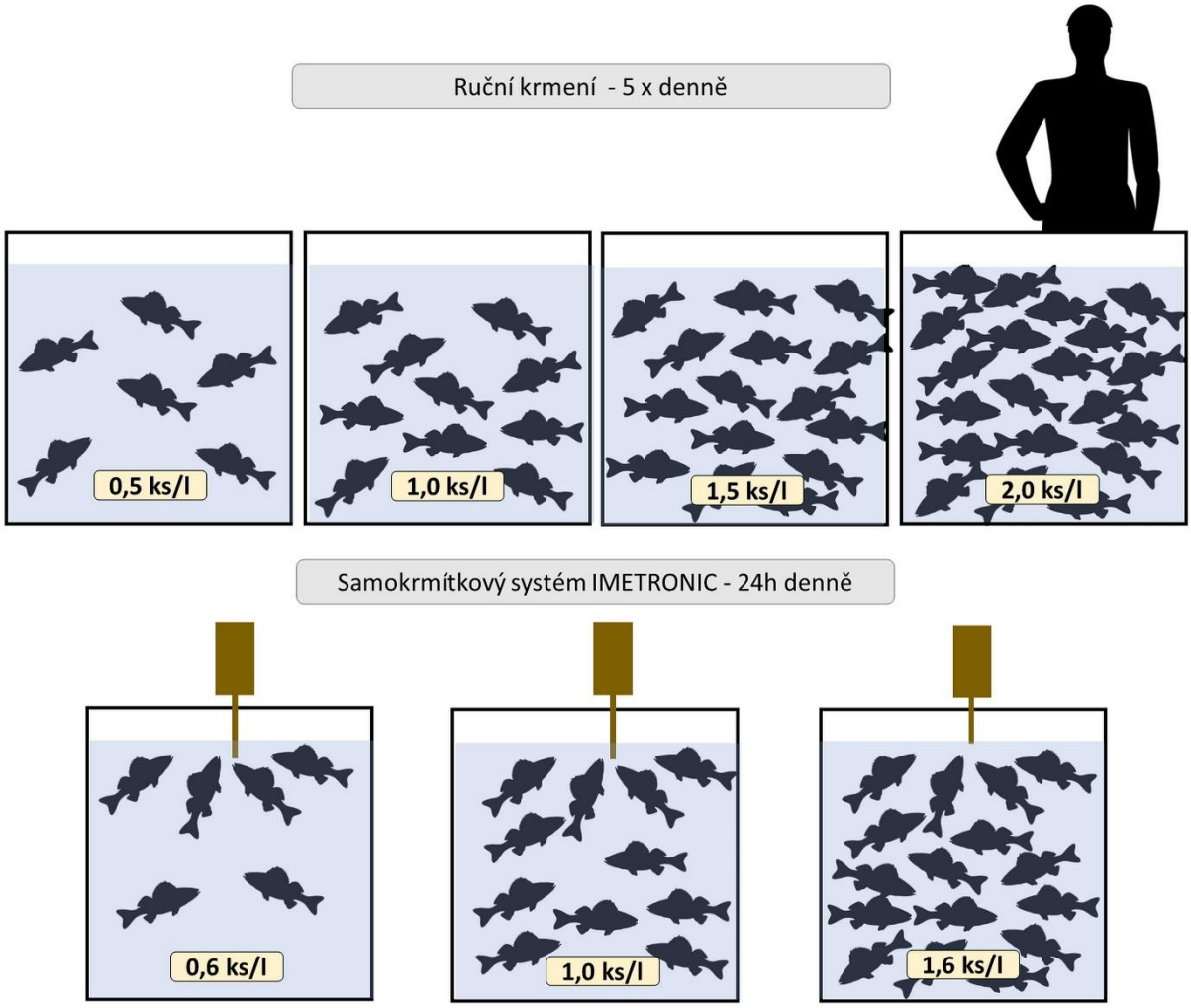


Obr. 7. Klasifikace pozorovaných projevů kanibalismu u candáta obecného. Situace (A) popisuje poškozování ocasní ploutve. Při projevu typu (B) dochází k poškozování prsních ploutví. Při situaci (C) dojde k vzájemnému zaklesnutí čelistí ryb soupeřících o krmivo. Kompletní pozření kořisti kanibalem znázorňuje situace (D). U typu (E) je kanibal udušen kořistí v tlamě. Reálné případy kanibalismu u candáta obecného (F).

Námi prezentovaná data ohledně kanibalismu v podstatě představují kanibalismus v podobě pozření kořisti kanibalujícím jedincem (Obr. 7). V literatuře je známo kategorizování na tři typy kanibalismu, a to především u karnivorních ryb. Domníváme se, že u skupin chovaných v nižších hustotách obsádek jsme naší metodikou nepostihli A-typ kanibalismu (Obr. 7) podobně jako je tomu například ve studii na kanici oranžovoskvrnném *Epinephelus coioides* (Takeshita a Soyano, 2009). Můžeme proto jen spekulovat, že velká část mortality pozorované ve skupinách s nižší hustotou obsádky mohla být způsobena právě C-typem kanibalismu. Nicméně tuto domněnku podporují i výsledky jiné práce na larvách a juvenilech

okouna říčního *P. fluviatilis* (Baras et al., 2003). Slabší stránkou studie je fakt, že nebyla otestována horní hranice hustoty obsádky, která by se projevila redukcí růstu či přežití ryb, tato znalost tedy zatím chybí.

Na tuto studii jsme plynule navázali další prací zaměřenou na efekt hustoty obsádky, kde jsme dle našich znalostí poprvé aplikovali vysoce sofistikovaný samokrmítkový systém IMETRONIC v rámci intenzivního odchovu okounovitých ryb krmených extrudovaným krmivem (Stejskal et al., 2019 – **Příloha 2**). Toto zařízení je sofistikovaným systémem pro distribuci krmiv a hodnocení počtu požadavků na krmivo, které je poté v uživatelem nastavitelném množství uvolňováno do nádrže s rybami. Systém je tvořen řídící jednotkou, sensorem a jednotlivými krmítky. Krmítko umožnuje dávkování malých a přesných množství krmiv. Software umožňuje vytvoření přehledu o chovu (např. počet nádrží, umístění, počet samokrmítek na nádrž), krmný protokol (počet požadavků ryb na krmivo, období inhibice, regulace, korekce) a kontrolu potravního chování ryb.



Obr. 8. Schematické znázornění experimentálních skupin v práci Stejskal et al. (2019) – **Příloha 2**.

Práce byla opět rozdělena na dva experimenty, přičemž v první části jsme testovali hustoty obsádek při ručním krmení a v druhé využití automatického samokrmítkového systému. Design experimentů tak zahrnoval vnější (člověkem – obsluhou) a vnitřní

(organismem – rybou) řízení režimu krmení (Obr. 8). Práce probíhala v porovnání s experimentem v **Příloze 1** na větších juvenilních rybách (průměrná počáteční hmotnost 19,1 a 25,4 g). V dříve publikovaných pracích bylo prokázáno, že neadekvátní hustota obsádky v intenzivních systémech může vést ke snížení využití krmiv rybami (Sharma a Chakrabarti, 1998), změnit rychlost metabolismu s ohledem na lipidy (Mommsen et al., 1999), sacharidy (Sangiao-Alvarellos et al., 2005) a proteiny (Costas et al., 2008). V nejobecnějším pohledu může vysoká hustota obsádky zhoršit kvalitu vody (Montero et al., 1999) snížením koncentrace kyslíku a zvýšením koncentrace amoniaku. Na druhé straně příliš nízká hustota obsádky je spojena s vysokými provozními náklady intenzivních chovů.

Z našich výsledků vyplývá, že průměrná kusová hmotnost se v průběhu experimentu, kdy si ryby samy žádaly o krmivo, zvýšila nejvíce u skupiny s hustotou obsádky 1,0 ks/l (konečná hmotnost 47,7 ± 2,8 g). Druhou nejrychleji rostoucí skupinou byla skupina s hustotou 0,6 ks/l (39,2 ± 3,7 g.). Nejmenší přírůstek byl zaznamenán u skupiny s nejvyšší hustotou obsádky na úrovni 1,4 ks/l (29,0 ± 3,4 g). Hmotnost nejrychleji rostoucí skupiny se v průběhu experimentu téměř zdvojnásobila, naproti tomu nejpomaleji rostoucí skupina zaznamenala přírůstek pouze na úrovni 14 % původní hmotnosti. V části práce, kde bylo krmivo aplikováno ručně, nebyla hustota na úrovní 1 ks/l rybami preferována, ale nejvyššího přírůstku bylo v rámci testovaných hustot obsádek dosaženo u ryb chovaných při 0,5 ks/l (48,5 ± 0,8 g). Vysvětlení lze najít v sociálním chování a procesu učení se žádat si o krmivo. Data počtu požadavků na krmivo (kvantifikace potravního chování) velmi dobře korespondovala s dosaženou finální hmotností ryb a specifickou rychlostí růstu (**Příloha 2**). Dále i celkové nejvyšší množství požadavků (0,41 ± 0,13 požadavků/rybu/den) na krmivo v hustotě obsádky 1 ks/l naznačuje, že právě tato úroveň je blízko optimu. Adekvátní krmný režim je nástrojem pro optimální růst ryb (Geay a Kestemont, 2015) a jeho prostřednictvím můžeme snížit hmotnostní heterogenitu chovaných obsádek ryb (Sun et al., 2016). Bylo popsáno, že restrikce krmiva může vést ke zvýšení objemu žaludku (Ruohonen a Grove, 1996). Optimální krmný režim tedy ovlivňuje welfare ryb a celkovou profitabilitu chovu (Alanärä a Strand, 2015), protože krmiva obvykle představují největší náklady v produkci akvakultury (Liu et al., 2016; Ahmed a Turchini, 2021). Krmné režimy s častějšími krmnými akcemi pozitivně ovlivnily růst mnoha druhů ryb (Wang et al., 1998). Nedávná studie na juvenilních okounech neprokázala rozdíly v rychlosti růstu při krmení třikrát denně nebo nepřetržitě, i když v nádržích s nepřetržitou dodávkou krmiva byla pozorována mírná (nesignifikantní) tendence k rychlejšímu růstu (Wysujack a Drahotta, 2017). Samokrmítkové systémy umožňují rybám skutečně řídit dobu, frekvenci a množství krmení. Zdá se, že mohou dokonce zvýšit pohodu chovaných ryb. Dále mohu být využity jako nástroj pro zkoumání vlivu různých faktorů na krmné chování ryb (Attia et al., 2012).

V obou částech této práce jsme se dále podrobně zaměřili na poškozování ploutví jakožto ukazatele pohody zvířat (**Příloha 2**). Agresivní chování mezi rybami jako konsekvence nepřiměřené hustoty obsádky může podporovat výskyt poškození ploutví (Latremouille, 2003). Plné porovnání mezi dvěma prezentovanými přístupy k aplikaci krmiva v **Příloze 2** není možné, protože se jednalo o dva časově oddělené experimenty na rybách různého původu. Nicméně rozdíl v ukazateli celkového poškození ploutví (TFLR) u ryb chovaných v hustotě 1 ks/l přináší otázku, zda lze poškození ploutví optimalizovat vhodným managementem krmení. Zmíněný ukazatel TFLR dosáhl hodnoty 1,00 ± 0,14 u ryb krmených ručně (5 × denně), zatímco

vyšší hodnoty 1,16 ± 0,05 znamenající nižší poškození ploutví, byly zaznamenány v experimentu, kde si ryby samy žádaly o krmivo. Poškození ploutví může zvýšit pravděpodobnost vzniku onemocnění a snížit estetickou přitažlivost ryb pro spotřebitele (Bosakowski a Wagner, 1994). Erodované ploutve mohou být nikou pro uchycení sekundárních patogenů a může dojít ke snížení osmoregulační schopnosti a narušení homeostáze. Možností, jak redukovat poškození ploutví je několik a zahrnují optimalizaci hydraulických poměrů v nádrži, krmných režimů a designu nádrže. Poškození ploutví a mortalitu lze definovat jako hlavní ukazatele welfare u intenzivně chovaných ryb (Ellis et al., 2002).

Obr. 9. Zhuštěná obsádka okouna říčního v RAS systému.

2.1.2. Charakterizace populací okounovitých ryb v intenzivní akvakultuře z pohledu fitness a chování.

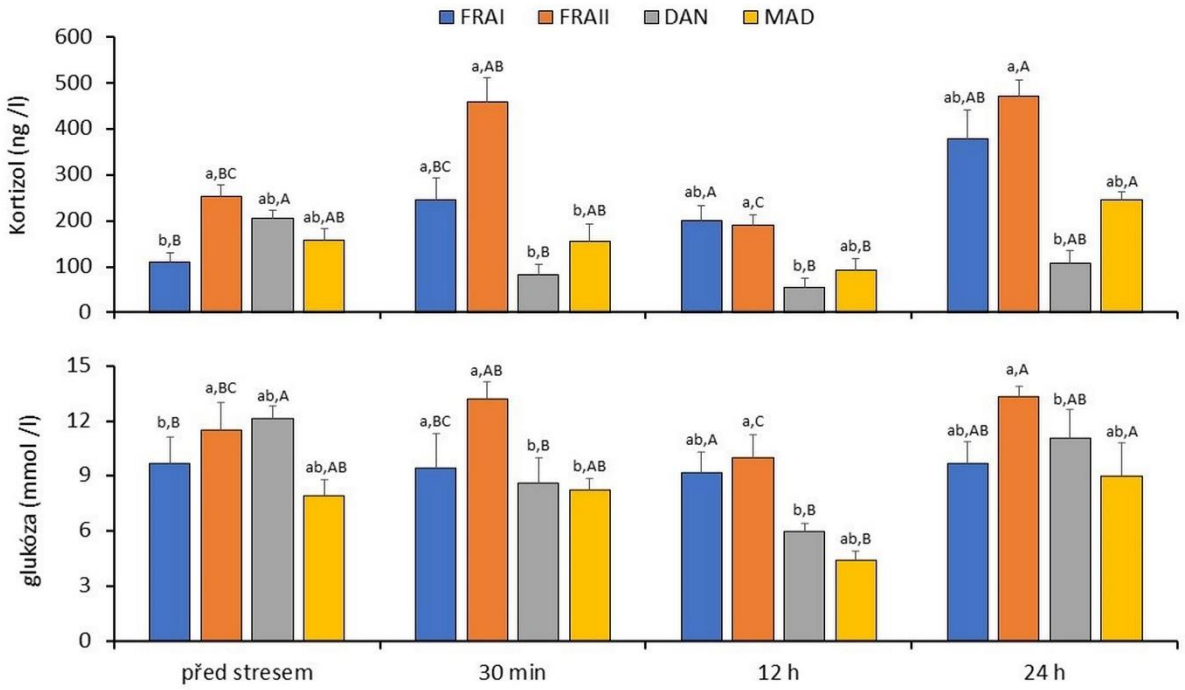
Intenzivní chovy okouna říčního a candáta obecného jsou stále ještě poměrně mladým odvětvím evropské akvakultury (Policar et al., 2019). Tyto druhy ryb se v současné Evropě také využívají k diverzifikaci sladkovodní akvakultury s cílem zvýšit konkurenceschopnost a rentabilitu produkčních podniků. V porovnání s dalšími faremně chovanými druhy (např. losos atlantský *Salmo salar* nebo pstruh duhový *O. mykiss*) u okouna říčního, ale i candáta obecného, chybí systematická selekční práce. Již v minulosti bylo i díky kolektivu autora reportováno, že různé evropské okouní populace z volných vod se liší v genetické variabilitě (Toomey et al., 2020; Vanina et al., 2019a). České, slovenské, finské a polské populace se dále liší ve specifické rychlosti růstu a růstové heterogenitě při intenzivním chovu v juvenilní periodě (Vanina et al., 2019a), i larvální periodě (Vanina et al., 2019b). Je známo, že populace chované a rozmnožované dlouhodobě (domestikované) podléhají přímým a nepřímým evolučním změnám způsobeným umělým výběrem (selekcí), pro požadované vlastnosti. V takových podmínkách se uplatňují vlivy příbuzenské plemenitby a genetického driftu, zejména když je efektivní velikost populace nedostatečná. V naší další práci (Gebauer et al., 2021 – **Příloha 3**) jsme se zaměřili na zjištění současného stavu intenzivně chovaných populací okouna říčního na čtyřech evropských farmách z pohledu morfologických

charakteristik, stavu poškození ploutví, somatických indexů, fitness, kritická rychlost plavání, stresová odolnost, chování a agresivita. Literatura uvádí, že mezi první rysy, které jsou ovlivněny domestikací, patří rysy chování (Kohane a Parsons, 1988).

Z našich výsledků je patrné, že porovnávané populace se nelišily v agresivitě hodnocené pomocí projevů, jako je atakování společně chovaných jedinců, pronásledování či okusování dalšího jedince v hejnu. Rozdíly byly pozorovány v celkové aktivitě ryb, přičemž populace z Dánska (DAN) vykazovaly výrazně vyšší aktivitu v porovnání s rybami z Francie (původem dvě farmy FRA I a FRA II) a Maďarska (MAD). Nicméně, tento výsledek následně nekorespondoval s fitness ryb v testu kritické rychlosti plavání měřené v plavacím tunelu Steffensenova typu, kde se ryby původem z Francie a Dánska vzájemně nelišily. Signifikantně nižší kritická rychlost plavání byla zaznamenána u ryb původem z maďarského chovu. Výzkum provedený Reinboldem et al. (2009) reportuje nižší plavecký potenciál u domestikovaných pstruhů duhových v porovnání s polodivokými pstruhy. Ve shodě s tímto zjištěním jsou i výsledky dalších autorů na morčáku evropském *Dicentrarchus labrax* (Benhaïm et al., 2012), kranasu americkém *Seriola dorsalis* (Wegner et al., 2018). Pasquet (2018) se domnívá, že domestikace snižuje plavecké výkony ryb, což dává do souvislostí s fyziologickými změnami v průběhu tohoto dlouhodobého procesu. Dále jsme se zaměřili na hodnocení explorativního chování po umístění nového objektu (kostka LEGO), přičemž bylo zjištěno, že převážná většina testovaných jedinců odpovídá zařazení do kategorie plachých. Pouze u dánské populace bylo zjištěno větší zastoupení středně aktivních a smělých jedinců. U stejné populace byly zjištěny nejnižší hodnoty latence (setrvání v klidu po umístění nového objektu do nádrže) v porovnání s ostatními testovanými populacemi. Rozdíly v nejbližší vzdálenosti od kostky stavebnice LEGO nebyly u testovaných populací prokázány. Literatura uvádí, že explorativní chování koreluje s vyšší aktivitou, agresivitou a nízkou reakcí na podněty prostředí (Dingemanse et al., 2007; Moretz et al., 2007). V naší studii jsme však takovou korelaci nemohli detekovat, protože testy aktivity, agresivity a stresové reakce na zhuštěné obsádky byly provedeny na různých rybách.

Pokud jde o další testované parametry, tak signifikantně vyšší hodnoty hepato-somatického indexu vykazovaly ryby z maďarské farmy (1,86 ± 0,46) a z francouzské farmy FRA I (1,44 ± 0,23). Ještě výraznější rozdíly byly zaznamenány u indexu periviscerálního tuku, kde nejvyšší hodnoty vykazovaly opět ryby z maďarské populace (5,34 ± 1,02). U kardio-somatického indexu se zjištěné hodnoty napříč populacemi nelišily. Dílčí rozdíly byly zaznamenány u poškození jednotlivých ploutví.

Ve vzorcích plazmy odebraných od ryb reprezentujících různé intenzivní chovy byly zjištěny jen nepatrné vlivy na biochemické ukazatele po provedení testu stresové zátěže snížením hladiny vody a zhuštěním obsádky (simulace aktivit na intenzivní farmě). Byly zjištěny jen nepatrné vlivy na hladiny metabolických enzymů ALT, AST, ALP a LDH po provedení testu stresové zátěže. Průkazné rozdíly byly zaznamenány u koncentrací kortizolu a glukózy, přičemž se zdá, že nejvíce reaktivními populacemi byla maďarská a francouzská FRA II (Obr. 10). Fevoldena et al. (2002) zjistili, že hladina kortizolu po vystavení stresu je významně děděna u jedinců pstruha duhového, kteří vykazovali nízkou citlivost na stres v podobě zvýšených hladin kortizolu. I tento fakt (*de facto* tolerance ryb vůči stresu) může být užitečný pro zvýšení produktivity akvakultury okounovitých ryb.



Obr. 10. Koncentrace stresových markerů před a po provedení testu stresové zátěže ve zhuštěné obsádce. Data uvedena jako průměr ± SEM. Indexy označují významný rozdíl (malá písmena v rámci populace, velká písmena mezi populacemi).

2.1.3. Vliv hypoxických podmínek na stresovou a imunitní odpověď

Dalším abiotickým faktorem, který jsme u v rámci intenzivního chovu okounovitých testovali, byla hladina kyslíku v odchovném systému, konkrétně vliv hypoxie na imunitní systém candáta obecného (**Příloha 4**). Je známo, že nedostatečná saturace kyslíkem (hypoxie) patří k jednomu z kritických faktorů při provozování těchto vysoce intenzivních odchovných systémů. Při technických problémech může v RAS i přes veškeré systémy zabezpečení a záložní zdroj elektrické energie dojít k akutním hypoxickým situacím. Navíc za určitých okolností může dojít vystavení ryb nízkým hladinám kyslíku po delší dobu. Zde je pak riziko ovlivnění pohody, zdravotního stavu ryb a imunitního systému, což má za následek zvýšenou náchylnost k onemocnění a v konečném důsledku i k ekonomickým ztrátám. Z druhého pohledu i dlouhodobější vystavení vysokým koncentracím kyslíku v řádu 140–150 % (hyperoxie) může způsobit stres u ryb a vést ke zvýšené náchylnosti k nemocem, redukci růstu a mortalitě ryb (Fridell et al., 2007). Obecně se v intenzivních akvakulturních systémech doporučuje udržovat minimální nasycení kyslíkem na úrovni 60 %, měřeno v odtoku z odchovné nádrže (Timmons a Vinci, 2007).

V naší práci jsme jako kontrolní normoxickou skupinu považovali ryby odchovávané v prostředí s koncentrací 8,3 mg/l a jako hypoxickou skupinu odchovávanou v koncentraci kyslíku 3,2 mg/l (40% nasycení). Frisk et al. (2012) nedávno definovali hypoxický stres pro candáta obecného na úrovni nižší než 40% nasycení kyslíkem (pro teplotní optimum v rozmezí 10,4 až 26,9 °C). Tato studie a dle našich informací ani žádná další nezkoumaly mechanismy, které jsou základem hypoxické stresové reakce. Hlubší hypoxie může způsobit oxidativní stres, snížený příjem potravy a vede ke zvýšení pravděpodobnosti onemocnění chovaných ryb

(Fridell et al., 2007; Ritola et al., 2002). U candáta obecného jsme vyhodnotili fyziologickou odpověď reakce ryb vystavených nízkým hladinám rozpuštěného kyslíku a překvapivě jsme nezjistili významné rozdíly v koncentraci glukózy, laktátdehydrogenázy a kortizolu v plazmě, stejně tak jako ve spleno-somatickém indexu. Hypotézou bylo i to, že nízká (neadekvátní) koncentrace kyslíku bude indukovat změny v proporci lymfoidních a myeloidních buněk v krvi a hlavové části ledviny. Výsledky ukázaly, že poměr těchto buněk v krvi se u ryb chovaných v hypoxii a normoxii během experimentu významně neměnil a byl na úrovni 95 % lymfocytů a 5 % myeloidních buněk. Jiná situace byla pozorována v kompozici buněk v hlavové ledvině. Během 28 dní experimentu v tomto orgánu poměr mezi myeloidními buňkami a lymfocyty v kontrolní skupině vykazoval pouze mírnou fluktuaci v rozmezí od 63 % do 79 % lymfocytů a 21 % až 37 % myeloidních buněk. Výraznější změny ve složení buněk byly pozorovány ve skupině vystavené nízké koncentraci kyslíku, a to především v počáteční fázi vystavení ryb hypoxii (první, sedmý a čtrnáctý den), kdy byl pozorován nárůst podílu myeloidních buněk, který dosáhl až 46 %.

Dále jsme hodnotili expresi osmi vybraných genů zapojených do imunitní a stresové odpovědi organismu k hypoxii. Obecně bylo několik genů méně exprimováno v játrech a hlavové ledvině při nízkých koncentracích kyslíku než v kontrolní skupině. V játrech dva z analyzovaných genů (*FTH1* a *NR3C1*) a v hlavové ledvině jeden gen (*EPAS1*) vykazovaly podobné vzorce přepisu. Zde byla úroveň exprese zmíněných genů podobná u obou skupin (normoxie *vs*. hypoxie) na začátku a na konci experimentu. Sedmý a čtrnáctý den skupina ryb v hypoxii vykazovala nižší hladiny transkriptů těchto genů. Dále čtyři geny (*HSP90AA1*, *FTH1* v hlavové ledvině a *NR3C1*, *HIF1A* v játrech) vykazovaly statisticky rozdílné počty transkriptů ve skupinách ryb chovaných v normoxii a hypoxii. Dále byl sledován průběh peritoneálního zánětu po předchozím vystavení hypoxii v porovnání s kontrolou. Za tímto účelem jsme použili již dříve osvědčený model popsaný kolektivem autorů Korytář et al. (2013). Další detaily studie jsou popsány v **Příloze 4**.

2.2. Optimalizace výživy ryb v RAS s využitím nových zdrojů proteinu a krmných aditiv

Navyšování světového výlovu z volných vod je již trvale neudržitelné, neboť dochází k postupnému vylovení populací a ohrožení výskytu některých druhů. Na rozdíl od produkce ryb z akvakultury, která má stále potenciál růst. Pro další navýšení produkce je ale nutný výzkum a vývoj nových technologií a strategií. Například v oblasti výživy ryb je nezbytný vývoj krmiv se specificky účinnými látkami podporujícími nejen růst ryb díky efektivnější konverzi živin, ale i zdravotní stav a odolnost ryb vůči nemocem a stravitelnost alternativ rybí moučky, které jsou v poslední době stále častěji zařazovány do krmných receptur.

2.2.1. Využití česneku kuchyňského jako krmného aditiva

V práci (Zare et al., 2021 – **Příloha 5**) jsme se zaměřili na možnost suplementace krmiv moučkou z česneku kuchyňského (*Allium sativum*). Po provedení krmného experimentu jsme nezjistili pozitivní vliv přídavku česneku (testované hladiny 10, 20 a 30 g/kg) na přežití, rychlost růstu, příjem a konverzi krmiva u okouna říčního. V obsahu sušiny, tuku a popelovin v mase ryb 87 dní krmených přídavkem česneku nebyly pozorovány žádné významné rozdíly.

U všech skupin suplementovaných česnekem byla zjištěna výrazně vyšší stravitelnost sušiny ve srovnání s kontrolou. Dále výrazně vyšší stravitelnost tuku byla zjištěna ve skupinách

s obsahem česneku 10 g/kg a 30 g/kg ve srovnání s kontrolní skupinou. Mezi skupinami nebyly pozorovány žádné rozdíly ve stravitelnosti bílkovin. Tyto výsledky jsou ve shodě se studií na mořčáku evropském (*Dicentrarchus labrax*) a labeu avanském (*Labeo rohita*) (İrkin a Yiğit, 2015; Sahu et al., 2007). Naopak pozitivní vlivy na růst byly pozorovány u druhů *Lateolabrax japonicus* a *Salmo caspius* a jsou dávány do souvislosti s působením bioaktivní sloučeniny česneku, včetně alliinu, allicinu a organických sloučenin síry, zejména thiosulfináty, které zvyšují trávení, příjem živin a růst (Lawson et al., 1998). V našem experimentu nebyly pozorovány významné rozdíly v aktivitě alaninaminotransferázy a aspartátaminotransferázy, koncentraci triglyceridů a celkového proteinu v krevním séru na konci krmného testu. Na všech testovaných úrovních byl přídavek česneku do krmiva spojen s významně nižšími hladinami cholesterolu v krevním séru. Dále výrazně vyšší hladiny albuminu byly detekovány ve skupinách G10 a G20 ve srovnání s ostatními skupinami.

Součástí této práce bylo i provedení stresové zkoušky, kterou byly manipulace s rybami a také snížení hladiny vody v odchovné nádrži. Po stresové situaci jsme se zaměřili na vyhodnocení koncentrace glukózy a kortizolu v krevním séru, jakožto primárních a sekundárních indikátorů stresu, před aplikací stresové zkoušky, bezprostředně po stresové situaci a v intervale 1, 6 a 24 h po stresové situaci. Kortizol je klíčový glukokortikoid u ryb a běžný indikátor stresu, který zvyšuje hladinu glukózy v krvi v reakci na stres (Barton, 2002). Bylo pozorováno, že průběh koncentrace kortizolu v krevním séru byl v kontrolní skupině bez přídavku česneku výrazně odlišný v porovnání se skupinami krmenými přídavkem česneku (podrobnosti v **Příloze 5**). Rovněž průběh koncentrace glukózy v krevním séru byl v kontrolní skupině odlišný od průběhu ve skupinách krmených přídavkem česneku. Z výsledů vyplývá, že přídavkem česneku lze mírnit průběh stresové reakce v závislosti na manipulačním stresu a stresu z vysoké obsádky. Kortizol a katecholaminy indukují glykolýzu a glukoneogenezi v hepatocytech, a tím zvyšují koncentraci glukózy v krvi ryb. Tato zvýšená hladina glukózy v krevním séru tedy rovněž indikuje vyšší úroveň stresu a zvyšuje energetický výdej ryb (Barton, 2002).

2.2.2. Možnosti náhrady rybí moučky hmyzí moučkou v intenzivních chovech ryb

Soubor prací zaměřených na výživu ryb z pohledu náhrad rybí moučky hmyzí moučkou uvozuje přehledový článek zaměřený na dopady používání těchto surovin na životní prostředí a zdroje proteinů původem z mořských ryb (Tran et al., 2022 – **Příloha 6**). Hmyzí moučka původem z různých druhů hmyzu se stala v posledních letech slibnou alternativou tradičních zdrojů proteinu v krmivech pro živočišnou produkci včetně akvakultury. Existuje dostatek studií, které potvrzují vhodnost hmyzí moučky jako částečné náhrady rybí moučky. Další práce naznačují, že hmyzí moučka může kompletně nahradit sójovou moučku bez negativního vlivu na růst ryb, konverzi krmiva, stravitelnost a kvalitu filet (Bruni et al., 2018; Magalhães et al., 2017; Renna et al., 2017). Dosud publikované přehledové články shrnuly a analyzovaly vlivy hmyzích mouček jako náhrad za rybí moučku na produkční parametry různých druhů ryb v rámci akvakultury (Gasco et al., 2019; Hua et al., 2021). Cílem naší přehledové práce bylo charakterizovat důsledky využívání hmyzích mouček jako zdroje krmiv pro vodní živočichy na životní prostředí. Recenzované publikace hodnotící hmyzí moučku jako náhradu rybí moučky v krmivech vodních živočichů byly analyzovány a po vytřídění použity pro výpočet ekonomického poměru FIFO (potřeba mořských ryb jako zdroje proteinu pro produkci 1 kg

akvakulturně odchovaných ryb), produkce pevného odpadu a dalších dopadů na životní prostředí.

Náš přehled zdůraznil a kvantifikoval značný dopad alternativních zdrojů proteinu (hmyzích mouček, mikrořas, bakteriálního proteinu) zejména pro potenciál globálního oteplování, spotřebu energie a vody. Současný stav připisujeme nedokonalosti výrobních technologií a nedostatečným kapacitám pro škálovatelnost produkce. Odhadujeme, že produkce hmyzu ve větších objemech by mohla snížit dopad na životní prostředí a zároveň konkurovat tradičním ingrediencím. Zároveň z přehledové práce nepřímo vyplývá, že hmyzí moučka má z alternativních surovin nejvyšší potenciál a prostor na zlepšení chovatelských a zpracovatelských technik, redukci nákladů a škálovatelnost. Především krmivo (schválené substráty) pro chované druhy hmyzu bylo identifikováno jako kategorie s nejvyšším podílem na kalkulovaných environmentálních parametrech a zde spatřujeme největší prostor pro zlepšení a testování vhodných substrátů, které v konečném důsledku přispějí ke zlepšení environmentálního přínosu hmyzích mouček. Největší část energie spotřebované na výrobu hmyzí moučky je spojena s nutností vyhřívání chovů, sušením hmyzu a zpracováním na moučku (De Boer et al., 2014). Jako strategické a energeticky účinné řešení se jeví instalovat zařízení na produkci hmyzu v rovníkovém klimatu (Salomone et al., 2017). Rovněž využití obnovitelné energie může tento negativní vliv redukovat přibližně o 25 % (Smetana et al., 2019).

Podle současného scénáře vývoje produkce ryb z akvakultury bude rybí moučka jako omezený zdroj proteinu blízko svým ekologickým limitům do roku 2037, což v kombinaci s neustálým růstem tržní ceny představuje velkou výzvu pro odvětví akvakultury (Shah et al., 2018). Redukce spotřeby ryb pro produkci rybí moučky a rybího tuku je stěžejní dlouhodobou strategií udržitelnosti populací ryb využívaných pro rybolov i jako zdrojů pro akvakulturu (Naylor et al., 2000). Podstatná nebo úplná náhrada rybí moučky za hmyzí moučku má potenciál snížit ukazatel eFIFO na <1,0 u většiny intenzivně chovaných taxonů ryb, z odvětví akvakultury (tj. spotřebitele) by se tak mohl stát čistý producent ryb (Kok et al., 2020). Další podrobnosti a detailní informace přináší **Příloha 6.**

2.2.2.1. Využití hmyzích mouček druhu Hermetia illucens v krmivech pro karnivorní ryby

Produkce hmyzí moučky výrazně snižuje tlak na životní prostředí a zdroje vody v porovnání se sójovou moučkou (Salomone et al., 2017, Smetana et al., 2019) a významně snižuje dopad na vodní ekosystémy trpící nadměrným lovem pelagických ryb za účelem výroby rybí moučky a rybího oleje (**Příloha 6**). Nicméně možnosti náhrady rybí moučky hmyzí moučkou jsou druhově specifické, a to jak z pohledu chovaných ryb, tak z pohledu použitého druhu hmyzu (**Příloha 7**). V současnosti je Evropskou regulí 2017/893 ze dne 24. 5. 2017 povoleno sedm druhů hmyzu. V další práci jsme v experimentálních krmivech pro okouna říčního testovali částečně odtučněnou moučku z bráněnky *Hermetia illucens*. Tento druh hmyzu je perspektivním druhem organismu pro cirkulární systémy nejen v akvakultuře. Larvy a pupy se živí v podstatě jakýmkoliv organickým substrátem, jako je odpad ze zeleniny, odpad z restaurací, krmivo pro kuřata, digestát z bioplynových stanic a další organické substráty rostlinného i živočišného původu (Spranghers et al., 2017). Larvy jsou schopny přeměnit tento odpad na hodnotnou biomasu bohatou na bílkoviny a tuky (van Huis et al., 2013). Nespornou výhodou chovu bráněnky v porovnání s dalšími druhy hmyzu je, že v dospělosti nepřijímá

potravu kvůli absenci ústního otvoru, tudíž není potenciálním přenašečem chorob (Smetana et al., 2019). Ve vlastním krmném experimentu jsme nenašli žádné významné rozdíly v přežití ryb, hmotnostní heterogenitě chovaných obsádek ryb, ani hematologických indexech. U odchovaných ryb rovněž nebyly pozorovány rozdíly v chemickém složení masa, především v sušině, obsahu bílkovin a tuků. Začlenění odtučněné hmyzí moučky z *H. illucens* na úrovni 60 % snížilo konečnou hmotnost těla ryb, specifickou rychlost růstu, příjem krmiva, retenci proteinu, koeficient kondice a hepato-somatický index. Podle Ringø et al. (2012) přítomnost vyšších koncentrací chitinu ve stravě zapříčiňuje redukci růstu ryb z důvodu snížení energetické dostupnosti a stravitelnosti živin. K podobným závěrům dospěl také Weththasinghe et al. (2021), který tvrdí, že nízká schopnost ryb využívat chitin jako zdroj energie redukuje růst ryb při vyšším obsahu hmyzí moučky v krmivech. Naše studie tedy ukázala, že obsah moučky z *H. illucens* do 400 g/kg v dietě okouna nemá významný vliv na tempo růstu. K podobným výsledkům došli Renna et al. (2017) na dalším karnivorním druhu, pstruhu duhovém, kde zastoupení 40 % hmyzí moučky v krmivech bylo úspěšně použito bez negativních vlivů na přežití ryb, rychlost růstu, kondici, somatické indexy a histomorfologii střeva. V naší práci jsme zjistili, že se zvyšujícím se zastoupením hmyzí moučky v krmivech ryb se ukazatel FIFO úměrně snižuje. Fish-in-fish-out ratio (FIFO) je praktickým indikátorem udržitelnosti životního prostředí a definuje, jaké množství odlovených ryb z volných vod zpracovaných na rybí moučku a olej je zapotřebí k vyprodukování jednoho kilogramu ryb na rybích farmách (**Příloha 6**).

Nově zaváděné suroviny pro výrobu krmiv, které mají sloužit pro výživu ryb pro humánní konzum, je nutno široce prostudovat a odhalit možné pozitivní i negativní důsledky na organismus chovaných ryb. Proto jsme v další práci (**Příloha 8**) testovali moučku z *H. illucens* v krmivech pro intenzivní chov candáta obecného a přinášíme poznatky o vlivech na histomorfologii a aktivitu oxidativních enzymů ve střevě a játrech. V designu tohoto experimentu jsme na rozdíl od předchozí práce (**Příloha 7**) začlenili do receptury významný podíl rostlinných zdrojů proteinu. V předchozí studii na okounu říčním činil procentní podíl rostlinných surovin 11–20 %, zatímco v práci na candátu obecném to bylo 42–48 %. Použití hmyzí moučky v krmivech nezměnilo aktivitu superoxid dismutázy v játrech a střevě, aktivitu katalázy v játrech, aktivitu glutathion peroxidázy ve střevě či aktivitu glutathion S-transferazy v játrech candáta. Významné zvýšení aktivity glutathion S-transferazy bylo pozorováno ve střevě candáta obecného krmeného stravou obsahující hmyzí moučku (u všech testovaných skupin). Je známo, že tyto antioxidační enzymy vykazují různou aktivitu v různých orgánech a játra jsou nejcitlivějším orgánem při změně diety v RAS (Policar et al., 2016). Pokud jde o detoxikaci ve střevě enzym superoxid dismutáza hraje zásadní roli (Tang et al., 2013).

V rámci morfometrie a histopatologie byly pozorovány jen marginální rozdíly mezi kontrolní skupinou a skupinami s odstupňovaným zastoupením hmyzí moučky. Na hranici významnosti (P = 0,065) bylo možné pozorovat trend zaznamenávající širší klky u skupiny s 36% zastoupením hmyzí moučky. Zařazení hmyzí moučky do krmiv pro candáta obecného tedy nevyvolalo žádné významné morfologické změny, což nenaznačuje negativní použití této suroviny pro fyziologii střev. Mírná až těžká a multifokální až difúzní jaterní vakuolární degenerace byla zaznamenána u všech skupin. Nicméně v kontrolní skupině a skupině s 9 % hmyzí moučky byla jaterní degenerace vyšší než v ostatních testovaných skupinách. Dosažené výsledky jsou v souladu s předchozími studiemi na různých druzích ryb krmených krmivy

s obsahem hmyzích mouček (Elia et al., 2018; Zarantoniello et al., 2019). Nepřítomnost střevních a jaterních zánětlivých změn může být spojena s protizánětlivými vlastnostmi kyseliny laurové (C12:0) a chitinu, které se v hmyzí moučce vyskytují.

Výsledek analýzy OTU (operational taxonomic units – taxonomické jednotky) neprokázal významné rozdíly v Shannonově indexu (ukazatel rozmanitosti společenstva) mezi testovanými skupinami. Naproti tomu diverzita střevních bakterií porovnaná Chao1 indexem mezi zjištěnými druhy vykazovala trend vyšší rozmanitosti a druhové pestrosti ve skupinách krmených hmyzí moučkou. Pomocí PCA analýzy vytvořené na úrovni rodů bylo také možné pozorovat určitý stupeň oddělení testovaných skupin. Dominantními OTU na úrovni kmenů byly Firmicutes bez ohledu použitou dietu. Candáti obecní v kontrolní skupině bez hmyzí moučky vykazovali vyšší početnost Proteobakterií (26 %), zatímco Bacteroidetes (7–13 %) byl převládajícím kmenem u ryb krmených krmivem s obsahem hmyzí moučky. Výsledky rovněž ukazují početnost rodu Clostridium u ryb krmených krmivy s 8 a 18 % hmyzí moučky. Zástupci rodu Clostridium jsou považovány za účinné mikroorganismy používané jako probiotika v akvakultuře (Nayak, 2010). Bylo prokázáno, že *Clostridium butyricum* mají schopnost patogenní inhibice u chovaných ryb (Gao et al., 2013). Bylo prokázáno, že moučka *H. illucens* moduluje bakteriální diverzitu a bohatost, které hrají zásadní roli ve výživě, imunologii a zdravotním stavu ryb, např. pstruha duhového (Bruni et al., 2018; Rimoldi et al., 2021). Bylo potvrzeno, že krmené druhy hmyzu jsou vhodné pro druhy ryb, které se přirozeně živí hmyzem (Gasco et al., 2020a).

2.2.2.2. Využití hmyzích mouček druhu Tenebrio molitor v krmivech pro karnivorní ryby

V předposlední práci zařazené v této habilitační práci (**Příloha 9**) jsme pro využití v krmivech testovali moučku z larev potemníka moučného (*T. molitor*). Dospělci tohoto druhu brouka nemohou být v krmivech použiti, protože obsahují chinony, které mohou reagovat s bílkovinami, a snížit tak jejich stravitelnost a nutriční hodnotu (Rohn et al., 2006). Larvy potemníků moučných jsou všežravé a mohou být krmeny rostlinami, ale i masem či peřím. Nejčastějším substrátem jsou však obilné otruby, mouka horší kvality, sójová mouka, sušené odstředěné mléko nebo kvasnice (Ramos-Elorduy et al., 2002). S cílem hlubšího poznání jsme významnost této hmyzí moučky v krmivech zkoumali pomocí techniky stabilních izotopů. Tato analýza je cenným nástrojem pro výzkum významnosti jednotlivých složek potravy především v ekologických studiích (Post, 2002). Konkrétně jsme v naší práci využili poměr stabilní izotopů dusíku a uhlíku ($\delta^{15}N$, resp. $\delta^{13}C$) a Bayesovské modely (Parnell et al., 2013).

Poměry stabilních izotopů hlavních proteinových složek krmiva (rybí moučka, moučka z *T. molitor*, kukuřičná mouka), které jsme použili v experimentálních krmivech, byly významně odlišné s logickou výjimkou u sójového proteinového koncentrátu a sójové moučky (tabulka 2). Poměr izotopu uhlíku $\delta^{13}C$ se mezi připravenými experimentálními krmivy nelišil, avšak zvyšující se zastoupení hmyzí moučky významně snížilo hodnotu izotopu dusíku $\delta^{15}N$. Dále jsme se zaměřili na diskriminační faktory $\Delta^{13}C$ mezi dietou a jednotlivými tkáněmi (krev, játra a svalovina). Diskriminační faktory $\Delta^{13}C$ v krvi (0,54–0,65 ‰) a játrech (2,65–3,35 ‰) nebyly významně ovlivněny použitou dietou. Nicméně $\Delta^{13}C$ ve svalovině byl významně nižší u diet obsahujících hmyzí moučku v porovnání s kontrolou. Použití hmyzí moučky významně zvýšilo diskriminaci $\Delta^{15}N$ ve všech tkáních, přičemž svalovina okouna vykazovala nejvyšší $\Delta^{15}N$ v porovnání s játry a krví. Prokázali jsme negativní korelaci mezi diskriminačním faktorem

krmivo-svalovina $\Delta^{15}N$ a růstem ryb. Hmyzí moučka není preferovaným zdrojem pro výstavbu svaloviny a krve a podíl hmyzí moučky byl disproporcionální se zvyšujícím se zastoupením hmyzí moučky v krmivu. Izotopové hodnoty testovaných tkání silně reflektovaly hodnoty příslušných krmiv, zejména pro $\delta^{15}N$, zatímco $\delta^{13}C$ byl mírně modifikován.

V praxi je *T. molitor* primárním konzumentem různých rostlinných substrátů (Cortes Ortiz et al., 2016) a je tedy více obohacen o izotopy ^{15}N a ^{13}C. To by mohlo vysvětlit vyšší ^{15}N izotopový „podpis" hmyzí moučky v porovnání se sójovou a kukuřičnou moučkou, které jsou klasifikovány jako producenti. Na druhou stranu rybí moučka získaná z mořských druhů ryb přináší podstatné obohacení o ^{15}N (Kusche et al., 2018). Další podrobnosti včetně grafů z Bayesovského modelování pro proporcionální příspěvek jednotlivých složek krmiva pro výstavbu svalové tkáně jsou uvedeny v **Příloze 9**. Toto byla první studie zkoumající izotopové „podpisy" a proporcionální příspěvek složek krmiva pro výstavbu tkání ryb krmených experimentálními krmivy, kde byla rybí moučka částečně nahrazena hmyzí moučkou.

Vzhledem k tomu, že náhrada rybí moučky hmyzí moučkou je nově testována v krmivech pro akvakulturu, tak zároveň je kladen i důraz na welfare ryb, jejich fyziologické ukazatele plavecké výkonnosti a metabolické funkce (**Příloha 10).** Plavání a metabolická aktivita jsou považovány za důležité proměnné fyziologické aktivity ryb (Allen et al., 2021). Kritická rychlost plavání (U_{crit}) a spotřeba kyslíku (MO_2) a energie nutná na přemístění (COT) jsou měřitelné parametry pro kvantifikaci výše uvedeného. Tyto parametry spolu s biochemickými markery mohou odrážet fyziologický stres (Brett, 1964) či výživný stav ryb (McKenzie et al., 1998). Poznatky naší práce se týkají vlivu experimentálních diet s obsahem hmyzí moučky z druhu *T. molitor* na metabolismus, plavecký výkon a výdej energie. Předchozí studie uvádějí, že zdroj bílkovin v krmivu ryb nemá vliv na plavecký výkon (Chai et al., 2013), výdej energie (Wilson et al., 2007) a spotřebu kyslíku ryb (Gerile a Pirhonen, 2017). V naší práci byla u okounů zjištěna kritická rychlost plavání na úrovni 97,42–117,23 cm/s nebo 5,43–6,49 BL/s (BL/s = délek těla za sekundu). Nicméně vlivy použití krmiv s obsahem hmyzích mouček na kritickou rychlost plavání, spotřebu kyslíku a vydanou energii nebyly prokázány. Jiné práce reportují, že především změny v profilu mastných kyselin ve svalovině mohou ovlivnit plavecký výkon lososa obecného (McKenzie et al., 1998), mořčáka evropského (Chatelier et al., 2006) a sivena arktického *Salvelinus alpinus* (Pettersson et al., 2010). V naší práci jsme zjistili, že vyšší množství kyseliny olejové negativně korelovalo s plaveckým výkonem okouna říčního, což je ve shodě s výsledky Wagnera et al. (2004), kteří uvádějí, že tato mastná kyselina může narušit činnost enzymu palmitoyltransferázy, jenž je odpovědný za zvýšení metabolismu červeného svalstva.

Dále jsme ryby testované na kritickou rychlost plavání podrobili hodnocení biochemických parametrů v plazmě před a po plavacím zátěžovém testu. Naše výsledky odhalily, že okouni, kteří prošli zátěžovými testy plavání, měli dvojnásobnou hladinu glukózy a desetinásobnou hladinu kortizolu v krvi v porovnání s netestovanými rybami, což je ve shodě s výsledky Jentoft et al. (2005). Míra kortizolu v krvi u netestovaných okounů (46,9–116,0) je srovnatelná s Acarete et al. (2004). Nebyly zaznamenány signifikantní rozdíly v koncentraci Na+, K+, triglyceridů a celkového proteinu v plazmě ryb před a po zátěži, rozdíly nebyly shledány ani při porovnání mezi experimentálními skupinami. Pozorovali jsme signifikantní zvýšení aktivity aspartátaminotransferázy u okounů říčních, kteří prošli zátěžovými testy v porovnání s netestovanými jedinci. Aspartátaminotransferáza je enzym, využívaný rybami

při poškození jaterních buněk (Gharaei et al., 2011), který zprostředkovává syntézu bílkovin a glukoneogenezi (Masola et al., 2008). Další podrobnosti o vlivu použité diety na biochemické ukazatele před a po zátěžových testech jsou uvedeny v **Příloze 10**.

3. Závěry

3.1. Význam výsledků pro vědní obor a možnosti směřování dalšího výzkumu

Příloha 1 je studie zaměřená na optimalizaci kombinace rybničního odchovu rychleného plůdku candáta obecného následného chovu v RAS. Práce se detailně zabývá vlivem počáteční velikosti plůdku, hustoty obsádky a protokolu pro převod ryb z přirozené potravy na kompletní krmivo. Doporučením je pro tento kombinovaný způsob produkce převádět rychlený plůdek o hmotnosti 0,42–0,55 g (celková délka těla 40–42 mm) při počáteční hustotě nasazení 8 ks/l. To odpovídá délce odchovu v rybnících na úrovni 4,6 týdnů (dle potravní nabídky).

Příloha 2 je druhou zařazenou studií optimalizující hustoty obsádek v rámci intenzivního chovu v RAS. Práce popisuje vliv hustoty obsádky na produkční parametry a poškození ploutví při ručním krmení a aplikaci krmiva speciálními samokrmítky v rámci dvou různých experimentů. Výsledky prokazují, že hustota obsádky a management krmení má významný vliv na růst a stav ploutví. Zároveň se potvrdila hypotéza, že používání samokrmítek umožnuje efektivní využití hustoty obsádek, pravděpodobně v důsledku snížené úrovně stresu. Výsledky studie lze využít k další optimalizaci intenzivní akvakultury okouna říčního.

V **Příloze 3** jsou prezentovány poznatky o chování (personalitě, agresivitě), morfologii, poškození ploutví, odolnosti vůči stresu a fitness okounů říčních pocházejících z různých RAS systémů v Evropě. Jedná se o výsledky prvního sledování tohoto typu u nově zavedeného druhu. S využitím získaných poznatků je šance na vylepšení metod intenzivního chovu a zároveň by tyto informace měly pomoci při domestikaci a selekčním programu pro potřeby moderní intenzivní akvakultury.

Interdisciplinární výzkum v **Příloze 4** poskytuje hlubší pochopení vlivu chronicky nízké saturace kyslíkem na candáta obecného chovaného v RAS. Práce nabízí bezprecedentní pohled na změny v imunokompetenci studovaných ryb a naznačují robustnost tohoto druhu akvakultury vzhledem k stresovým faktorům intenzivní akvakultury v RAS.

Příloha 5 obsahuje informace o možnostech suplementace krmiv moučkou z česneku kuchyňského v krmivech s ohledem na růst, stravitelnost hematologii a odolnost ryb vůči stresu. Přídavek česneku pozitivně ovlivnil stravitelnost tuků, hladinu cholesterolu a průběh stresové reakce v závislosti na manipulaci s rybami. Testovaná moučka z česneku má potenciál stát se funkčním doplňkem pro krmiva, protože v dávce 10 g/kg vykazuje příznivé účinky. Zařazení této ingredience do krmiv lze doporučit jako alternativní strategii pro posílení rezistence ryb vůči stresu.

Publikace v **Přílohách 6, 7, 8, 9 a 10** představují blok studií zabývajících se možnostmi využití hmyzí moučky. V přehledové práci (**Příloha 6**) jsme extrahovali data z různých publikovaných studií (144 recenzovaných publikacích), abychom vyhodnotili dopady produkce a použití hmyzí moučky v krmivech na životní prostředí, včetně potenciálu globálního oteplování, využití energie, využití půdy, spotřebu vody, acidifikaci, eutrofizaci, ekonomický FIFO ukazatel (míru využívání mořských zdrojů) a produkci pevného odpadu. Publikace tak poskytuje ucelený pohled na trendy, které jsou v současnosti považovány za vhodnou strategii minimalizace dopadů akvakultury na omezené zdroje proteinu v mořích.

V **Příloze 7** byl zhodnocen vliv inkorporace částečně odtučněné moučky z *H. illucens* na produkční parametry, chemické složení a profil mastných kyselin produkovaných ryb, složení těla, hematologické parametry a environmentální dopady použití krmiva s různým obsahem hmyzí moučky. Růst byl redukován při začlenění více než 60 % moučky z *H. illucens*, ale 40 % začlení lze doporučit. Částečné nahrazení rybí moučky hmyzí moučkou bude v budoucnu stále významnější strategií, jak krýt proteinové nároky akvakultury jako odvětví živočišné výroby.

Další práce **(Příloha 8**) ze série studií zaměřených na výživu ryby popisuje efekty diet s obsahem odtučněné hmyzí moučky *H. illucens* v chovu candáta obecného. Aplikace krmiv obsahujících moučku z *H. illucens* pozitivně modulovala rozmanitost střevní mikroflóry, zejména při zařazení této moučky až na úroveň 18 %, což odpovídá 50% náhradě rybí moučky. Tato úroveň náhrady rovněž zvýšila abundanci bakterií rodů *Clostridium*, *Oceanobacillus*, *Bacteroides* a *Faecalibacterium*. Inkorporace moučky z *H. illucens* má vliv na aktivitu oxidativních enzymů v játrech a ve střevě, ale histologická struktura střeva zůstává neovlivněna.

V další práci zařazené do habilitace (**Příloha 9)** je prezentována dle našich informací první studie zaměřená na odhalení významu inkorporace hmyzí moučky jako zdroje proteinu do krmiv pro karnivorní druh ryby, v tomto případě okouna říčního, pomocí analýzy stabilních izotopů uhlíku a dusíku. Práce odhaluje i významnost rybí moučky a rostlinných ingrediencí, především sóji. Na základě studie doporučujeme 25% nahrazení rybí moučky hmyzí moučkou pro zajištění optimálního růstu ryb a zdraví jater. Vyšší podíl hmyzí moučky nevyvolal vyšší relativní příspěvek k vývoji tří tkání (svalovina, játra a krev). Práce přináší originální data, kompletně nový úhel pohledu na využívání hmyzích mouček a naznačila další směr výzkumu, který by měl být více zaměřen na možnosti kombinace hmyzích složek krmiv s přídavkem chitinázy za účelem zvýšení využitelnosti krmiv.

V poslední práci zařazené do habilitace (**Příloha 10)** poodhalujeme vlivy použití hmyzí moučky z potemníka domácího na schopnost a kapacitu plavání, spotřebu kyslíku, využití energie a fyziologickou odezvu organismu v testech na kritickou rychlost plavání. Naše studie ukazuje, že využití hmyzí moučky v krmivech pro okouna říčního nikterak negativně neovlivňuje fyziologii plavání, spotřebu kyslíku a náchylnost ke stresu.

3.2. Využití dosažených výsledků při výuce

Teoretické i praktické poznatky, které jsou součástí této habilitační práce, byly využity při výuce studijního předmětu s názvem „Recirkulační akvakulturní systémy“ v magisterském studijním programu Fakulty rybářství a ochrany vod Jihočeské univerzity v Českých Budějovicích. Na získávání některých výsledků prezentovaných v této práci se významně podílela dřívější studentka doktorského studia Ing. Markéta Prokešová, Ph.D. a aktuální (v době přípravy habilitační práce) studenti doktorského studia Ing. Jan Matoušek, MSc. Hung Quang Tran a MSc. Mahyar Zare. Dále byli zapojeni tři studenti magisterského studia, a sice Bc. Ondřej Tomášek, Bc. Jan Volský a Bc. Jakub Čejka. V rámci bakalářské práce dílčí téma zpracoval Marek Kodras. Výše jmenovaní studenti pracovali pod vedením autora habilitační práce s výjimkou bakalářské práce Marka Kodrase, kde autor figuroval v pozici konzultanta. Dále byli zapojeni studenti doktorského programu, kde autor habilitační práce nebyl v pozici školitele, a sice MSc. Tatyana Gebauer, Ph.D., Ing. Jiří Křišťan, Ph.D., MSc. Nadine Schäfer a MSc. Oleksandr Movchan.

3.3. Využití dosažených výsledků pro praxi

Současným trendem je stále častější využívání RAS k produkci násadových a tržních ryb v produkčním rybářství. Technologie RAS vyžaduje efektivní péči, udržování optimálních parametrů prostředí a druhově specifický management chovu a pro tyto aspekty lze využít poznatků prezentovaných v **Přílohách 1–4**. Z druhového hlediska jsem se v habilitační práci věnoval především okounu říčnímu a candátu obecnému. Tyto druhy ryb se v současné Evropě také využívají k diverzifikaci sladkovodní akvakultury s cílem zvýšit konkurenceschopnost produkčních podniků a podnítit zájem spotřebitelů o kvalitní rybí maso, které právě tyto druhy nabízí.

Znalost vlivu alternativních krmných ingrediencí pro posílení imunitního systému a stresové odolnosti ryb může vyústit v zařazení těchto ingrediencí do krmiv a může představovat alternativní strategii pro posílení rezistence ryb vůči stresovým situacím a s tím spojenými sekundárními problémy. Použité alternativní suroviny jsou běžně k dispozici, a proto jsou potencionálními uživateli především krmivářské a zpracovatelské firmy, které intenzivně hledají suplementy krmiv posilující odolnost chovaných ryb. Výsledky ze studií s náhradami rybí moučky představují rozšíření znalostí o účinku použitých surovin v krmivech a efekty na kvalitu masa ryb, produkční charakteristiky, stravitelnost živin, kompozici mikroorganismů ve střevě a dopady využívání těchto krmiv na životní prostředí. Zařazení hmyzích mouček do krmiv může být v praxi alternativní strategií pro ekologickou intenzifikaci chovů ryb a snížení závislosti na mořských zdrojích proteinu. Potencionálními uživateli výsledků jsou především krmivářské a chovatelské firmy, které využijí tyto intenzivně environmentálně přijatelné náhrady rybí moučky jako zdroje proteinu pro krmiva v akvakultuře s potenciálem modulovat střevní mikroflóru včetně dalších benefitů.

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5. Seznam prací autora zahrnutých do habilitační práce

Součástí habilitační práce je celkem 10 prací publikovaných v mezinárodních vědeckých časopisech s oponentním řízením. Všechny práce mají přidělen indikátor IF v databázi Web of Science společnosti Thomson Reuters. Publikace jsou číslovány podle odkazů v jednotlivých kapitolách průvodního textu. Hodnoty IF (rozsah IF: 0,960–10,592) a umístění v příslušené kategorii (Q1–Q3) u jednotlivých publikací jsou uvedeny k příslušnému datu publikování článků v jednotlivých časopisech. U každé publikace je rovněž uveden aktuální citační ohlas (SCI) v době psaní této habilitační práce.

Příloha č. 1: Policar, T., **Stejskal, V.,** Křišťan, J., Bossuyt J., Bláha, M., 2013. The effect of fish size and density on the weaning success in pond cultured pikeperch (*Sander lucioperca* L.) juveniles. Aquaculture International 21: 869–882. (IF 2013 = 0.960; SCI 2022 = 34; Q3)

Příloha č. 2: Stejskal, V., Matoušek, J., Prokešová, M., Podhorec, P., Křišťan, J., Policar, T., Gebauer, T., 2020. Fin damage and growth parameters relative to stocking density and feeding method in intensively cultured European perch (*Perca fluviatilis* L.). Journal of Fish Diseases 43: 253–262. (IF 2020 = 2.767; SCI 2022 = 4; Q2)

Příloha č. 3: Gebauer, T., Gebauer, R., Palińska-Żarska, K., Císař, P., Movchan, O., Tomášek, O., Prokešová, M., Matoušek, J., Hliwa, P., Król, J., Żarski, D., Rebl, A., **Stejskal, V.,** 2021. Assessment of behavioural and physiological traits as indicators of suitability for European perch aquaculture. Aquaculture 544: 737048. (IF 2020 = 4.242; SCI 2022 = 0; Q1)

Příloha č. 4: Schäfer, N., Matoušek, J., Rebl, A., **Stejskal, V.,** Brunner, R.M, Goldammer, T., Verleih, M., Korytář, T. 2021. Effects of hypoxic challenge with additional intraperitoneal stimulation on the immune status of pikeperch (*Sander lucioperca* L. 1758). Biology 10: 649. (IF 2021 – 5.079; SCI 2022 = 0; Q2)

Příloha č. 5: Zare, M., Tran, H. Q., Prokešová, M., **Stejskal, V.,** 2021. Effects of garlic *Allium sativum* powder on nutrient digestibility, haematology, and immune and stress responses in Eurasian perch *Perca fluviatilis* juveniles. Animals 11: 2735. (IF 2020 = 2.752; SCI 2022 = 0; Q2)

Příloha č. 6: Tran, H.Q., Doan, H.V., **Stejskal, V.,** 2022. Environmental consequences of using insect meal as an ingredient in aquafeeds: a systematic view. Reviews in Aquaculture 14: 237–251. (IF 2020 – 10.592; SCI 2022 = 2; Q1)

Příloha č. 7: Stejskal, V., Tran, H.Q., Prokešová, M., Gebauer, T., Giang, P.T., Gai, F., Gasco, L., 2020. Partially defatted *Hermetia illucens* larva meal in diet of Eurasian perch (*Perca fluviatilis*) Juveniles. Animals 10: 1876. (IF 2020 = 2.752; SCI 2022 = 12; Q2)

Příloha č. 8: Tran Q.H., Prokešová, M., Zare, M., Gebauer, T., Elia, A.C., Colombino, E., Ferrocino, I., Caimi, C., Gai, F., Casco, L., **Stejskal, V.,** 2021. How does pikeperch *Sander lucioperca* respond to dietary insect meal *Hermetia illucens*? Investigation on gut microbiota, histomorphology, and antioxidant biomarkers. Frontiers in Marine Science 8: 680942. (IF 2020 = 4.912; SCI 2022 = 0; Q1)

Příloha č. 9: Tran, Q.H., Kiljunen, M., Doan, H.V., **Stejskal, V.,** 2021. European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): From a stable isotope perspective. Aquaculture 545: 737265. (IF 2020 = 4.242; SCI 2022 = 1; Q1)

Příloha č. 10: Tran Q.H., Doan, V.H., **Stejskal, V.,** 2021. Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*. Aquaculture 539: 736610. (IF 2020 = 4.242; SCI 2022 = 3; Q1)

6. Český abstrakt

Stejskal, V., 2021. Optimalizace intenzivního chovu a výživy ryb v recirkulačních akvakulturních systémech. Habilitační práce, Jihočeská univerzita v Českých Budějovicích, Fakulta rybářství a ochrany vod, Vodňany: 192 s.

Habilitační práce je tvořena souborem 10 publikací v časopisech s impakt faktorem. V první části je práce zaměřena na optimalizaci abiotických faktorů v recirkulačních akvakulturních systémech, především velikosti nasazovaných ryb, hustě obsádky a způsobu krmení. Dále jsou prezentována data o rozdílech v chování, kondici ploutví, fitness, agresivitě a stresové odolnosti u okounů říčních (*Perca fluviatilis*) původem z intenzivních podmínek různých evropských chovů využívajících recirkulační technologii. Druhá část práce se věnuje optimalizaci výživy ryb z pohledu použití mouček rostlinného původu, a především hmyzích mouček jako významné alternativy k rybí moučce. Jsou popsány pozitivní účinky při začlenění moučky česneku setého *Allium sativa* do krmiv pro zmírnění následků stresových situací. Blok studií zaměřených na využívání hmyzích mouček je uvozen přehledovou prací sumarizující vlivy trendu inkorporace hmyzích mouček na environmentální parametry, jako je produkce skleníkových plynů, spotřeba vody, spotřeba energie, acidifikace, eutrofizace, potřeba půdy, produkce pevného odpadu, emise fosforu, emise dusíku a potřeba krmných ryb pro produkci jednotky faremně chovaných ryb. Dále je možnost náhrady rybí moučky hmyzí moučkou, jakožto jedna z neslibnějších alternativ pro současné zdroje proteinu, studována z mnoha různých pohledů. Pozornost je věnována především moučkám druhů *Hermetia illucens* a *Tenebrio molitor* a jejich účinnost v krmivech pro okouna říčního a candáta obecného (*Sander lucioperca*). Práce se v této části zabývá vlivy těchto alternativních krmiv na produkční charakteristiky, fyziologii chovaných ryb včetně spotřeby kyslíku a fitness, hematologické parametry, odolnost vůči stresu, histomorfologii jater a střeva, antioxidační odezvu a modulaci společenstva střevní mikroflóry. Analýza stabilních izotopů je použita pro objasnění významnosti jednotlivých komponent krmiv včetně hmyzí moučky pro výstavbu tkání jater, krve a svaloviny.

Klíčová slova: Intenzivní chov, pohoda zvířat, stres, mikrobiom, výživa, stravitelnost, hmyzí moučka, krmná aditiva

7. Anglický abstrakt

Stejskal, V., 2021. Optimization of intensive fish culture and nutrition in recirculating aquaculture systems. Habilitation thesis, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Vodňany: 192 p.

The habilitation thesis is composed of a group of ten papers published in high-impact journals. The work in the first section focuses on optimizing abiotic parameters in recirculating aquaculture systems, particularly the initial body size of stocked fish, stocking density, and feeding strategy. Furthermore, data on behavior differences, fin condition, fitness, aggressiveness, and stress resistance of European perch (*Perca fluviatilis*) originating from intensive conditions of various European farms using recirculation technology are presented. The second part of the work is devoted to the optimization of fish nutrition in terms of the use of meals of plant origin, and especially insect meals as important alternatives to fish meals. The benefits of using garlic *Allium sativa* meal in feeds to help alleviate the impact of stressful situations are addressed. A review of the effects of insect meal incorporation on environmental parameters such as greenhouse gas production, water consumption, energy consumption, acidification, eutrophication, land demand, solid waste production, phosphorus emissions, nitrogen emissions, and forage fish required for the production of a unit of farmed fish introduces the block of studies focused on the use of insect meal. In addition, the possibility of replacing fishmeal with insect meal, which is one of the most promising alternatives to existing protein sources, is being researched from a range of viewpoints. Particular attention is paid to *Hermetia illucens* and *Tenebrio molitor* meals and their effectiveness in feed for perch and pikeperch (*Sander lucioperca*). The effects of these alternative feeds on production characteristics, farmed fish physiology, including oxygen consumption and fitness, hematological parameters, stress resistance, histomorphology of the liver and intestine, antioxidant response, and modulation of the intestinal microflora community are discussed in this section. Individual feed components, such as insect meal, are studied using stable isotope analysis to determine their importance in the development of liver tissue, blood, and muscle.

Key words: Intensive culture, animal welfare, stress, microbiome, nutrition, digestibility, insect meal, feed additives

8. Poděkování

Rád bych poděkoval všem kolegům z Fakulty rybářství a ochrany vod Jihočeské Univerzity v Českých Budějovicích, Univerzity v Turíně, Leibnizova ústavu pro biologii hospodářských zvířat, Institutu Stanislawa Sakowicze pro vnitrozemský rybolov, Polské akademie věd, University v Jyväskylä a University v Chiang Mai, kteří mi v mé vědecké práci pomáhali. Jmenovitě bych chtěl poděkovat prof. Ing. Janu Kouřilovi, Ph.D. a doc. Ing. Tomáši Policarovi, Ph.D. za výraznou pomoc v počátcích vědecké kariéry. Habilitační práce mohla vzniknout díky studiím finančně podpořeným následujícími agenturami a projekty:

Ministerstvo školství mládeže a tělovýchovy

- projekt CENAKVA CZ.1.05/2.1.00/01.0024 – Jihočeské výzkumné centrum akvakultury a biodiverzity hydrocenóz
- projekt LM2018099 Velké výzkumné infrastruktury CENAKVA – Jihočeské výzkumné centrum akvakultury a biodiverzity hydrocenóz (CZ.1.05/2.1.00/19.0380)
- projekt LO1205 – Udržitelnost excelence centra akvakultury a biodiverzity hydrocenóz

Národní agentura pro zemědělský výzkum

- projekt NAZV QI 101C033 – Vývoj a optimalizace metod intenzivního chovu candáta obecného (*Sander lucioperca*) a okouna říčního (*Perca fluviatilis*) v ČR
- projekt NAZV QK1810296 – Využití alternativních komponent a inovativních postupů ve výživě ryb
- projekt NAZV QK 1920326 – Akvakultura reofilních druhů ryb
- projekt NAZV QK 1820354 – Technická a technologická inovace intenzivních chovů ryb založená na nových znalostech umožňující efektivní a stabilní produkci

Operační program Výzkum, vývoj a vzdělávání

- projekt CZ.02.1.01./0.0/0.0/16_025/0007370 – Reprodukční a genetické postupy pro uchování biodiverzity ryb a akvakulturu
- projekt CZ.02.1.01/0.0/0.0/16_019/0000869 – Udržitelná produkce zdravých ryb v různých akvakulturních systémech – PROFISH

Grantová agentura Jihočeské univerzity v ČB

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- Grant #: MV-II.1-RM-001

9. Přílohy

Příloha č. 1

Policar, T., **Stejskal, V.,** Křišťan, J., Bossuyt J., Bláha, M., 2013. The effect of fish size and density on the weaning success in pond cultured pikeperch (*Sander lucioperca* L.) juveniles. Aquaculture International 21: 869–882. (IF 2013 = 0.960; SCI 2022 = 34; Q3)

Aquacult Int (2013) 21:869–882
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The effect of fish size and stocking density on the weaning success of pond-cultured pikeperch *Sander lucioperca* L. juveniles

Tomas Policar · Vlastimil Stejskal · Jiri Kristan · Peter Podhorec · Viktor Svinger · Martin Blaha

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Abstract The effect of initial fish size (small with TL = 40.3 ± 2.3 mm and $W = 0.42 \pm 0.15$ g, medium with TL = 56.2 ± 2.7 mm and $W = 1.66 \pm 0.4$ g, and big with TL = 71.0 ± 3.2 mm and $W = 2.95 \pm 0.65$ g) and stocking density of identical fish with TL = 40.3 ± 2.3 mm and $W = 0.42 \pm 0.15$ g (1; 2; 4; 8 fish l^{-1}) on weaning success was evaluated in pond-cultured pikeperch. The trial was divided into weaning (12 days) and post-weaning (16 days) periods. Small juveniles reached significantly higher specific growth rate (SGR = 1.6 ± 0.2 % day^{-1}) and survival rate ($S = 81.7 \pm 2.7$ %) and lower cannibalism ($C = 3.0 \pm 0.75$ %) compared to medium and large juveniles (SGR = 0.3–0.5 % day^{-1}, $S = 65.3$–76.5 %, $C = 6.5$–7.5 %) during the weaning period. The higher survival rate was found at the two higher densities ($S = 72.0$–79.1 %) during the weaning period. The lowest survival rate ($S = 38.9 \pm 2.7$) was observed at the lowest fish density. Fish stocking density did not affect growth, condition, or cannibalism rate during the weaning period. Similar trends of growth, survival, and cannibalism of weaned juveniles were observed during the post-weaning period. A mass weaning trial verified experimental results showing small pikeperch juveniles to reach satisfactory growth rate (SGR = 1.4 ± 0.1 and 7.2 ± 0.2 % day^{-1}), survival ($S = 78.7 \pm 3.0$ % and 97.6 ± 1.0 %), and cannibalism ($C = 4.0 \pm 1.5$ % and 2.5 ± 1.0 %) rates during the weaning and post-weaning periods. No body or fin deformities of weaned juveniles were observed.

Keywords Artificial food · Growth · Pikeperch · Pond · Recirculation aquaculture system · Survival

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Introduction

The European pikeperch *Sander lucioperca* L. with delicate flesh and attractiveness as a game fish is among the most valuable freshwater fish in Europe (Kestemont and Melard 2000; Schulz et al. 2007) and highly sought by the European market (Dil 2008; Setälä et al. 2008). The major supply of market-size pikeperch is provided by natural fisheries. Unfortunately, pikeperch catches are drastically decreasing in Europe due to overfishing and the decline of wild stock (Dil 2008; Müller-Belecke and Zienert 2008). Currently, the pikeperch market is fluctuating and undersupplied in most European countries (Dil 2008). Intensive pikeperch farming in recirculation aquaculture systems (RAS) has been developed during the past 15 years, mainly in the Netherlands, Finland, Denmark, and France (Philipsen 2008; Van Mechelen 2008; Fontaine 2009). A list of currently operating pikeperch farms is presented in Table 1.

European intensive pikeperch farming has mainly utilized a controlled culture cycle including broodstock nutrition (Wang et al. 2009a), the induction of normal (Demska-Zakes and Zakes 2002) or out-of-season spawning (Zakes and Szczepkowski 2004; Ronyai 2007; Zakes 2007; Müller-Belecke and Zienert 2008), egg stripping and artificial fertilization (Ronyai 2007) or natural egg production and fertilization (Demska-Zakes and Zakes 2002), controlled egg incubation, hatching (Ronyai 2007), larvae (Ostaszewska et al. 2005; Kestemont et al. 2007; Lund et al. 2011) and juvenile nursery (Zakes et al. 2004; Schulz et al. 2005, 2007, 2008; Luchiari et al. 2006, 2009; Wang et al. 2009b), and ongrowing culture with rapid growth rate (Molnar et al. 2004; Ronyai and Csengeri 2008). This production cycle has been associated with a lower per cent of spawning fish, reduced fertilization, and hatching rates, and high larval deformity and mortality compared to wild or pond-cultured pikeperch (Schlumberger and Proteau 1996; Demska-Zakes and Zakes 2002; Kestemont et al. 2007; Wang et al. 2009a). Currently, intensive pikeperch farms provide insufficient numbers of high-quality larvae and juveniles for ongrowing (Philipsen, personal communication), limiting production and profit (Schram 2008). The combination of pond and RAS culture of pikeperch for juvenile production intended for ongrowing can address this situation (Malison and Held 1992; Ruuhijarvi and Hyvarinen 1996; Zakes and Demska-Zakes 1996; Zakes 1997a, b). Pond culture of pikeperch larvae and juveniles provides high-quality and more or less stable production (Hilge and Steffens 1996; Zakes 1999; Peterka et al. 2003; Adamek and Opacak 2005; Musil and Kouril 2006). The weaning of pond-cultured juveniles to ensure adaptation of fish to artificial food, and high survival rate during weaning has been improved with the innovative feeding techniques

Table 1 Current operating intensive pikeperch farms in Europe

Countries	Pikeperch farms
The Netherlands	Excellence fish farm Lont en s van baaren Van Slooten Aquaculture Viskweekcentrum
Finland	Kidus Savon Taimen Hanka-Tainen
Denmark	Aquapri Lyksvad Fish farm
Czech Republic	Fish Farm Bohemia
France	SARL Asialor

(Zakes and Demska-Zakes 1998; Ljunggren et al. 2003; Altun et al. 2008) and optimization of water temperature (Zakes 1997b, 1999). Zakes (1997a, b, 1999), Zakes and Demska-Zakes (1996), Szkudlarek and Zakes (2002), and Molnar et al. (2004) have studied the effect of fish size and stocking density of pond-cultured pikeperch on the survival and growth rate during weaning. However, all mentioned studies were experimental works without the effect on the pikeperch farms. The aim of this study was to evaluate the effect of differing fish size and stocking density on weaning success in pond-cultured pikeperch juveniles and to investigate an optimal protocol for weaning of pond-cultured pikeperch juveniles for practical application.

Materials and methods

Broodstock reproduction

Twenty-four pond-cultured pikeperch broodstock (12 females TL = 563.4 ± 52.0 mm, W = 1902.5 ± 405 g and 12 males TL = 545.0 ± 45 mm, W = 1702.0 ± 301 g) were used for production of larvae. Fish, at a sex ratio 1:1, were kept in 1 × 1 × 0.8 m cages within flow-through tanks. All broodstock were injected intramuscularly with a single dose of human chorionic gonadotropin (hCG) at 500 IU per kg of fish body weight before the spawning period, when oocyte maturity reached stage III. Other details of spawning protocol were according to Demska-Zakes and Zakes (2002), and Kristan et al. (2012). After natural spawning and fertilization, cages with artificial nests containing fertilized eggs were moved into 12 tanks within the RAS of the University of South Bohemia, Faculty of Fisheries and Protection of Waters (USB FFPW). Each nest was incubated separately under controlled conditions (T = 15.5 ± 0.75 °C, oxygen level = 97 ± 1.8 %, pH = 7.5 ± 0.1, $NH_3 < 0.02$ mg l^{-1}, $NO_2^- < 0.01$ mg l^{-1}).

Pond culture of larvae and juveniles

Swimming larvae were harvested and counted from cages 2 days after hatching on May 5, 2009. Larvae (n = 798,000) were transported in twelve 50-l polyethylene bags (66,500 larvae in each bag) with oxygen environment (20 l water and 30 l oxygen) for stocking into two fish production ponds at Fisheries Nove Hrady Ltd. Hadac pond (48°48′6.243″N, 14°49′1.696″E), with an area of 2.66 ha, and Bejkovna pond (48°48′12.188″N, 14°48′53.242″E), with an area of 1.33 ha, were prepared prior to stocking according to studies by Musil and Kouril (2006) and Stejskal et al. (2009). Initial larval density in both ponds was 200,000 larvae per ha. In total, 532,000 and 266,000 larvae were stocked into Hadac and Bejkovna ponds, respectively.

Pikeperch larvae and juveniles were reared under natural pond conditions for 42 days, with inspections conducted at 14-day intervals. Size and condition of pikeperch, zooplankton levels, and water quality (water temperature = 17.3 ± 1.0 °C, oxygen level = 64.0 ± 4.5 %, pH = 6.99 ± 0.35 mg l^{-1}, NH_4 = 0.24 ± 0.1 mg l^{-1} and NO_3^- = 0.54 ± 0.2 mg l^{-1}) were evaluated according to the method of Peterka et al. (2003). Juvenile culture was completed in both ponds on the same date, when the decreased natural food supply, cannibalism, and bi-modal fish size were observed (Hilge and Steffens 1996; Peterka et al. 2003).

Pikeperch juveniles were harvested in outlet channel with special net cage according to experience gained from the studies Stejskal et al. (2009) and Policar et al. (2011). Care was

taken in manipulation, and juveniles were transported in a solution of 1 g NaCl l^{-1} to RAS of USB FFPW.

Grading and transfer of juveniles to RAS

Juveniles were categorized into three size groups (small: TL = 40.3 ± 2.3 mm, W = 0.42 ± 0.15 g; medium: TL = 56.2 ± 2.7 mm, W = 1.66 ± 0.4 g; and large: TL = 71.0 ± 3.2 mm, W = 2.95 ± 0.65 g) and stocked into 21 cylindrical tanks (volume 185 l) in the RAS. Water temperature was maintained at the ambient temperature in ponds (19.5 °C) for the duration of the trial.

Experiment 1. The effect of initial fish size on weaning success

Three initial fish size groups, small (S), medium (M), and large (L), were assessed during the weaning and post-weaning periods. Each size group was tested in triplicate. Nine tanks with identical fish density (8 juveniles l^{-1}) were stocked with 13,320 juveniles. The size groups represented initial biomass densities of S = 3.36 g l^{-1}; M = 13.28 g l^{-1}; L = 23.6 l^{-1}.

Experiment 2. The effect of initial fish density on weaning success

Four fish initial densities ($D1$ = 1 fish l^{-1}; $D2$ = 2 fish l^{-1}; $D3$ = 4 fish l^{-1}; and $D4$ = 8 fish l^{-1}) were evaluated during weaning and post-weaning periods. Each density level was tested in triplicate. In total, twelve tanks were stocked with 8,325 small juveniles (TL = 40.3 ± 2.3 mm; W = 0.42 ± 0.15 g) at the four densities, which resulted four initial biomass densities: $D1$ = 0.42 g l^{-1}; $D2$ = 0.84 g l^{-1}; $D3$ = 1.68 g l^{-1}; $D4$ = 3.36 g l^{-1}.

At the end of the experiments, the effect of initial fish size and initial fish density on growth, survival, cannibalism, and fish condition was assessed.

Verification of experimental results by mass weaning

Mass weaning was conducted 1 year after the initial experiments under conditions of fish farm USB FFPW. Size (TL = 42.4 ± 2.4 mm, W = 0.55 ± 0.23 g) and initial fish density (8 fish l^{-1}) found in the original trial to produce optimal results were used for the mass weaning verification of experimental results. Identical reproduction, pond culture, and weaning protocols as in the original trial were followed. Six 800-l tanks within the RAS of USB FFWP were used. The same parameters (growth, survival, cannibalism, and fish condition) were assessed at the end of weaning and post-weaning periods.

Weaning and post-weaning period in juveniles

A weaning schedule comprised of weaning (12 days) and post-weaning period (16 days) was employed in all parts of the study. Weaning, the point at which all surviving juveniles fully accepted artificial food under the controlled conditions of the RAS, was conducted by the following procedure.

Optimal environmental conditions for pikeperch culture (water temperature = 22.0 ± 1.5 °C, oxygen level = 85 ± 15 %, light regime = 16L:8D, light intensity = 100 lx at water surface) (Hilge and Steffens 1996) were initiated during the second day after stocking into RAS. Fish were not fed during the two first days after stocking.

Frozen chironomid larvae—red worms (*Chironomus plumosus*)—were fed on days three and four. A combination of frozen chironomid larvae (75 %) and commercial starter Inicio Plus from BioMar Ltd. (25 %) was fed on days 5 and 6. Subsequently, an increasing proportion of commercial starter in the daily ration was applied every 2 days of the weaning period (50 % chironomid larvae: 50 % starter on days 7 and 8, and 25 % chironomid larvae: 75 % starter on days 9 and 10). Juveniles were fed 100 % commercial starter on days 11 and 12. All foods were offered by hand every 30 min during the light phase ad libitum according to the feeding behaviour of fish.

All surviving juveniles accepted artificial starter after day 12, completing the weaning period. A post-weaning period of 16 days was used as verification of completed weaning.

Dead fish were removed and counted during every day of the weaning and post-weaning periods. At the end of each period, surviving fish were harvested and counted, and a representative sample of 33 juveniles was collected from each tank. Cannibal specimens were separated by a fish sorter and removed from each group during the manipulation with fish at the end of each period. Survival rate (S in %) was calculated as $S = (J_2/J_1) \times 100$, where J_2 is number of surviving juveniles, and J_1 is number of stocked juveniles. Cannibalism rate (C in %) was calculated as $C = [(J_1 - \text{SDJ} - J_2)/J_1] \times 100$, where SDJ is the sum of dead juveniles removed from the tank during each period. Individual body weight (W) was measured by a Mettler electronic balance (model AE 200) to the nearest 0.1 g, and TL was measured by a calliper to the nearest 0.1 mm. Specific growth rate [$\text{SGR} = 100\ \text{t}^{-1} \ln (W_2 W_1^{-1})$, where W_1 and W_2 are initial and final body weights in g, and t is growing period in days] and condition level [Fulton's condition coefficient $\text{FC} = (W/\text{TL}^3) \times 100$, where W is final body weight and TL is final total length of fish] were calculated after biometric analysis of fish from representative samples.

Protein, fat, and energy analysis of feeds was conducted by the Food Research Institute of Prague according to CSN 467092 standard. Fatty acid composition was analysed according to Mraz and Pickova (2009) with a Varian CP3800 gas chromatograph (Stockholm, Sweden). Fatty acids were identified by comparison with the standard mixture GLC-68A (Nu-check Prep, Elysian, MN) and using retention times. Peak areas were integrated with the Star chromatography Workstation software version 5.5 (Varian AB, Stockholm, Sweden). An internal standard 15-methylheptadecanoate (Larodan Fine Chemicals AB, Malmo, Sweden) was used for calculation of the absolute amount of individual fatty acids (Table 2).

Statistical analysis

All data of growth (TL, W, SGR), condition (FC), and survival (S and C) are presented as mean (± SE), and statistical assessment was performed by Statistica software 6.1 (StatSoft, Inc. Czech Republic). One-way analysis of variance ANOVA ($P < 0.05$) followed by Tukey's multiple comparison test (TL, W, and FC) or nonparametric Kruskal–Wallis test (SGR, S, and C) was used for comparison of growth, condition, and survival among the three size and four density groups at the end of the weaning and post-weaning periods and mass weaning.

Results

The effect of initial fish size on weaning success

During the weaning period, small juveniles reached significantly higher SGR (1.6 ± 0.2 % day^{-1}) and survival rate ($S = 81.7 \pm 2.7$ %) compared to growth (SGR = 0.3–0.5 %

Table 2 Composition of feeds used for all experiments

Parameter	Chironomid larvae	Starter Inico Plus
Diameter (mm)	9–13	1.1–1.5 (1:1)
Protein[a]	65	55
Fat[a]	1	20
Fatty acids[b]		
12:0	0.1	0.2
14:0	3.2	5.2
15:0	2.2	0.4
16:0	20.3	16.4
17:0	1.6	0.2
18:0	6.6	2.5
20:0	0.7	0.2
22:0	0	0
14:1	1.5	0.1
16:1 ($n-7$)	13.9	7.8
17:1	2.1	0
18:1 ($n-9$)	15.1	12.9
18:1 ($n-7$)	4.6	1.7
20:1 ($n-9$)	0	3.8
22:1	0	6.2
24:1	0	0.4
18:2 ($n-6$)	13.6	22.6
18:3 ($n-6$)	0.6	0
20:4 ($n-6$)	2.4	0.3
18:3 ($n-3$)	3.4	2.4
18:4 ($n-3$)	0.4	2.4
20:5 ($n-3$)	7.7	7.3
22:5 ($n-3$)	0	0.6
22:6 ($n-3$)	0	6.6
$\sum$SFA	34.7	25.0
$\sum$MUFA	37.2	33.0
$\sum$PUFA	28.1	42.1
$n-3$	11.5	19.3
$n-6$	16.6	22.8
$n-6/n-3$	1.4	1.2
Net energy[c]	15.1	20.0

[a] Per cent dry matter

[b] Per cent total fatty acid fraction

[c] MJ kg^{-1} in dry matter

day^{-1}) and survival rate ($S = 65.3$–76.5 %) of medium and large juveniles. A higher cannibalism rate was found in large and medium juveniles ($C = 7.5 \pm 2.5$ % and 6.5 ± 2.5 %) compared to small ones ($C = 3.0 \pm 0.75$ %) during weaning (Table 3). No difference in growth, survival, and cannibalism rate was observed between small and medium juveniles or between medium and large groups during the post-weaning period. A difference in growth, survival, and cannibalism rate was found only between small and large juveniles (Table 4). Fulton's condition coefficient was similar in all groups during both weaning and post-weaning periods. A higher SGR of pikeperch juveniles was found in

Table 3 Effect of initial fish size and density on growth, survival, and cannibalism in pikeperch *Sander lucioperca* L. juveniles during the weaning period

Parameter	Experiment 1			Experiment 2			
	S	*M*	*L*	*D*1	*D*2	*D*3	*D*4
Initial *W* (g)	0.42 ± 0.15^{a}	1.66 ± 0.4^{b}	2.95 ± 0.65^{c}	0.42 ± 0.15^{a}	0.42 ± 0.15^{a}	0.42 ± 0.15^{a}	0.42 ± 0.15^{a}
Initial TL (mm)	40.3 ± 2.3^{a}	56.2 ± 2.7^{b}	71.0 ± 3.2^{c}	40.3 ± 2.3^{a}	40.3 ± 2.3^{a}	40.3 ± 2.3^{a}	40.3 ± 2.3^{a}
Final *W* (g)	0.51 ± 0.10^{a}	1.76 ± 0.34^{b}	3.05 ± 0.55^{c}	0.56 ± 0.12^{a}	0.57 ± 0.15^{a}	0.61 ± 0.13^{a}	0.58 ± 0.15^{a}
Final TL (mm)	42.5 ± 2.1^{a}	58.3 ± 2.5^{b}	73.5 ± 3.1^{c}	43.1 ± 2.5^{a}	43.8 ± 2.4^{a}	45.2 ± 3.0^{a}	44.2 ± 2.2^{a}
SGR (% day^{-1})	1.6 ± 0.2^{b}	0.5 ± 0.05^{a}	0.3 ± 0.02^{a}	2.4 ± 0.2^{a}	2.5 ± 0.3^{a}	3.1 ± 0.3^{a}	2.7 ± 0.3^{a}
Final FC	0.7 ± 0.05^{a}	0.9 ± 0.1^{a}	0.8 ± 0.06^{a}	0.7 ± 0.04^{a}	0.7 ± 0.05^{a}	0.7 ± 0.07^{a}	0.7 ± 0.05^{a}
S (%)	81.7 ± 2.7^{c}	76.5 ± 3.5^{b}	65.3 ± 3.2^{a}	38.9 ± 2.7^{a}	57.3 ± 3.5^{b}	72.0 ± 2.2^{c}	79.1 ± 2.1^{c}
C (%)	3.0 ± 0.75^{a}	6.5 ± 2.5^{b}	7.5 ± 2.5^{b}	4.5 ± 1.5^{a}	3.0 ± 0.8^{a}	3.0 ± 0.75^{a}	2.5 ± 0.5^{a}

Within a column in experiment 1 and 2, values without a letter in common are significantly different ($P < 0.05$) among treatments

Table 4 Effect of initial fish size and density on growth, survival, and cannibalism in pikeperch (*Sander lucioperca* L.) juveniles during the post-weaning period

Parameter	Experiment 1			Experiment 2			
	S	*M*	*L*	*D*1	*D*2	*D*3	*D*4
Initial *W* (g)	0.51 ± 0.10^{a}	1.76 ± 0.34^{b}	3.05 ± 0.55^{c}	0.56 ± 0.12^{a}	0.57 ± 0.15^{a}	0.61 ± 0.13^{a}	0.58 ± 0.15^{a}
Initial TL (mm)	42.5 ± 2.1^{a}	58.3 ± 2.5^{b}	73.5 ± 3.1^{c}	43.1 ± 2.5^{a}	43.8 ± 2.4^{a}	45.2 ± 3.0^{a}	44.2 ± 2.2^{a}
Final *W* (g)	1.68 ± 0.3^{a}	3.2 ± 0.3^{b}	4.8 ± 0.5^{c}	1.38 ± 0.5^{a}	1.42 ± 0.4^{a}	1.44 ± 0.4^{a}	1.4 ± 0.4^{a}
Final TL (mm)	58.3 ± 2.5^{a}	77.2 ± 3.7^{b}	85.2 ± 3.8^{c}	54.2 ± 2.7^{a}	58.2 ± 2.4^{a}	59.1 ± 2.8^{a}	57.9 ± 2.1^{a}
SGR (% day^{-1})	7.5 ± 0.8^{b}	4.9 ± 0.2^{ab}	3.8 ± 0.3^{a}	7.5 ± 0.4^{a}	7.6 ± 0.4^{a}	7.2 ± 0.5^{a}	7.3 ± 0.4^{a}
Final FC	0.8 ± 0.04^{a}	0.7 ± 0.05^{a}	0.8 ± 0.06^{a}	0.9 ± 0.05^{a}	0.7 ± 0.04^{a}	0.7 ± 0.04^{a}	0.7 ± 0.05^{a}
S (%)	96.0 ± 2.5^{b}	90.3 ± 3.5^{ab}	86.1 ± 3.7^{a}	82.1 ± 3.0^{a}	84.7 ± 2.7^{a}	91.4 ± 2.0^{b}	96.5 ± 1.9^{b}
C (%)	2.0 ± 0.5^{a}	4.7 ± 2.0^{ab}	6.0 ± 2.2^{b}	2.5 ± 1.0^{a}	2.0 ± 0.75^{a}	1.5 ± 0.5^{a}	1.5 ± 0.5^{a}

Within a column in experiment 1 and 2, values without a letter in common are significantly different ($P < 0.05$) among treatments

the post-weaning period compared to the weaning period. No body or fin deformity of weaned juveniles was found in any group during both experiments.

The effect of initial fish density on the weaning success

No differences in growth, fish condition, and cannibalism rate were found among the initial fish density groups. However, significant differences in survival rate were observed. The lowest survival rate ($S = 38.9 \pm 2.7$ %) was seen at the lowest stocking density ($D1$), and the highest survival rates were evident at higher densities ($D3 = 72.0 \pm 2.2$ % and $D4 = 79.1 \pm 2.1$ %) after the weaning period (Table 3). Statistically similar fish condition, growth, and cannibalism rates were recorded for all fish densities after the post-weaning period. Higher survival rate (91.4–96.5 %) was found in the two higher fish densities ($D3$ and $D4$) compared to densities $D1$ and $D2$ (82.1 and 84.7 %, respectively) (Table 4). No body or fin deformities of weaned juveniles were found.

Verification of experimental results by mass weaning

A high survival rate was found after the weaning and post-weaning periods (78.7 ± 3.0 % and 97.6 ± 1.0 %, respectively) of the mass juvenile weaning under fish farm conditions. Cannibalism rate was 4.0 ± 1.5 % and 2.5 ± 1.0 % after the weaning and post-weaning periods, respectively. Lower SGR (1.4 ± 0.1 % day^{-1}) was found during the weaning period and higher SGR (7.2 ± 0.2 % day^{-1}) during the post-weaning period. Fulton's condition coefficient after weaning was lower (FC = 0.6 ± 0.15) compared to FC 0.8 ± 0.2 after the post-weaning period. No body or fin deformities of weaned juveniles were observed during this part of our study. These results verified experimental results indicating that small pikeperch juveniles under higher fish density show acceptable growth and survival during and after weaning (Table 5).

Discussion

The transition of pond-cultured pikeperch juveniles to controlled conditions and artificial food is a critical aspect for increasing efficiency of pikeperch culture (Zakes 1999; Ljunggren et al. 2003). Pond culture of pikeperch juveniles provides high-quality fish without deformities at low production cost (Policar et al. 2011). However, this system has limitations regarding fish survival and growth rate, which are dependent on factors including weather conditions, water quality and temperature, natural food supply, cannibalism, and predators (Hilge and Steffens 1996; Zakes 1999; Bodis and Bercsenyi 2009). Zakes and Demska-Zakes (1998) reported higher growth rate of intensively reared juveniles fed artificial food compared to those fed zooplankton. Weaning of pond-cultured juveniles to commercial feed has been developed in Europe over the past 15 years as an effective tool for increasing fish supply and diversification of European intensive aquaculture. Most studies have shown low survival rates of 42.2–47.5 % in weaned pikeperch juveniles after transition (Skudlarek and Zakes 2002; Molnar et al. 2004), while Zakes (1999) obtained survival rates of 62.5–82.0 % in juveniles with initial TL = 39.4 mm and $W = 0.53$ g. These results are similar to those of the present study, which were verified by mass weaning.

A successful weaning protocol for pond-cultured percids requires acceptable first food. In general, first food for optimal transition of percids from natural to artificial food should

Table 5 Growth, survival, and cannibalism with mass weaning of juvenile pikeperch *Sander lucioperca* L.

Period	Initial *W* (g)	Initial TL (mm)	Final *W* (g)	Final TL (mm)	SGR (% day^{-1})	Final FC	*S* (%)	*C* (%)
Weaning	0.55 ± 0.1	42.4 ± 2.4	0.65 ± 0.15	48.5 ± 3.5	1.4 ± 0.1	0.6 ± 0.15	78.7 ± 3.0	4.0 ± 1.5
Post-weaning	0.65 ± 0.15	48.5 ± 3.5	1.54 ± 0.35	59.0 ± 4.0	7.2 ± 0.2	0.8 ± 0.2	97.6 ± 2.0	2.5 ± 1.0

be non-moving, flavourful, and attractive to weaned fish (Policar et al. 2009). Ljunggren et al. (2003) successfully used agglomerated marine larvae feed for weaning of pond-cultured juvenile pikeperch without any supplemental feed. Previous studies of pikeperch weaning have employed *Tubifex* (Molnar et al. 2004), *Neomysis integer*, *Daphnia* sp., chironomid larvae and yolk (Ljunggren et al. 2003), and *Daphnia magna* or *Moina branchiata* (Zakes 1999). Wedekind (2008) suggested the use of frozen chironomid larvae as the most effective transition food for weaning of pond-cultured pikeperch juveniles.

The success of weaning in percids is also dependent on factors such as water temperature (Zakes 1997b, 1999) in addition to initial quality, size, and stocking density of the pond-cultured juveniles (Zakes1997a; Policar et al. 2009). The present study as well as Zakes (1997b, 1999) showed optimal water temperature for weaning pikeperch juveniles to be 22 °C. Only high-quality, healthy, and undamaged pond-cultured juveniles should be used for weaning. Fish should be harvested with care in outlet channel below the pond during favourable weather conditions such as cloudy or rainy with maximum air temperature 18–20 °C (Policar et al. 2011).

Prior to the present study, trials of weaning success in pikeperch used low initial fish density (1.37–3.5 pikeperch juveniles l^{-1} or 0.60–2.65 g l^{-1}) and limited initial fish size (body weight = 0.25 ± 0.06; 0.53 ± 0.06; 0.65 ± 0.11 and 0.91 ± 0.04 g) (Zakes and Demska-Zakes 1996; Zakes 1997a, 1997b, 1999; Szkudlarek and Zakes 2002; Molnar et al. 2004). This is the first investigation of impact of initial size and stocking density.

The highest survival rate during the weaning period was observed in small juveniles (81.7 %) at the highest stocking density (79.1 %). These results were verified by the mass weaning trial, which resulted in a survival rate (78.7 %) similar to the previous experiments. We recommend the use of small pond-cultured pikeperch juveniles (TL = 40–42 mm and W = 0.42–0.55 g) reared in ponds for 4–6 weeks for the weaning procedure. For weaning and intensive aquaculture older and bigger pikeperch juveniles (TL = 110–130 mm and W = 6.5–16 g) harvested from ponds during autumn may not be suitable. Low survival rate (25–30 %) was observed during weaning of these older pikeperch juveniles in our unpublished study.

Generally, lower survival rate was found during the weaning (38.9–81.7 %) compared to the post-weaning period (82.1–96.5 %) in all tested groups. Lower mortality of juveniles during post-weaning period confirmed adaptation of weaned fish to artificial feed and controlled rearing conditions.

An acceptable rate of cannibalism was observed during the weaning (3.0–7.5 %) and post-weaning (1.5–6.0 %) periods for all parts of the study. Zakes (1999), Szkudlarek and Zakes (2002), and Molnar et al. (2004) reported higher cannibalism during and after weaning of pond-cultured pikeperch juveniles (6.25–41.7 %) compared to our study. Precise size grading of juveniles at the beginning of the study and the removing of cannibal specimens from each group after weaning reduced cannibalism. Size grading of perch juveniles has been used to control cannibalism during intensive culture (Melard et al. 1996; Wallat et al. 2005). Grading of perch has not been shown to increase production efficiency of intensively reared perch (Melard et al. 1996). During the weaning and post-weaning periods, small juveniles were associated with lower cannibalism than were medium and large juveniles. Zakes (1999) did not find differences of cannibalism rate in small and large fish fed artificial food and reared at 22 and 24 °C, respectively. However, the cannibalism rate in that study was significantly higher (15.5–16.6 %) than in the present experiment. Initial stocking density was not shown to affect the cannibalism rate during the weaning and post-weaning period of the present study. Szkudlarek and Zakes (2002) and Molnar

et al. (2004) also did not find an effect of initial fish density on cannibalism during and after weaning.

An effect of initial fish size on growth rate was confirmed during the weaning and the post-weaning periods in the present study. Small fish demonstrated higher SGR compared to medium and large fish. This confirmed higher growth and better adaptation of smaller fish to new conditions. Generally, our results showed lower SGR during the weaning period compared to SGR in the post-weaning period. Slower growth rate during weaning can be caused by the adaptation of fish to new food and rearing conditions.

No differences in condition (FC) and no body or fin deformities were observed in any group during the study. Kestemont et al. (2007) reported deformities in intensively reared pikeperch larvae after weaning. Fin deformity and damage in perch juvenile culture under RAS was described by Stejskal et al. (2011). The condition of weaned pikeperch juveniles was consistently good without any morphological abnormalities during the present study.

Conclusion

The weaning of small pond-cultured pikeperch juveniles (TL = 40–42 mm and $W = 0.42$–0.55 g) at a density of 8 fish per litre can ensure effective production of pikeperch juveniles for ongrowing in European intensive pikeperch farms.

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Fin damage and growth parameters relative to stocking density and feeding method in intensively cultured European perch (*Perca fluviatilis L.*)

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Abstract

We evaluated the relationship of stocking density to survival, growth performance and fin condition of European perch *Perca fluviatilis* with hand feeding and self-feeders. Hand-fed perch (body weight 19.1 ± 5.1 g and total length 107 ± 9 mm) were reared at 0.5, 1.0, 1.5 and 2.0 fish/L. Self-feeding perch (body weight 25.4 ± 3.9 g and total length 128 ± 7 mm) were reared at stocking densities of 0.6, 1.0 and 1.4 fish/L. Pond-reared perch served as a comparison group for fin damage assessment. We found no differences in survival rate among stocking densities with either feeding method. Hand-fed fish displayed the highest weight gain and SGR at stocking density of 0.5 fish/L. The self-feeding fish showed a non-linear association of weight gain with stocking density with the highest growth at 1.0 fish/L. Fin length was noticeably greater in pond-reared fish compared with RAS-reared fish regardless of feeding method. In both experiments, fin length relative to standard length showed a negative relationship with stocking density, with pectoral fins showing the greatest effect. Fin condition deteriorated with increasing stocking density, and growth was highest at 0.5 and 1.0 fish/L in hand-fed and self-feeding fish, respectively.

KEYWORDS
fin erosion, intensive culture, *Perca fluviatilis*, self-feeders, stocking density, welfare

1 | INTRODUCTION

Intensive culture of fish requires high production and stocking density in order to recover capital investment and operational costs (Schneider, Blancheton, Varadi, Eding, & Verreth, 2006). The issue of fish welfare in intensive commercial production systems has gained increasing attention by government authorities and fish farmers as well as consumers in recent decades (Ashley, 2007; Ellingsen et al., 2015; Huntingford et al., 2006; Martins et al., 2012). High stocking density may trigger issues of fish welfare (Martins et al., 2005; Stejskal et al., 2018), yet no limits or criteria regarding crowding in farmed fish have been established (North et al., 2006).

Based on meta-analysis of 43 studies focusing on the welfare of farmed rainbow trout *Oncorhynchus mykiss*, Ellis et al. (2002) reported a wide range of proxies used to document fish welfare (growth, survival, condition factor, feed conversion ratio, coefficient of weight variation, haematocrit, fin condition) and concluded that inappropriate stocking density has a negative impact on fish welfare. High-density culture of rainbow trout showed adverse effects on growth, nutritional condition, feed conversion ratio and fin condition (Bosakowski & Wagner, 1994a, 1994b; Wagner, Intelmann, & Routledge, 1996).

Fin damage at high culture density has been reported for Atlantic salmon *Salmon salar* (Noble, Mizusawa, Suzuki, & Tabata, 2007; Pelis & McCormick, 2003), walleye *Sander vitreum* (Clayton, Stevenson,

& Summerfelt, 1998) and pikeperch *Sander lucioperca* (Policar et al., 2016). Fin damage/erosion is considered an indicator of decreased fish welfare (Ellis et al., 2009; North et al., 2006) and has implications for the economics of fish farming (Hoyle et al., 2007). Fin damage or absence of fins could negatively impact acceptance by consumers when fish are sold whole, gutted or live, and fish with damaged fins are confirmed to have lower economic value than those with intact fins (Hoyle et al., 2007).

The major sources of fin erosion include abrasion by rough surfaces, nutritional deficiencies (Lellis & Barrows, 2000), bacterial infection and aggressive interactions among fish (Hoyle et al., 2007; Latremouille, 2003). While the first mentioned sources can be mitigated with good husbandry practices, the aggressive behaviour of a fish species is a complex phenomenon dependent on multiple factors including stocking density (Ellis et al., 2002) and feeding strategy (Adams, Turnbull, Bell, Bron, & Huntingford, 2007; Azzaydi, Madrid, Zamora, Sánchez-Vázquez, & Martínez, 1998; Kaushik, 2000).

As fish feed represents 30%–50% of operational cost (Kaushik, 1990), the feed used and method of providing it are crucial to sustaining economic viability of intensive farming. Currently, three major strategies are available for feed distribution: hand feeding, automatic feeders and self-feeders. The main advantage of the self-feeder is the dispensing of a precise quantity of feed according to fish demands, which reduces losses through uneaten feed and costs of filtration (Stewart et al., 2012). Self-feeders have been demonstrated to improve growth performance and fish feed conversion ratio (Noble et al., 2007) as well as to decrease stress (Endo, Kumahara, Yoshida, & Tabata, 2002) and level of fin damage (Stewart et al., 2012). This suggests that intensive culture with self-feeders can employ higher stocking density without compromising fish welfare. However, no information on fin condition relative to stocking density and feeding method is currently available.

European perch *Perca fluviatilis* is currently considered a candidate for diversification of European aquaculture due to high market demand (Kestemont & Mélard, 2000; Stejskal, Kouril, Musil, Hamackova, & Policar, 2009; Watson, 2008) and their successful production in intensive recirculating aquaculture systems (Toner, 2015) with stocking densities to 60 kg/m^3 (Kestemont et al., 2003; Mélard, Kestemont, & Grignard, 1996). The European perch is in demand by consumers throughout Europe and can be supplied as fillets or whole fish (Tamazouzt, Dubois, & Fontaine, 1993). Therefore, research into fin erosion in intensively cultured European perch is crucial. Stejskal, Policar, Křišťan, Kouřil, and Hamáčková (2011) described higher fin erosion in fish reared in RAS compared with pond-cultured, but fin damage of European perch relative to stocking density and feeding methods has not been adequately explored (Stejskal et al., 2011).

The goal of the present study was to determine the effect of stocking density and feeding method on growth, survival and level of fin erosion in European perch cultured in RAS, as well as to compare fin damage in RAS with that of pond-cultured European perch. We hypothesized that fish reared with self-feeding systems can tolerate higher stocking density with acceptable growth parameters and less fin damage.

2 | MATERIALS AND METHODS

2.1 | Experiment I: Effect of stocking density with hand feeding

Newly hatched European perch larvae were stocked into experimental fish ponds and harvested after 150 days as autumn fry. Harvested juveniles (body weight 7.9 ± 4.1 g) were transferred into two 1000-L fibreglass tanks with a recirculation system for a 1-day adaptation phase, according to Policar et al. (2013), after which they were reared for 6 weeks under the same conditions before initiating the trial.

Experiment I was conducted in 12 glass aquaria (350 × 400 × 500 mm, water volume 50 L) in an RAS of total volume 2,970 L. A total of 600 specimens, weight 19.1 ± 5.1 g and total length 107 ± 9 mm, were separated into four groups of stocking densities 0.5 fish/L (H0.5), 1.0 fish/L (H1.0), 1.5 fish/L (H1.5) and 2.0 fish/L (H2.0) in triplicate with initial biomass of 9.5 kg/m^3, 19 kg/m^3, 28.5 kg/m^3 and 38 kg/m^3, respectively.

Commercial feed of pellet size 2.0 mm (Ecolife 60, BioMar) containing crude protein 47%, crude fat 14%, crude fibre 3% and total phosphorus 1.01% (manufacturer's data) was provided manually at 2-hr intervals (five feedings/day) from 08.00 to 16.00 hr with the ration according to Fiogbé & Kestemont (2003). Feed was preweighed, and fish were fed to satiation. Uneaten food was siphoned daily and counted to calculate feed intake. Duration of the trial was 112 days.

Twenty pond-reared European perch (body weight 52.1 ± 6.2 g, total length 161 ± 6 mm) obtained from the fish farm Rybářství Nové Hrady s.r.o., Czech Republic, served as a comparison group for fin damage assessment (Stejskal et al., 2011). The pond perch were reared extensively in polyculture with common carp *Cyprinus carpio* with no commercial feed provided.

2.2 | Experiment II: Effect of stocking density under self-feeding feeding regime

Newly hatched larvae were stocked into experimental fish ponds as in Experiment I, and autumn fry was harvested after 170 days. Harvested juveniles (body weight 9.4 ± 4.0 g) were placed in four 700-L fibreglass tanks in a recirculation system for a 1-day habituation period according to Policar et al. (2013), after which they were reared for 6 weeks under the same conditions before initiating the trial. The trial (duration 63 days) was conducted in 12 plastic tanks (250 × 350 × 900 mm, water volume 60 L) in an RAS of 3,960 L total volume. A total of 720 perch (initial body weight 25.4 ± 3.9 g and total length 128 ± 7 mm) were divided into three groups with stocking densities of 0.6 L^{-1} (S0.6), 1.0 L^{-1} (S1.0) and 1.4 fish/L (S1.4) in four replicates with initial biomass of 14.5 kg/m^3, 25 kg/m^3 and 35.4 kg/m^3, respectively.

Fish were provided commercial feed of pellet size 1.9 mm (INICIO Plus, BioMar) containing crude protein 51%, crude fat 15%, crude fibre 2.4% and total phosphorus 1.1% (manufacturer's data) using a

24-hr self-feeding system with a string sensor for fish feed demand (IMETRONIC). Prior to the trial, fish were trained to operate the sensor over a 1-week period in which they were fed near the sensor by an automatic feeder. The self-feeders were adjusted to release 1 g of feed per demand, with the number of demands limited to one per min to avoid hedonic behaviour. Number of demands for feed was automatically recorded using POLY Files software (IMETRONIC). Mean daily food demand per fish was calculated using number of fish per tank. Feed demand (FD) was calculated as mean number of daily demands throughout the experiment (63 days). Uneaten food was siphoned daily and counted to calculate feed intake.

Thirty pond-reared European perch (body weight 47.5 ± 7.2 g and total length 158 ± 6 mm) obtained from the fish farm Školní rybářství Protivín s.r.o. were used for comparison of fin damage. The pond perch were reared extensively in polyculture with common carp *Cyprinus carpio* with no commercial feed provided.

2.3 | Culture conditions

In both experiments, water temperature, pH and dissolved oxygen were monitored at 08.00 and 15.00 hr daily with a multimeter HQ40d (Hach Lange) (Table 1). Ammonia, nitrate and nitrite concentrations were analysed at 3-day intervals with kits (HACH, LCK 304, LCK 339, LCK 341) using a HACH DR 2,800 portable spectrophotometer (Hach Lange). Light intensity ranged from 100 to 140 lux at the water surface with a consistent 12L:12D photoperiod.

2.4 | Assessment of fin condition

At the conclusion of Experiments I (day 112) and II (day 63), 20 (12 tanks, n = 240) and 30 specimens per tank (12 tanks, n = 360), along with 20 and 30 pond-reared fish, were sampled, respectively. Fish were mildly anaesthetized using clove oil (0.3 ml/L) and weighed (OHAUS Explorer EX224M, NJ, USA) to the nearest 0.1 mg. Anaesthetized fish were photographed in the left and right lateral and ventral views using a Canon DR 5,300 camera fixed to a tripod (Figure 1). Images were processed with in MicroImage v.4.0. (NIH) to measure total length, standard length, fin length, body height and body width.

TABLE 1 Water parameters

Parameter		Experiment 1	Experiment 2
Temperature	°C	22.7 ± 1.4	23.3 ± 1.1
Oxygen	mg/L	6.7 ± 1.6	6.1 ± 1.2
pH		6.8 ± 0.5	7.1 ± 0.6
Nitrite-N	mg/L	0.72 ± 0.41	0.62 ± 0.32
Nitrate-N	mg/L	58.8 ± 27.3	38.8 ± 17.4
Ammonia-N	mg/L	1.47 ± 0.92	1.07 ± 0.83

Note: Data are expressed as mean ± standard deviation.

2.5 | Calculations and statistical analysis

Data were used to calculate the following variables:

$$\text{Survival}, S(\%) = (N_F - N_I / N_F) \times 100$$

$$\text{Specific growth rate}(\%), \text{SGR} = \left[(\ln B_F - \ln B_I) \times d^{-1}\right] \times 100$$

$$\text{Weight gain}, \text{WG} = W_F - W_I$$

$$\text{Coefficient of weight variation}, \text{CV} = (SD / W_M) \times 100$$

$$\text{Fulton's condition factor}, K = (\text{W}/\text{TL}^3) \times 100$$

$$\text{Feed conversion ratio}, \text{FCR} = \text{F}/(\text{W}_F - \text{W}_I)$$

$$\text{Body height index}, \text{BHI} = \text{TL} \times \text{H}^{-1}$$

$$\text{Body width index BWI} = \text{TL} \times \text{WD}^{-1}$$

where N_I is the initial number of fish; N_F is the number of fish at the end of the experiment; B_I is the initial fish biomass; B_F is the fish biomass at the end of the experiment; $\ln B_F$ is natural logarithm for final body weight; $\ln B_I$ is natural logarithm for initial body weight; SD is standard deviation of fish subsample; W_M is the mean body weight of fish in a given tank; TL is total length; H is body height; WD is body width; d is duration of the experiment in days; F is the weight of consumed feed; and WG is weight gain. Initial relative lengths of fins for both experiments are presented in Table 2.

Relative length (RL) of the first dorsal, second dorsal, caudal, anal, pectoral and pelvic fins was calculated using the following formula:

$$\text{Fin relative length}, \text{RL} = \text{TL}_F \times \text{SL}^{-1}$$

where FL is the total fin length (mm) and SL is the standard length of the specimen (mm).

The sum of the total length of all fins to standard body length ratio (TRFL) was calculated as an indicator of fin damage using the formula.

$$\text{TRFL} = (\text{FL}_{RPF} + \text{FL}_{LPF} + \text{FL}_{RVF} + \text{FL}_{LVF} + \text{FL}_{SDF} + \text{FL}_{FDF} + \text{FL}_{CF} + \text{FL}_{AF}) \times \text{SL}^{-1}$$

where FL_{RPF} is length of the right pectoral fin, FL_{LPF} is length of the left pectoral fin, FL_{RVF} is length of the right ventral fin, FL_{LVF} is length of the left ventral fin, FL_{SDF} is length of the second dorsal fin, FL_{FDF} is length of the first dorsal fin, FL_{CF} is length of the caudal fin, and FL_{AF} is length of the anal fin.

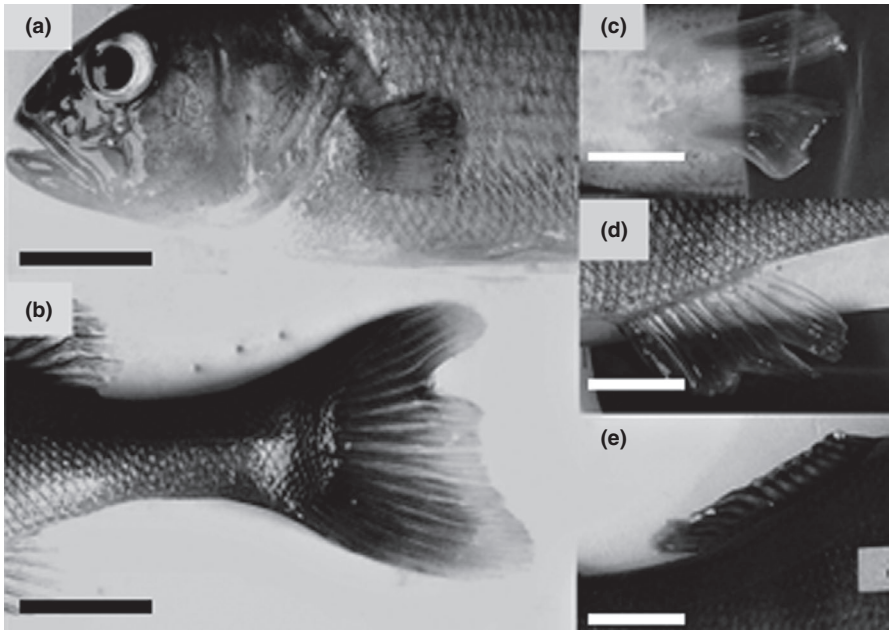

FIGURE 1 Fin damage in European perch: pectoral (a), caudal (b), ventral (c), anal (d) and second dorsal fins (e). Scale bar represents 10 mm

TABLE 2 Initial relative length of right pectoral fin (RL_{RPF}), left pectoral fin (RL_{LPF}), right ventral fin (RL_{RVF}), left ventral fin (RL_{LVF}), second dorsal fin (RL_{SDF}), first dorsal fin (RL_{FDF}), caudal fin (RL_{CF}) and anal fin (RL_{AF}) expressed as relative fin length

	RL_{RPF}	RL_{LPF}	RL_{RVF}	RL_{LVF}	RL_{FDF}	RL_{SDF}	RL_{CF}	RL_{AF}
Exp. I	0.16 ± 0.02	0.16 ± 0.02	0.21 ± 0.03	0.21 ± 0.03	0.19 ± 0.04	0.14 ± 0.02	0.15 ± 0.02	0.12 ± 0.03
Exp. II	0.17 ± 0.02	0.15 ± 0.03	0.20 ± 0.02	0.20 ± 0.03	0.18 ± 0.04	0.12 ± 0.02	0.14 ± 0.02	0.10 ± 0.03

Data were analysed using STATISTICA v.10.1 for Windows (StatSoft). Prior to analyses, all measured variables were checked for normality and homoscedasticity of variance (Cochran, Hartley, Bartlett test). One-way ANOVA was used to analyse differences in measured variables among experimental groups. When a difference was detected ($p < .05$), Tukey's multiple range test was applied. Arcsine transformation was used for relative fin lengths and TRLF.

3 | RESULTS

3.1 | Experiment I

In Experiment I, survival rates were >86% in all groups with no significant ($p = .148$) differences among stocking densities (Table 3). Final mean body weight was significantly ($p < .001$) higher in H0.5 than in other tested groups, reflecting higher SGR (Table 3), with no differences among other groups (Figure 2). The highest TRFL was found in pond-reared fish. Total relative fin length decreased ($p < .001$) with increased stocking density, with significantly higher TRFL in H0.5 compared with H1.5 and H2.0 (Figure 2).

No significant differences in CV, BHI or BWI were found among test groups (Table 3). The highest FCR was observed in groups H0.5 and H1.0. The K was highest in H0.5 and significantly differed from H1.5 and H2.0.

No obvious fin damage was observed in the pond-reared fish. The relative length of pectoral, ventral, second dorsal, caudal and anal fins was found to be lower in RAS-reared fish compared with pond-reared, regardless of stocking density (Figures 2 and 3) means that those fins were most damaged in intensive conditions.

Comparisons of relative fin length revealed no significant differences among RAS groups in right ($p = .330$) and left ventral ($p = .507$), first dorsal ($p = .232$) or caudal fins (Figure 3). However, a clear trend ($p < .001$) of decreasing relative length of pectoral fins with increased stocking density was observed. The relative length of the anal fin showed the highest ($p < .001$) values in H 1.5 and H 2.0 and lowest in H 0.5 and H 1.0 (Figure 3). Moreover, relative length of the anal fin showed the highest ($p < .002$) values in H 0.5 and H 1.5.

3.2 | Experiment II

Survival rate ranged from 89.7% to 93.2% and did not differ among groups (Table 4). Mean final body weight was significantly ($p < .001$) higher in S1.0 than in the other two groups, and highest TRFL ($p = .002$) was seen in S0.6 (Figure 4). Significantly lower mean final body weight and TRFL were found at the highest stocking density, S1.4.

The significantly higher SGR and FD were observed in S1.0 compared with S0.6 and S1.4. Significantly lower WG and CV were

TABLE 3 Effect of stocking density on production parameters of hand-fed European perch 1

	Stocking density (fish/L)					
Parameter	**0.5**	**1.0**	**1.5**	**2.0**	***F***	***p***
S (%)	91.7 ± 2.4	94.2 ± 4.2	90.0 ± 3.6	86.3 ± 1.0	2.35	.149
CV (%)	30.7 ± 8.2	24.9 ± 2.8	26.0 ± 0.3	25.7 ± 3.1	0.96	.458
SGR (%)	0.85 ± 0.02^{a}	0.56 ± 0.03^{b}	0.47 ± 0.03^{bc}	0.46 ± 0.03^{c}	124.99	<.001
WG (g)	29.4 ± 1.0^{a}	16.2 ± 1.2^{b}	13.0 ± 1.1^{c}	12.7 ± 1.0^{c}	158.65	<.001
FCR	0.9 ± 0.1^{a}	1.1 ± 0.1^{a}	1.5 ± 0.1^{b}	1.7 ± 0.2^{b}	23.91	<.001
K	1.18 ± 0.03^{a}	1.11 ± 0.03^{ab}	1.10 ± 0.03^{b}	1.09 ± 0.02^{b}	7.41	.011
BHI	4.15 ± 0.19	4.27 ± 0.15	4.36 ± 0.18	4.39 ± 0.20	1.04	.427
BWI	7.53 ± 0.61	7.67 ± 0.32	7.90 ± 0.25	7.83 ± 0.34	0.51	.684

Note: Data are expressed as mean ± standard deviation ($n = 3$). Different superscripts within a row indicate significant differences ($p > .05$, ANOVA).
Abbreviations: BHI, body height index; BWI, body width index; CV, coefficient of weight variation; FCR, feed conversion ratio; K, Fulton's condition factor; S, final survival; SGR, specific growth rate; WG, weigh gain.

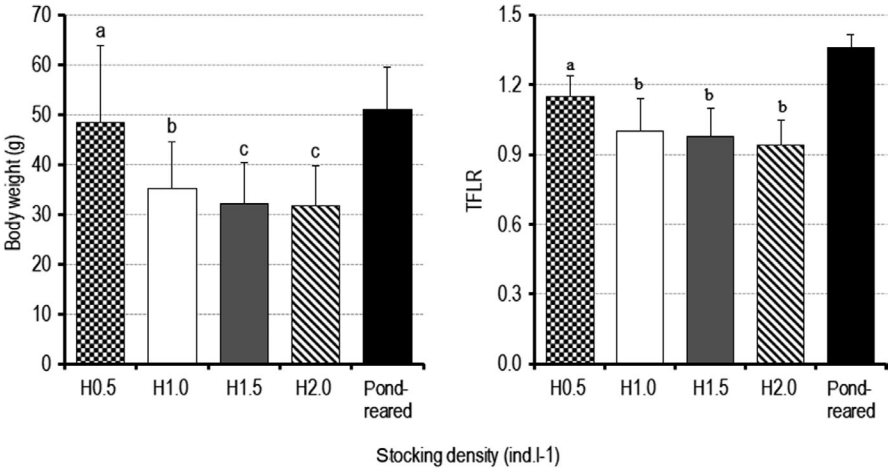

FIGURE 2 Effect of stocking density on final body weight and total fin length/standard length (TRFL) of European perch with hand feeding. Fish were reared at 0.5 (H0.5), 1.0 (H1.0), 1.5 (H1.5) and 2.0 fish/L (H2.0). Data are expressed as mean ± standard deviation ($n = 60$; $n = 20$ for pond-reared). Columns with different superscripts differ significantly ($p > .05$, ANOVA)

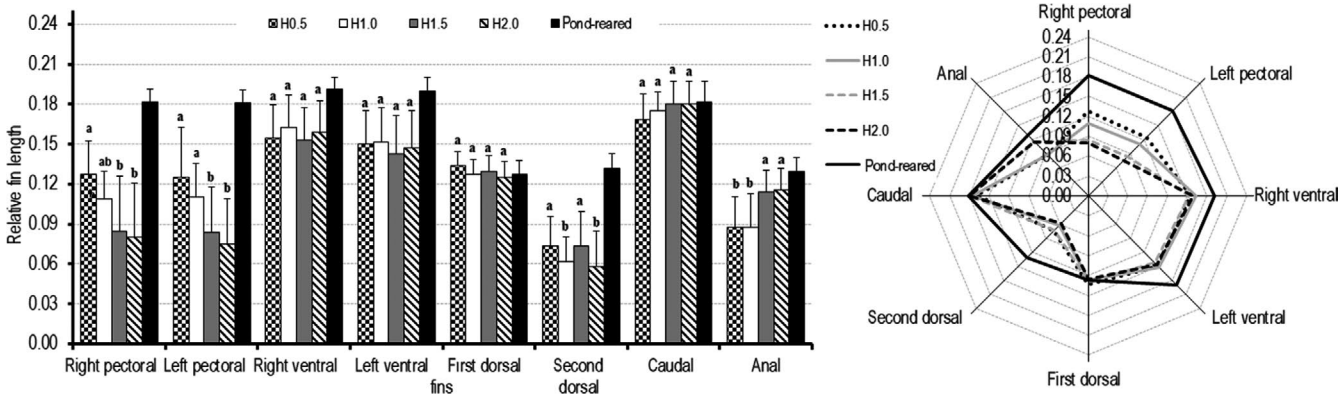

FIGURE 3 Effect of stocking density on relative fin length. Right pectoral fin (RPF), left pectoral fin (LPF), right ventral fin (RVF), left ventral fin (LVF), second dorsal fin (SDF), first dorsal fin (FDF), caudal fin (CF) and anal fin (AF) with hand-feeding and pond-reared controls. Data are expressed as mean ± standard deviation ($n = 60$). Bars with the same superscript do not differ significantly ($p > .05$, ANOVA)

observed in S1.4. No significant difference in K was found among test groups (Table 4).

In Experiment 2, relative fin length was generally lower in RAS-reared perch than in pond-reared fish (Figure 5). Significant differences ($p < .001$) were found only in right and left pectoral fins, with a clear trend of decreased relative length with increased stocking density. Similarly to Experiment 1, pectoral, ventral and caudal fins were most damaged in intensive conditions.

Parameter	Stocking density (fish/L) 0.6	1.0	1.4	*F*	*p*
S (%)	92.4 ± 2.7	93.3 ± 3.0	89.3 ± 2.2	2.54	NS
CV (%)	26.7 ± 2.2[a]	28.6 ± 7.1[a]	19.1 ± 2.9[b]	6.42	.018
SGR (%)	0.91 ± 0.21[b]	1.36 ± 0.06[a]	0.47 ± 0.12[c]	39.74	<.001
WG (g)	20.3 ± 6.1[b]	34.1 ± 1.9[a]	8.6 ± 2.4[c]	44.66	<.001
FCR	0.98 ± 0.10[b]	0.96 ± 0.07[b]	1.22 ± 0.16[a]	6.37	.019
K	1.15 ± 0.05[b]	1.24 ± 0.02[a]	1.06 ± 0.03[c]	63.12	<.001
FD	0.20 ± 0.04[b]	0.41 ± 0.13[a]	0.22 ± 0.08[b]	8.80	.008

Note: Different superscripts within a row indicate significant differences ($p > .05$., ANOVA). FD is mean number of demands for feed per fish, per day.

Abbreviations: CV, coefficient of weight variation; FCR, feed conversion ratio; K, Fulton's condition factor; S, final survival; SGR, specific growth rate; WG, weight gain.

TABLE 4 Effect of stocking density on production parameters of self-feeding European perch. Data are expressed as mean ± standard deviation ($n = 3$)

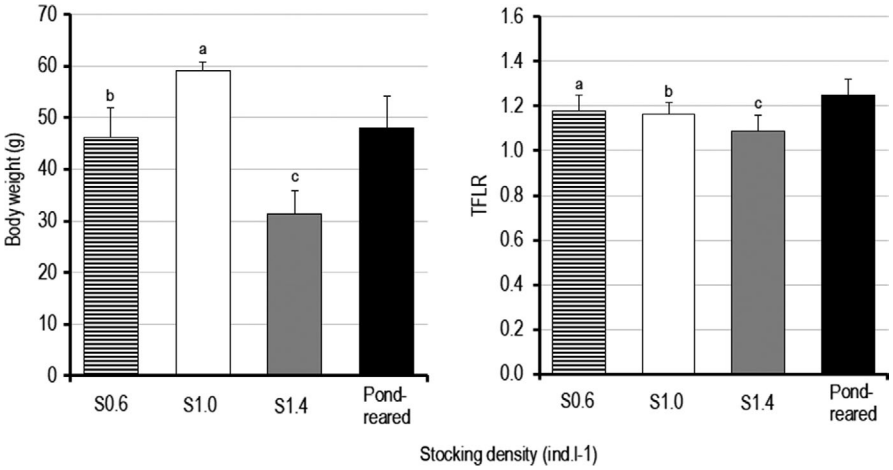

FIGURE 4 Effect of stocking density on final body weight and total fin length/standard length ratio (TRFL) of European perch in a self-feeding system and pond-reared. Fish reared at 0.6 (S0.6), 1.0 (S1.0) and 1.4 fish/L (S1.4). Data are expressed as mean ± standard deviation ($n = 120$ for S0.6, S1.0, S1.4, $n = 30$ for pond-reared). Bars with the same superscript do not differ significantly ($p > .05$, ANOVA)

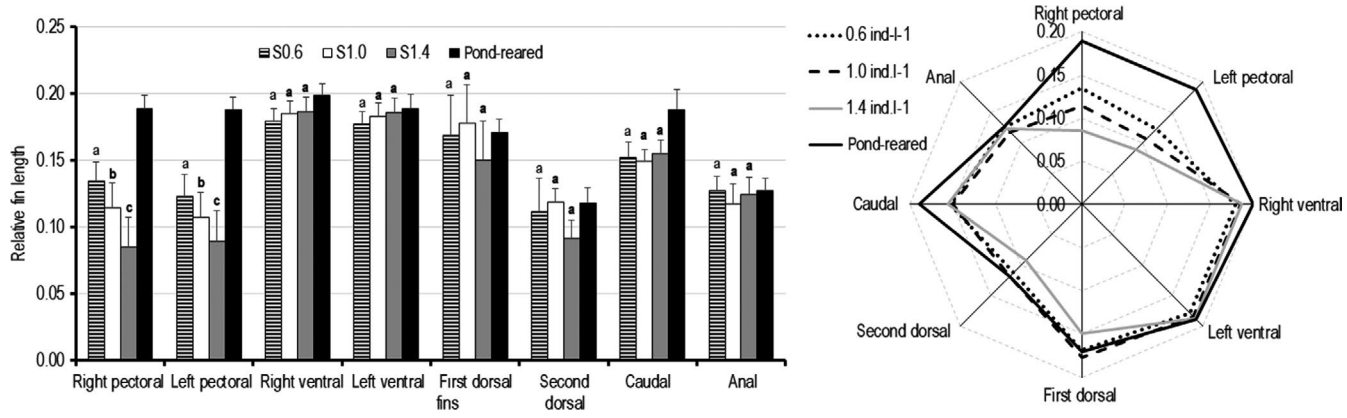

FIGURE 5 Effect of stocking density on relative fin length of the right pectoral fin (RPF), left pectoral fin (LPF), right ventral fin (RVF), left ventral fin (LVF), second dorsal fin (SDF), first dorsal fin (FDF) and anal fin (AF) in a self-feeding system. Data are expressed as mean ± standard deviation ($n = 120$). Bars with the same superscript do not differ significantly ($p > .05$, ANOVA)

4 | DISCUSSION

Fish welfare is an important issue in intensive aquaculture (Stubbe Solgaard & Yang, 2011). Rearing fish at high stocking densities may compromise fish welfare and decrease survival and growth rates (Ellis et al., 2002; Sirakov & Ivancheva, 2008; Stejskal et al., 2018; Stejskal, Policar, Křišťan, Kouřil, & Hamáčková, 2016; Yarahmadi, Miandare, Hoseinifar, Gheysvandi, & Akbarzadeh, 2015) and exacerbate fin erosion due to aggressive encounters, competition for food (Calabrese et al., 2017; Huntingford & Adams, 2005; Latremouille, 2003), water quality deterioration (Ellis et al., 2002) and bacterial

infections (Ellis et al., 2008; Turnbull, Richards, & Robertson, 1996). Fin erosion also has an impact on consumer perception of farmed products at the fish market (Cooke, 2001; Wall, 2001). Previous studies have documented that rearing fish with appropriate stocking density and feeding method can significantly improve survival and growth rates and decrease the level of fin erosion (Alanärä & Brännäs, 1997; Noble et al., 2007; Rubio et al., 2004).

Research has documented stocking density and survival rate to be inversely related (Baskerville-Bridges & Kling, 2000; Sirakov & Ivancheva, 2008). In contrast, we did not find differences in survival among our test groups with either feeding method. This is in agreement with studies of rainbow trout (Wallat, Tiu, Rapp, & Moore, 2004), sea bass *Dicentrarchus labrax* (Sammouth et al., 2009) and tiger grouper *Epinephelus fuscoguttatus* (Salari, Saad, Kamarudin, & Zokaeifar, 2012) and suggests that the densities used in our study did not trigger water quality deterioration or lethal bacterial infections. However, we observed significant differences in weight gain and SGR of European perch associated with stocking density (Tables 2 and 3). Hand-fed fish displayed the highest growth and highest SGR at 0.5 fish/L, the lowest tested density. The highest growth at lower stocking density was also found in hand-fed Nile tilapia *Oreochromis niloticus* (El-Sayed, 2002), Japanese flounder *Paralichthys olivaceus* (Bolasina, Tagawa, Yamashita, & Tanaka, 2006) and turbot *Scophthalmus maximus* (Irwin, O'Halloran, & FitzGerald, 1999), with the primary source of low growth at higher densities attributed to social/behavioural factors causing chronic stress with increasing plasma cortisol concentration in fish.

The self-feeding fish showed a non-linear relationship between stocking density and weight gain with the highest growth at 1.0 fish/L. This supports our hypothesis that self-feeding fish show tolerance to higher stocking densities. The lowest WG was observed at 1.4 fish/L. Lower weight gain at higher stocking densities was also reported in rainbow trout by Alanärä (1996) and Alanärä and Brännäs (1996) who identified reduced feeding activity at high stocking densities and suggested stress as a chief cause. This may also be the case in our study, as we documented lower numbers of feed demands (0.22 demand fish^{-1} day^{-1}) in S1.4 compared with other groups, suggesting that fish were not limited by the number of self-feeders nor by feed dose, but most likely suffered from stress due to crowding, triggering loss of appetite. It has been reported that broad dispersion of feed pellets may encourage feed intake and, consequently, increase growth rate with reduced size heterogeneity (Alanärä & Brännäs, 1996; Jørgensen, Baardvik, Eliassen, & Jobling, 1996). Reduction in feeding activity may be due to the establishment of dominance hierarchies, with dominant fish monopolizing available food sources and suppressing feeding in non-dominant fish (Boujard, Labbé, & Aupérin, 2002; McCarthy, Gair, & Houlihan, 1999). However, dominance hierarchies are frequently formed at lower stocking densities due to greater available territory (Alanärä & Brännäs, 1996; Jørgensen et al., 2007; Wallace, Kolbeinshavn, & Reinsnes, 1988). This is corroborated by our finding of lowest CV at 1.4 fish/L and the highest at 0.6 fish/L in self-feeders, as studies have suggested high CV as indicative of the establishment of hierarchies (Brett, 1979; Park, Renukdas, Luna, & Roy, 2015; Rowland, Mifsud, Nixon, & Boyd, 2006). This makes it unlikely that low growth rate at high density is the result of dominance hierarchy (Jørgensen et al., 2007). In the present study, CV of groups S0.6, S1.0 and S1.4 differed significantly. Hence, dominance hierarchies may not be the chief cause of lower growth at lowest and highest densities in self-feeding fish.

Intensive fish farming practice frequently exposes fish to a range of stressors such as sorting, transport, handling, vaccinations, crowding and starving (or inappropriate feeding), which do not exist for wild fish (Adams et al., 2007; Martins et al., 2012; North et al., 2006; Stewart et al., 2012; Wall, 2001; Yarahmadi et al., 2015). Currently, the range of cultured fish as well as culture systems is widely increased meaning that optimizing of feeding technique is difficult to precise for appropriate species, size category and culture conditions (Zhou, Xu, Lin, Sun, & Yang, 2018). However, the self-feeding systems served good option for farming practice in line with lower levels of some welfare indicators (Attia et al., 2012). Main advantage of such systems is that they allow fish to choose their feeding time and frequency, which is not ensured in other feeding systems (automatic feeders, hand feeding) and fed according to their biological rhythms (Benhaïm, Ferrari, Colchen, Chatain, & Bégout, 2017; López-Olmeda, Noble, & Sánchez-Vázquez, 2012).

Rearing fish in intensive aquaculture at high densities can contribute to fin erosion and, consequently, to fin length (Ellis et al., 2002; North et al., 2006). In our study, fin length was noticeably greater in the pond-reared group compared with RAS-reared fish regardless of feeding method. This conforms to results of Stejskal et al. (2011) who documented no fin damage or reduction in fin length in pond-reared European perch versus RAS-reared. Bosakowski and Wagner (1994a) reported 10%–50% shorter ray fins in hatchery-reared rainbow trout, cutthroat trout *Oncorhynchus clarkii* and brown trout *Salmo trutta* compared with captured wild fish.

In both experiments, we found reduction of TRFL with increased density (Figures 1 and 4), confirming a correlation between TRFL and stocking density regardless of feeding regime. According to Ellis et al. (2008), bacterial and fungal disease could be a cause of fin erosion and length reduction; however, we observed no visible signs on European perch fins in either experiment, and a high survival rate was recorded in all tested groups (Tables 2 and 3). Moreover, bacterial and fungal disease, as well as nutritional deficiency and abrasion by rough surfaces, would affect fin condition similarly in all tested groups, as all fish were reared in the same RAS. Therefore, it is likely that the fin damage was the result of more frequent fin nipping at high stocking density. Social/behavioural interactions due to stress (Baker & Ayles, 1990) when rearing at high stocking density have been reported as major sources of fin damage (Hoyle et al., 2007; Latremouille, 2003).

In both experiments, we observed a trend of decrease in relative length of pectoral fins with increasing stocking density. This agrees with results of Stejskal et al. (2011) who found intensively cultured European perch to exhibit up to 52% reduction in pectoral fin length. Therefore, reduction of pectoral fin length in both experiments

was attributed to high stocking density and consequent increased interaction among fish. In contrast, Bosakowski & Wagner, 1994a, 1994b documented that up to 46% of intensively cultured salmonids showed no damage to pectoral fins, but a damaged dorsal fin was present in 40%–74%, suggesting that fin erosion is species-specific. We observed difference in relative length of the dorsal fins only in the second dorsal fin of group H2.0. This result corroborates the study of Stejskal et al. (2011).

Our results showed that the rearing density and feeding method exert a significant impact on European perch growth and fin condition and confirmed our hypothesis that self-feeding systems can accommodate higher stocking density, possibly due to decreased stress. The level of fin erosion was considerably higher in RAS-reared groups versus pond-reared, with both pectoral fins affected. Results of the study can be used for further improvement of intensive European perch aquaculture.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Assessment of behavioural and physiological traits as indicators of suitability for European perch aquaculture

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ABSTRACT

Domestication and selective breeding can mitigate current bottlenecks in European perch aquaculture. Monitoring the condition and stress tolerance of European perch stocks from different recirculating aquaculture systems is of general interest to set the baseline values of promising candidates for further breeding processes. We recorded morphometric, behavioural (critical swimming speed, activity, aggressiveness, propensity to approach a novel object), and physiological parameters (plasma cortisol, glucose, ion concentrations, enzyme activity levels) after stress induction across four European perch stocks obtained from different aquaculture facilities in France (I and II), Denmark, and Hungary. The European perch stock from Denmark revealed the population with the most pronounced activity pattern. This was reflected by the highest relative swimming speed and a high percentage of bold-exploratory behaviour, which coincides with increased aggressive interactions within this stock. Additionally, we detected a higher tolerance to adverse environmental challenges in the perch stock from Denmark compared with the stocks from France and Hungary. The observed characteristics suggest that the stock from Denmark has a higher potential in the future framework of selective-breeding assessment. Further in-depth research is required to elucidate which traits and genetic as well as epigenetic components accelerate the domestication processes of European perch aquaculture and promote selective breeding processes.

Abbreviations: AF, anal fin length; BW, body weight; CF, caudal fin length; CSI, cardiosomatic index; DPV, the distance between pectoral and ventral fins; DVA, the distance between ventral and anal fin; ED, eye diameter; FDF, first dorsal fin length; GSI, gonadosomatic index; HL, head length; Hmax, maximum body height; Hmin, minimum body height; HSI, hepatosomatic index; LPF, left pectoral fin length; LVF, left ventral fin length; PAD, preorbital anal fin distance; PDD, distance from anterior most part of the body to the first dorsal fin ray; POH, distance from the posterior margin of the orbit to the end of the two orbitals; PRD, distance from the anterior most part of the body to the front margin of the orbit; PVD, the distance from the anterior most part of the body to the first ventral fin ray; PVSI, perivisceral fat index; RPF, right pectoral fin length; RVF, right ventral fin length; SDF, second dorsal fin length; SL, standard length; SSI, spleenosomatic index; TFLR, total fin length ratio.

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1. Introduction

Globally, aquaculture is the fastest-growing food production sector, and further growth is expected (Bostock et al., 2010). In contrast, the European (EU) sector has been stagnating for the last few decades (FAO, 2019) due to several bottlenecks, such as a shortage of suitable sites, the ecological carrying capacity of existing ones (Simard et al., 2008), the limited availability of fresh water, and strict regulations (Badiola et al., 2012; Nielsen et al., 2016). To cope with this, recirculating aquaculture systems (RASs) have been established as a viable sustainable option. However, RASs are characterized by high capital and operating costs (De Ionno et al., 2006; Martins et al., 2010; Dalsgaard et al., 2013) that significantly reduce the profits from farming low-value species in RASs. Since 75% of EU aquaculture production relies on low/medium value species, such as common carp, *Cyprinus carpio,* and rainbow trout, *Oncorhynchus mykiss* (Chiu et al., 2013; Dalsgaard et al., 2013; Żarski et al., 2017), the diversification of EU aquaculture with high-value/high-demand species such as European perch, *Perca fluviatilis,* pikeperch, *Sander lucioperca,* the African catfish, *Clarias gariepinus,* and burbot, *Lota lota,* is inevitable (Watson, 2008; Wang et al., 2009; Kucharczyk et al., 2018; Kucharczyk et al., 2019).

Key advantages of intensive RAS include the efficient use of water, energy, and land (Badiola et al., 2012); high productivity facilitated by fully controlled environments for fish; optimal feeding strategies (Helfrich and Libey, 1991); and full disease control (Summerfelt et al., 2009). On the other hand, high stocking densities and the artificial nature of rearing tanks can impair water quality and compromise fish health, including increased stress (Davidson et al., 2014), impaired swimming (Davidson et al., 2011), and fin damage/erosion (Ellis et al., 2008; Stejskal et al., 2011; Stejskal et al., 2020), which may negatively affect the consumer's acceptance (Hoyle et al., 2007). In recent decades, interest in fish health has gained increasing attention from government authorities and fish farmers and led to raised consumer awareness (Huntingford et al., 2006; Ashley, 2007).

European perch is often indicated as a suitable candidate for diversification of European intensive aquaculture (Watson, 2008; Toner, 2015). In the EU, European perch domestication was initiated 27 years ago (Fontaine and Teletchea, 2019). Today, the fourth level of domestication has been achieved, where the entire life cycle is closed in captivity. However, some bottlenecks in the farming of European perch still remain (Kestemont et al., 2003; Mandiki et al., 2007; Król et al., 2015) since no selective breeding programmes have been put in place (Teletchea and Fontaine, 2014), which could significantly expand the European perch aquaculture, as is the case for many other aquaculture fish species (Gjedrem, 2012; Gjedrem et al., 2012). The successful selective breeding process requires the identification of desirable aquaculture traits which positively correlate with fish health and profitability. Previous studies showed that breeding fish with higher growth, later sexual maturity, disease resistance, and elevated meat quality were successful for Atlantic salmon (*Salmo salar*), rainbow trout, and common carp (Gjedrem, 2012; Teletchea and Fontaine, 2014). However, these results were achieved through single-trait approaches. According to Toomey et al. (2020c), the multi-trait approach considers many desirable production traits for future selective breeding programmes and, thereby, reveals the best alternative. It has been identified for European perch that population(s) presenting optimal larvae development (e.g. swim bladder inflation, deformity rate), higher growth rate and survival, lower heterogeneity, lower levels of aggression and cannibalism, and higher stress tolerance and disease resistance have higher domestication potential. It is noteworthy that some traits are interlinked. For example, European perch individuals with insufficiently inflated swim bladders grow slowly and trigger different levels of heterogeneity (Baras et al., 2003). On the other hand, higher size heterogeneity can facilitate cannibalism and increase the level of mortality (Król and Zieliński, 2015). Consequently, some traits are less crucial for the selective breeding assessment framework. These mechanistic relationships may be overlooked since many traits are not simply reflected by a specific biological function. Therefore, the identification and assessment of potential traits for the incipient selective breeding process is a complex task and should be addressed through repeated appropriate methodological approaches to capture multiple traits. Additionally, most of the above-mentioned key traits are population-specific and influenced by geographic origin and genetic background (Mandiki et al., 2004; Toomey et al., 2019; Vanina et al., 2019a; Vanina et al., 2019b; Toomey et al., 2020b). The impact of geographic origin and genetic background on particular morphological traits of European perch have been analysed previously (Mandiki et al., 2004; Pimakhin and Zák, 2014; Vanina et al., 2019a; Vanina et al., 2019b). Nonetheless, behavioural and physiological population-specific differentiation and its potential for selective breeding goals have been poorly investigated to date (Mandiki et al., 2004; Toomey et al., 2019).

Indeed, behavioural patterns and physiological biomarkers are the most used criteria to assess the condition of fish in farming systems (Gjedrem and Baranski, 2010). Physiological parameters indicate the health status of fish in response to stimuli in RAS environments (e.g. density, nutrition, water quality) (Fazio, 2019), and behaviour patterns affect these values (e.g. lower cortisol is associated with higher aggression) (Castanheira et al., 2013). Furthermore, a recent study compared the inter-populational structure and aggressive interactions across different European perch groups (Toomey et al., 2019) and recommended that the population with lower aggressiveness and/or higher stress tolerance to RAS conditions is well suited for domestication and subsequent selective breeding (Toomey et al., 2020a; Toomey et al., 2020c).

In the present study, parameters indicating the health condition of European perch from different aquaculture facilities with different rearing management in France, Denmark, and Hungary were defined. These parameters included morphometric traits, fin condition, behavioural patterns including (a) critical swimming speed, (b) activity, (c) aggressiveness, and (d) fish boldness, and physiological parameters including (a) cortisol, (b) glucose, (c) ion content, and (d) enzyme activities were evaluated to set baselines for the most promising candidates for future selective breeding programmes.

2. Material and methods

2.1. Fish acquisition and husbandry

Four stocks of European perch juveniles (300 ind. Per stock) were obtained from Danish (D, 41.4 ± 6.7 g), Hungarian (H, 36.0 ± 7.4 g), and two French commercial RAS farms (FI and FII, 43.0 ± 7.3 g and 85.9 ± 20.4 g, respectively). The fish were transported in late November 2019 in oxygenated polyethylene bags (filled with 1/3 of water, 2/3 oxygen) placed in thermo-boxes and delivered to the RAS of the Faculty of Fisheries and Protection of Waters, University of South Bohemia (Czech Republic).

After gradual water temperature acclimatization (1 °C per hour), each stock was kept in a separate grey tank (net water volume 800 L) with a water exchange rate of 1.5 tanks per hour at a density of two individuals per L^{-1} set within the same RAS. The concentration of oxygen (above 90% of saturation), pH (7.0 ± 0.3), and temperature (23.0 ± 1.0 °C) were monitored twice daily throughout the whole experiment with a multimeter (Hach Lange HQ40d, Germany). Concentrations of ammonium and nitrites (lower than 0.05 mg/L^{-1}) were measured every second day with a portable spectrophotometer (DR 2800, Hach Company, USA). The light intensity was set at 400 lx at the water surface (DT-8809, Cem, China) and the photoperiod was constant at 12 L:12D. To minimize stress, each stock was fed with commercial pellets obtained together with the fish from their respective farms. The fish were fed by automatic fish feeders (Eheim Twinfeeder, model 2582, Germany) three times a day, at 7:30 a.m., 2:00 p.m., and 7:15 p.m., with a daily ration of 1.5% of the biomass. The fish were adapted for two weeks before

initiating the experiment. They were not fed for 24 h before each test conducted in this study. There was a seven-day interval between each test.

2.2. Morphometry, fin condition, somatic indexes

After two weeks of adaptation, 100 individuals from each stock (400 ind. in total) were randomly sampled. Fish were mildly anaesthetsized using clove oil (200 μL in 10 L of water) and weighed using a precision balance (OHAUS Explorer EX224M, NJ, USA) with an accuracy of up to 0.1 mg. Anaesthetized fish were photographed (Canon DR 5300) from both lateral sides and the ventral side. The images were processed using ImageJ software (Rueden et al., 2017).

Relative lengths (RLs) of the second dorsal, caudal, anal, both pectoral, and both pelvic fins were calculated using the following formula (Stejskal et al., 2020):

- $RL\text{particular fin} = L\text{particular fin} \times SL^{-1}$

where L is the length of the particular fin (mm) and SL is the standard length of the respective specimen (mm).

The sum of the total lengths of all fins to standard body length ratio (FLR) was calculated as a complex indicator of fin damage using the formula below:

- $FLR = (LRPF + LLPF + LRVF + LLVF + LSDF + LCF + LAF) \times SL^{-1}$

where LRPF is the length of the right pectoral fin, LLPF is the length of the left pectoral fin, LRVF is the length of the right ventral fin, LLVF is the length of the left ventral fin, LSDF is the length of the second dorsal fin, LFDF is the length of the first dorsal fin, LCF is the length of the caudal fin, and LAF is the total length of the anal fin (See Fig. 1).

Somatic indices were evaluated on 20 randomly selected individuals per stock (80 ind. in total), which were anaesthetized with an overdose of clove oil (Hamackova et al., 2006). Somatic indexes were calculated using the following formulas:

- HSI, % = 100 × liver weight/BW
- GSI, % = 100 × gonad weight/BW
- PVSI, % = 100 × fat weight/BW
- SSI, % = 100 × spleen weight/BW
- CSI, % = 100 × heart weight/BW

2.3. The critical swimming speed test (U_{crit})

A Steffensen-type swimming tunnel with a respirometer (volume of 10 L, Loligo System Inc., Viborg, Denmark) with a testing chamber size of 10 × 10 × 38 cm was used for measurement of the critical swimming speed. In total, 50 randomly selected individuals per stock (200 fish in total) were tested. Fish were gently netted from the rearing tanks and measured with a digital calliper (L: total length, H: body height, W: body width, ± 0.01 mm) before running the test. The fish were then placed into the chamber for 20 min acclimatization with a constant water velocity of 5 cm/s^{-1}. After acclimatization, water velocity was increased every minute in a constant stepwise progression of 2 cm/s-1 until the fish was exhausted. The tested fish was considered exhausted in the event that it floated back to the back grid of the swimming chamber and stayed in this position for at least 10 s.

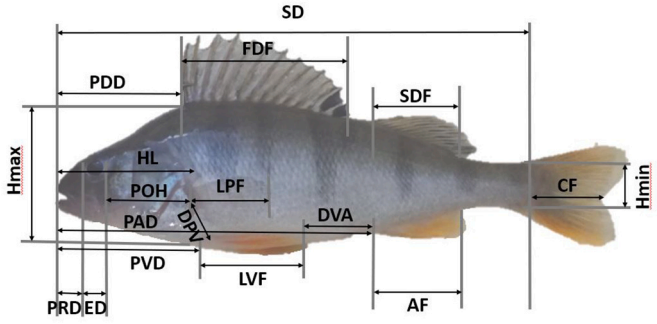

Fig. 1. Morphometric indices of fins investigated in this study.

Then, critical swimming speed was calculated according to Brett (1965). The water in the swimming tunnel was replaced with dechlorinated aged tap water ($O_2 \geq 90\%$, pH 7 ± 0.5, T 23 ± 1 °C) between every single fish testing. All individuals were used only once to avoid bias caused by experience.

2.4. Crowding challenge test

In order to induce a stress response in the fish, we decreased the water level of each 800 L tank (one by one) to 5 cm above the dorsal fins of the fish for 30 min. Thereafter, the water volume was increased again to the original water level. Blood samples were collected using heparinized Omnifix-F Solo 1 mL syringes (Braun, Melsungen, Germany) from the caudal blood vessels of anaesthetized fish (MS-222; 200 μg in 10 L water). Seven randomly selected individuals per stock were sampled at four different time points: before the water level decreased (control), and at 30 min, 12 h, and 24 h after the water level returned to the original level (n = 112 fish). The blood samples were kept in Eppendorf tubes on ice for less than 20 min before centrifugation (10,000 ×g/10 min at 5 °C). Afterwards, plasma was collected and stored at −80 °C until further analyses (Stafila laboratory, Czech Republic). Blood physiological parameters such as the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and the concentrations of glucose and monovalent potassium ions (K^+) were assessed using an Abbott Architect c8000 clinical chemistry analyser and assay kits (Abbot Diagnostics, Illinois, USA). Cortisol concentration was analysed using a reagent kit and automated immune analyser Immulite 2000 XPi (Siemens Healthineers, Siemens Healthcare GmbH, Erlangen, Germany).

2.5. Novel object test

The novel object test was performed according to the method described in Jones and Godin (2010). In total, 360 individuals were used in the test (90 ind. Per stock). The fish were gently netted from the rearing tanks and transferred into white rectangular tanks (one fish per tank; H × W × L = 37 cm × 57.5 cm × 73.5 cm) filled with dechlorinated aged tap water and left for 15 min acclimatization. Afterwards, a small yellow Lego block (LEGO 6176 DUPLO Basic Brick) representing a novel object was placed in the centre of the tank, and the behaviour of a fish was recorded (DS-2CD2043G0-I camera type, Hikvision, Hangzhou, China) for 30 min. The water in the experimental tanks was replaced with dechlorinated, aged tap water ($O_2 \geq 90\%$, pH 7 ± 0.5, T 23 ± 1 °C) between every single fish testing. All individuals were used only once to avoid bias caused by experience.

Three parameters were determined from the video records: the number of fish approaches to the Lego block (closer than 5 cm), the closest distance of the fish from the Lego block (within 5 cm), and the latency of the closest approach to the Lego block. These parameters were determined per each individual of 90 individuals of each stock. The videos were processed semiautomatically using an in-house image processing script implemented in Matlab. In each video, the region of the image that is out of the water was manually labelled by the image mask. The fish was detected as the largest object after edge detection in the mask. The Lego block was detected as a yellow object by thresholding the image by colour. The distance between the fish and the Lego block was calculated by finding the smallest distance between objects' edges in pixels. Then, the pixel distance value was converted into centimetres.

Pixel size was previously calculated from the known box size. The time of the closest approach was calculated from the camera frame rate.

2.6. Open field test

The open field test was performed according to the method described in Závorka et al. (2017). In total, 360 individuals were used in the test (90 ind. per stock). Fish were gently netted from the rearing tanks and placed into round white experimental tanks (one fish per tank; H x D = 53 cm × 68 cm) and left for 15 min acclimatization. The individual activity was video recorded (DS-2CD2043G0-I camera type, Hikvision, Hangzhou, China) for 10 min after acclimatization. The distance moved during the 10-min test was measured as the activity of each individual. The water in the experimental tanks was replaced with dechlorinated, aged tap water ($O_2 \geq 90\%$, pH 7 ± 0.5, T 23 ± 1 °C) between every single fish testing. No individual fish was repeatedly used in testing.

Video recordings were semiautomatically processed by the same method as for the processing of the novel object test videos. The script detects the centroid of the fish. The distance swam was calculated based on the fish centroids and the frame rate of the camera.

2.7. Group aggressiveness test

For the group aggressiveness test, we used 20 individuals per stock in triplicates. The fish were gently netted from the house tanks and placed in round white testing tanks (20 fish per tank; H × D = 53 × 68 cm) for one day of acclimatization in water ($O_2 \geq 90\%$, pH 7 ± 0.5, T 23 °C ± 1 °C). After acclimatization, aggressive interactions between 20 fish were video (DS-2CD2043G0-I camera type, Hikvision, Hangzhou, China) recorded for 30 min. In order to standardize the individual hunger level, the fish were not fed for the duration of the test. After each replication, the water in the experimental tanks was replaced with dechlorinated aged tap water ($O_2 \geq 90\%$, pH 7 ± 0.5, T 23 °C ± 1 °C), and the experimental tanks were thoroughly rinsed with tap water. Aggressive behaviours were assessed according to Toomey et al. (2019): (a) chase movement towards the conspecific, resulting in the conspecific's flight from the aggressor; (b) attack movement towards the conspecific, without actually biting the conspecific; and (c) the number of bites directed at the conspecific's body. Each type of aggressive behaviour was calculated relative to the initial number of individuals in the tank.

2.8. Statistical analysis

Morphometric indices did not meet the assumption of PCA, discriminant analysis, and parametric ANOVA and, therefore, single indices were compared using the Kruskal–Wallis H test with multiple comparisons of means as a post-hoc test. Somatic indices and weight showed non-normal data distributions and, therefore, were also compared using the Kruskal–Wallis H test. In the novel object test, the number of approaches was classified as shy (0 approaches), intermediate (1–5 approaches), and bold (≥ 6 approaches). The novel object test parameters, activity levels, aggression parameters, and blood plasma indices did not meet the assumption of the parametric test (MANOVA, ANOVA) and were analysed using the Kruskal–Wallis H test as above. The data on the critical swimming speed (U_{crit}) met the assumptions of an ANCOVA and, therefore, the ANCOVA was run to determine the effect of different European perch stocks on relative U_{crit} with SL as the covariable. The data are presented as mean ± standard deviation. Significance values have been adjusted with the Bonferroni correction for multiple tests.

The data were analysed in SPSS ver. 25 (SPSS Inc., Chicago, IL, USA). Radar charts were plotted in OriginPro (OriginPro 8, OriginLab Corporation, USA).

3. Results

3.1. Body weight, somatic, and morphometric indices

Body weight (BW) differed significantly among the European perch stocks. The FII stock revealed the highest BW, followed by the FI and D stocks, while the H stock showed the lowest BW (Table 1). The highest HSI was observed in the H and FI stocks and the lowest in the FII and D stocks, while the FI was not statistically differentiated from FII. The opposite trend was observed for GSI, where the D and FII stocks displayed significantly higher values compared with the H stock, while the FI stock did not significantly differ from other stocks. The PVSI was significantly higher in the H stock compared to the three other stocks. A significantly higher SSI was found in D and H as compared with FII. The CSI did not differ among the investigated stocks of European perch. (See Table 1)

All morphometric indices were significantly different across at least two of the four stocks, except for the distance between the pectoral and ventral fins (Fig. 2; Appendix S1).

3.2. Relative fin lengths

A comparison of relative fin lengths revealed significant differences among the different European perch stocks (Fig. 3; Appendix S2). The right pectoral fin was statistically longer in European perch from the FII and D stocks, while the left pectoral fin differed only between the FI and H stocks. In general, right pectoral fins showed lower values (greater erosion) as compared with left pectoral fins within a particular stock. The right and left vertical fins showed the same statistical difference, where the FI and D stocks were statistically differentiated only from the H stock. The *sec*ond dorsal fin was longest in the FI stock, while the lowest anal and caudal fins were found in the FII and D stocks, respectively. The relative total fin length showed a statistical difference only between the FI and H stocks.

3.3. Critical swimming speed

After adjustment for SL, there was a statistically significant difference in the relative U_{crit} between the European perch stocks; $F(3, 195) = 2.717$, $p = 0.046$, partial $\eta^2 = 0.04$. Post hoc analysis showed that the U_{crit} was significantly greater in the D stock versus the H stock (mean difference of 0.516 BL/s^{-1}, $p = 0.041$; 95% CI 0.012 to 1.02). The FI and FII stocks did not statistically differ from the other groups (Fig. 4).

3.4. Novel object test

The D stock showed a higher presence of intermediate and bold individuals and, consequently, a lower proportion of shy individuals. Moreover, the shy individuals from the D stock showed lower values of latency and closest distance compared with shy individuals from the other stocks in the study. Intermediate and bold individuals did not

Table 1
Body weight (in grams; 200 individuals per stock) and somatic indices (in %; 20 individuals per stock) of four European perch stocks.

	FI	FII	D	H
W	43.0 ± 7.3^{b}	85.9 ± 20.4^{a}	41.4 ± 6.7^{b}	36.0 ± 7.4^{c}
HSI	1.44 ± 0.23^{ab}	1.15 ± 0.22^{bc}	0.98 ± 0.27^{c}	1.86 ± 0.46^{a}
GSI	0.54 ± 0.26 X^{ab}	0.87 ± 0.60 Z^{a}	1.00 ± 0.61 Y^{a}	0.41 ± 0.21 Y^{b}
PVSI	2.51 ± 1.04^{b}	2.92 ± 1.03^{b}	2.96 ± 1.07^{b}	5.34 ± 1.02^{a}
SSI	0.09 ± 0.02^{ab}	0.08 ± 0.01^{b}	0.10 ± 0.03^{a}	0.10 ± 0.03^{a}
CSI	0.16 ± 0.04	0.15 ± 0.03	0.15 ± 0.03	0.14 ± 0.03

HSI—hepatosomatic index; GSI—gonadosomatic index; PVSI—perivisceral fat index; SSI—splenosomatic index; CSI—cardiosomatic index. X-5, Y-2, Y-3 individuals with indeterminate gonads. Different letters within a row indicate a significant difference ($p \leq 0.05$).

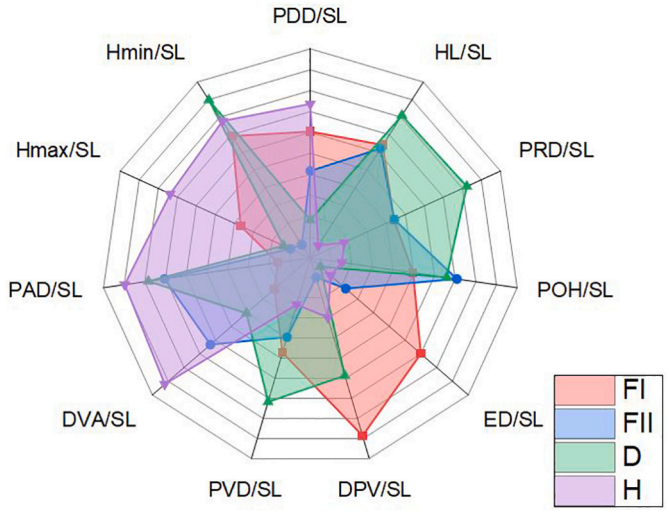

Fig. 2. Visualization of morphometric indices among four European perch stocks.

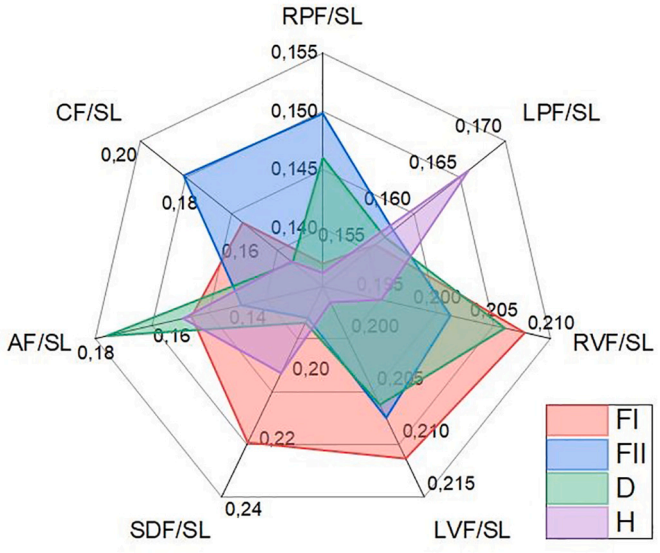

Fig. 3. Visualization of relative fin lengths among four European perch stocks.

show any significant differences in latency and closest distance among the tested stocks. The number of approaches towards the Lego block was not significantly different (Table 2).

3.5. Open field test and aggressiveness challenge

Distributions of activity scores were similar for European perch from the FI, FII, and H stocks but not for the D stock (Table 3). Median activity scores were statistically significantly different between the groups ($H(3) = 25.543$, $p < 0.001$).

Distributions of attack scores were not similar for all groups, as assessed by visual inspection bar plots. The mean ranks of attack scores were 6.00, 6.83, and 9.67 for the H, FI, and D stocks, respectively, but the differences were not statistically significant ($\chi^{2(3)} = 5.310$, $p = 0.15$). Zero aggressive interactions were observed in the FII stock (Table 4).

3.6. Crowding challenge

The crowding challenge (induced by lowering the water level for 30 min) had only a minor impact on the plasma parameters investigated in

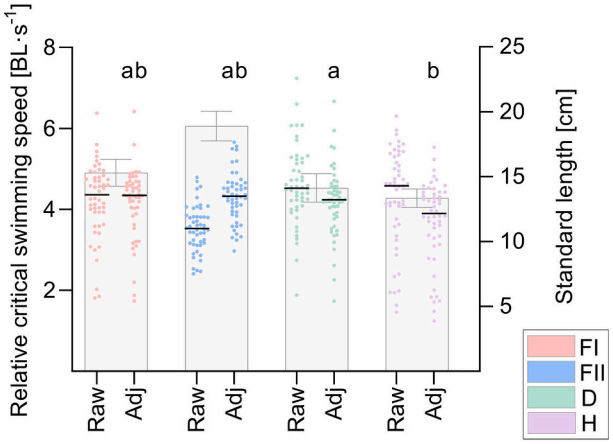

Fig. 4. Unadjusted (Raw) and adjusted (Adj) relative critical swimming speeds (U_{crit}; coloured dot plots, left ordinate) of the four European perch stocks, as indicated below the scheme. Standard length (grey columns, right ordinate) served as a covariate to adjust the relative U_{crit} values. Superscript letters indicate significantly different adjusted values ($p < 0.05$; $n = 50$ individuals per stock).

Table 2
Differences among European perch stocks in measured variables of exploratory behaviour (shy, inter, bold) evaluated with novel object test ($p < 0.05$; $n = 90$ per stock).

		%	Latency (sec)	Closest distance (cm)	Approaches
D	Shy	75.6	158.8 ± 53.8^{c}	15.2 ± 1.0^{b}	1.99 ± 0.67
	Inter	15.5	0.9 ± 0.22	2.9 ± 0.5	
	Bold	8.9	10.7 ± 8.5	0.5 ± 0.1	
FI	Shy	90	568.8 ± 81.6^{b}	25.3 ± 1.2^{a}	2.10 ± 1.12
	Inter	5.6	12.9 ± 12.6	1.8 ± 0.7	
	Bold	4.4	52.5 ± 34.2	1.7 ± 1.0	
FII	Shy	91.1	937.3 ± 90.0^{a}	26.5 ± 0.8^{a}	0.40 ± 1.73
	Inter	5.6	0.1 ± 0.0	1.1 ± 0.7	
	Bold	3.3	190.4 ± 187.0	1.7 ± 0.8	
H	Shy	86.7	645.1 ± 90.5^{b}	28.0 ± 1.1^{a}	2.04 ± 1.15
	Inter	8.9	73.5 ± 71.1	0.6 ± 0.2	
	Bold	4.4	33.6 ± 11.8	1.9 ± 0.9	

Table 3
Open field test scoring of activity among European perch stocks ($n = 90$ individuals per stock).

Parameters	FI	FII	D	H
n	90	90	90	90
Activity (cm)	1.24 ± 1.80^{b}	1.35 ± 2.13^{b}	2.31 ± 2.51^{a}	0.72 ± 0.81^{b}

Table 4
Quantification of aggressive interactions among European perch stocks ($n = 60$ individuals per stock).

Parameters	FI	FII	D	H
Chase	0	0	0	0
Attack	0.67 ± 1.15	0	2.33 ± 2.31	0.67 ± 0.58
Bite	0	0	0	0

the four perch stocks (Appendix S3). Thirty minutes after the challenge, plasma cortisol levels showed a significant ($p < 0.05$) 2.3-fold increase in individuals of stock FI, while K^{+} contents were significantly ($p < 0.05$) ~3-fold elevated in individuals of stock FII. Concomitantly, no changes ($p > 0.05$) were detected in individuals from stocks D and H. Remarkably, the cortisol levels in all control and treatment groups reached high values (above 80 ng per millilitre plasma) and, thus, exceeded the

physiological range. The same applies to glucose values, which were above a concentration of 6 mmol per litre plasma across all groups. The activities of the metabolic enzymes ALT, AST, ALP, and LDH remained almost unchanged, except for the reduced levels of ALT, AST, and LDH in individuals from stock D 12 h after the crowding challenge (compared to controls).

4. Discussion

The domestication process usually considers species as a unity, neglecting geographic backgrounds and population-specific adaptation histories, which could be important for domestication and selective breeding programmes (Lecocq et al., 2018). Previous life history challenges (e.g. gene flow disruption, local adaptation) may have lasting effects on behaviour and physiology and subsequently the economic attractiveness of wild population(s) (Lecocq et al., 2016; Polverino et al., 2018). A variety of proxies should be applied to assess the suitability of European perch cohorts for aquaculture, including behavioural patterns (e.g. critical swimming speed, activity, aggressiveness, fish boldness) together with physiological parameters (e.g. levels of cortisol, glucose, and enzyme activity) (Huntingford et al., 2006; Ellis et al., 2012; Martins et al., 2012; Molnár et al., 2018). Based on the criteria, the population(s) that display(s) desired behavioural patterns and an elevated tolerance to intensive aquaculture conditions are considered attractive for the selective breeding process (Castanheira et al., 2017; Toomey et al., 2020a; Toomey et al., 2020b).

In our study, four European perch stocks differed in their stress tolerance and behaviour under challenging conditions. Previous studies showed a negative linear relationship between body length and relative U_{crit} for many fish species (Brett, 1965; Hammer, 1995; Mateus et al., 2008; Xiong et al., 2014; Hou et al., 2018; Hachim et al., 2020). However, this is not in line with our results. Despite body size differences, the relative U_{crit} was not statistically different between FI and FII individuals, but it differed significantly among perch in the D and H stocks (Table 5). It was expected that the H stock with the lowest SL would reveal the highest U_{crit}. Conversely, the H stock showed the lowest U_{crit}. This low U_{crit} value could result from the high fat (PVSI) content in the peritoneal cavity (Table 5) (Grigorakis et al., 2002). Although all our stocks were kept under the same rearing conditions, it was possibly caused by suboptimal feed (fish were fed with feeds obtained from their original facilities). The high peritoneal fat content in the H group could indicate that the feeds or feeding regimes being used in their place of origin might not be optimal. Suboptimal feeding conditions promote metabolic disorders. In cultured gilthead sea bream, *Sparus aurata*, for instance, high HSI co-occurred with PVSI indices under suboptimal feeding conditions (Grigorakis et al., 2002). Although we do not have sufficient information on the original fish husbandry to make any assessment, we cannot exclude suboptimal rearing conditions on the farm, which could have affected not only behavioural but also physiological characteristics (López-Olmeda et al., 2012).

The relative U_{crit} is most likely associated with multifold behavioural and physiological responses (Koolhaas et al., 1999; Carbonara et al., 2012). For instance, the relative U_{crit} of the D stock could be explained by the exhibition of a generally higher exploratory behaviour and lower latency time of shy individuals as compared with other stock (Table 2). Moreover, the D stock exhibited a higher proportion of bold individuals who, according to the literature, show higher levels of activity and swimming locomotion (Table 5) (Pottinger and Carrick, 2001). Some authors regard boldness as a genetically manifested personality trait (Øverli et al., 2005), which drives divergent behaviour and, consequently, determines the degree of fitness (Øverli et al., 2005; Thomson et al., 2011). For instance, first-generation laboratory-reared tropical poecilid (*Brachyraphis episcopi*) showed similar bold behaviour to their wild parents, suggesting its heritable component (Brown et al., 2007). In contrast, hatched brown trout (*Salmo trutta*) showed a higher bold response to the novel object than wild ones, indicating that boldness was not related to genetics but promoted with hatchery selection (Sundström et al., 2004). Additionally, Sundström et al. (2004) documented that neither fish size nor growth rate allow conclusions on bold behaviour. This matches with our study, where FII stock with the highest weight showed the lowest percentage of bold individuals (Table 5). Johnsson et al. (1996) supposed that the husbandry in the predator-free hatchery environment relaxes antipredator behaviour and makes fish more willing to explore their environments. Unfortunately, we have no information on the previous husbandry conditions of the European perch stocks analysed, including their genetic selection level and parental broodstocks, to rule out their potential impacts on bold behaviour. Therefore, it remains unclear if boldness is genetic- or selection-specific or a combination. Nonetheless, many studies share the consensus that bold individuals are more likely to become dominant than their shy conspecifics (Sundström et al., 2004; Chapman et al., 2011; Colléter and Brown, 2011). These dominant individuals have higher feeding motivation (Wilson et al., 1993), higher willingness to explore and take a risk (Armstrong et al., 1997; Brick and Jakobsson, 2002), and higher tolerance to stressful environments (Brown et al., 2005), suggesting that dominant individuals may have higher potential for selection during the domestication process.

Bold behaviour has been reported to correlate with higher activity, higher aggression, and a low response to environmental stimuli (Pottinger and Carrick, 2001; Sih et al., 2004; Dingemanse et al., 2007; Moretz et al., 2007). In our study, we could not detect such a correlation, since the open field test, aggressiveness, and crowding challenges were performed on different individuals. The activity levels of three different European perch populations were already investigated by Toomey et al. (2019). Since the activity did not differ among populations, Toomey et al. considered this trait as less suitable for selection. On the contrary, our open field test revealed the highest activity level of individuals from stock D (Table 5). The different results on activity could reflect the techniques and methods employed, although the open field test is a common measure of activity in fish (Adriaenssens and Johnsson, 2013; Závorka et al., 2017) and other animals (Hall and Ballachey, 1932; Boyer et al., 2010). Fish activity could be measured more effectively in husbandry tanks than in a novel experimental arena or together with a novel object test (in the case of bold personality investigation). There is a perception that individuals are forced into the open field test with no opportunity to escape or, on the contrary, are free to explore the open field (Walsh and Cummins, 1976). Therefore, it seems that this test alone is less informative, and methods of using the open field test as a component of fish fitness evaluation for any future European perch domestication process should be reconsidered.

Stocks that tend to be bolder, taking a higher interest in exploration, may also be aggressive. Relative aggressive interactions clearly showed a higher number of attacks in European perch from the D stock; however, the differences were not statistically significant among stocks (Table 5). Aggressiveness is not desired in the hatchery environment due to the establishment of the dominance hierarchy. It is believed that dominant individuals are more aggressive (Mélard et al., 1996;

Table 5
Table summarizing the main results on the traits of four European perch stocks.

	FI	FII	D	H
W (g)	43.0 ± 7.3^{b}	85.9 ± 20.4^{a}	41.4 ± 6.7^{b}	36.0 ± 7.4^{c}
SL (cm)	15.3 ± 1.04^{b}	18.89 ± 1.14^{a}	14.14 ± 1.1^{b}	13.3 ± 0.72^{c}
PVSI	2.51 ± 1.04^{b}	2.92 ± 1.03^{b}	2.96 ± 1.07^{b}	5.34 ± 1.02^{a}
U_{crit} ($BL \cdot s^{-1}$)	4.19 ± 0.13^{ab}	4.48 ± 0.26^{ab}	4.24 ± 0.15^{a}	3.72 ± 0.19^{b}
Bold ind. (%)	4.4	3.3	8.9	4.4
Approaches	2.10 ± 1.12	0.40 ± 1.73	1.99 ± 0.67	2.04 ± 1.15
Activity (cm)	1.24 ± 1.80^{b}	1.35 ± 2.13^{b}	2.31 ± 2.51^{a}	0.72 ± 0.81^{b}
Attack number	0.67 ± 1.15	0	2.33 ± 2.31	0.67 ± 0.58
Cortisol (ng/ml)	251.89 ± 24.97^{bc}	458.87 ± 51.84^{ab}	191.29 ± 21.91^{c}	470.52 ± 35.54^{a}

Sneddon, 2003), which reduces the overall health score (Martins et al., 2012) and increases cannibalism with subsequent mortality. However, several parameters of hatchery practices may trigger aggressive behaviour, including a) size heterogeneity, b) stocking density, c) photoperiod and light intensity, and d) feeding regime (see Kestemont et al., 2003 and references therein). Furthermore, it was proven that frequent removal of predicted aggressive/cannibalistic fish from the husbandry tank improved neither survival nor growth rate, since their removal promotes a new dominance hierarchy in European perch. Since aggressive behaviour could be correlated with other desirable aquaculture traits (e.g. competitiveness, higher metabolic and locomotor activity, and a lower stress response to environmental stimuli), we suggest considering this trait as useful/informative for overall fish fitness estimation in the comparative analyses of the multiply traits approach.

In order to assess the response of the four European perch stocks to different stocking densities, we recorded the plasma concentrations of the stress hormone cortisol and other associated physiological parameters, which may be altered in the course of cortisol-induced metabolic processes (i.e. glucose and the enzymatic activities of ALT, AST, ALP, and LDH) (Mommsen et al., 1999). Thirty minutes after the crowding challenge, the mean glucose levels reached high values (> 10 mg/dl) in all groups, though non-significantly different from the other time points. We detected in parallel an approximately two-fold increase in the cortisol concentration of European perch from the FI and FII stocks. We note in this context that the biomass in the FII tanks was doubled compared to the other tanks, due to the two-fold higher weight of the individual FII European perch (Appendix S3), which inevitably exerted a higher degree of density. However, a stocking density of 2 individuals per L^{-1} with a net water volume of 800 L for each tank is considered optimal for European perch (Policar et al., 2015). Stock D showed the smallest alterations across the examined parameters and seems to be less responsive to environmental stimuli and treatments compared to stock H. This could be one of the reasons for the low relative swimming speed of group H (Fig. 4). Studies on rainbow trout established that the individual concentration of cortisol is determined by genetics (Barton and Iwama, 1991; Pottinger et al., 1992; Ortuño et al., 2002). According to Fevolden et al. (2002), a relatively high heritability of cortisol levels was found in a progeny of rainbow trout, where the respective strain displayed low stress responsiveness under prolonged environmental stress. This information about tolerance to stress (e.g. low cortisol level) may be useful for a productive breeding process in European perch aquaculture.

Finally, the stock from D is the northernmost stock among the populations investigated in the present study. Its overall performance could be modulated by relating this population to the fast pace of life syndrome theory (fast POLS). Fast POLS populations are more competitively successful, and, correspondingly, their higher metabolic rates allow bold individuals to sustain greater muscular activity, which induces changes in locomotor activity (Øverli et al., 1999; Castanheira et al., 2017; Molnár et al., 2018). Hence, the higher level of aggressive interactions seen in stock D could be a part of their personality, which need not necessarily lead to a stress response. The difference in the fast-slow continuum among fish populations could be determined by the total number of resources allocated to either survival or reproduction during their life history (Stearns and Rodrigues, 2020). Biro and Stamps (2008) stated that higher success in competing for resources in fast POLS populations is reflected by faster growth performance and earlier reproduction compared with populations at the slow end of the POLS continuum. However, we have no information on the genetic and phenotypic plasticity (Pigliucci et al., 2006) of the investigated perch stock, their past environmental circumstances (Devevey et al., 2010), or the parental broodstock (Olin et al., 2012), but, apparently, differing geographic background plays a role in population-specific responses to different challenges.

5. Conclusion

This study evaluated morphometric parameters, the critical swimming speed, the aggressiveness, activity, and propensity to approach a novel object, and the stress response to assess the potential of individuals from four European perch stocks. The differences were especially evident in two European perch stocks from D and H. Despite a possible discrepancy in their previous husbandry circumstances, European perch from stock D revealed a higher level of locomotion, boldness, and activity, together with aggressiveness and tolerance to the challenge during the period of crowding, unlike its conspecifics from stocks FI, FII, and H. These characteristics could be more beneficial for future fish selection and the domestication process. Further research should consider information on the origin of fish and focus on the genetic and phenotypic plasticity of the individual stocks as well as on adapted fish farming protocols. These observations may pave the way for subsequent genomic and/or epigenetic analyses of the investigated stocks (Skaala et al., 2005; de Los Ríos-Pérez et al., 2020) to identify the determinants of desirable traits and facilitate the future selective breeding process in European perch aquaculture.

Author contributions

VS, KP-Ż, and DŻ conceived the original idea. VS carried out the stock sampling. TG and RG performed the novel object test, the open field test, and the group aggressiveness test. RG performed the statistical analyses. PC and OM processed all video data. OT performed and processed data from the critical swimming test. MP and JM carried out the blood sampling. AR processed and described data from the stress challenge test. TG wrote the article. TG, RG, KP- Ż, PC, OM, OT, MP, JM, PH, JK, DŻ, AR and VS discussed the results and contributed to the final manuscript, revising it critically for important intellectual content.

Compliance with ethical standards

This study was performed in compliance with the terms of the Czech legislation (section 29 of Act No.246/1992 Coll., on Protection of animals against cruelty, as amended by Act No. 77/2004 Coll.) and animal experiments have been approved by the Ministry of Education, approval ID: MSMT-18301/2018-2.

Author contributions

Name of the author and e-mail ID	Types of contribution
Tatyana Gebauer	wrote the MS, performed the novel object test, the open field test, and the group aggressivity test
Radek Gebauer	performed the novel object test, the open field test, and the group aggressivity test, performed the statistical analyses, contributed and revised the final MS
Katarzyna Palińska-Żarska	conceived the original idea, contributed and revised the final MS
Petr Císař	processed all video data
Oleksandr Movchan	processed all video data
Ondřej Tomášek	performed and processed data from the critical swimming test, contributed and revised the final MS
Markéta Prokešová	carried out the blood sampling, contributed and revised the final MS
Jan Matoušek	carried out the blood sampling, contributed and revised the final MS
Piotr Hliwa	contributed and revised the final MS
Jarosław Król	contributed and revised the final MS
Daniel Żarski	conceived the original idea, contributed and revised the final MS
Alexander Rebl	processed and described data from the stress challenge test, contributed and revised the final MS
Vlastimil Stejskal	conceived the original idea, carried out the stocks sampling, contributed and revised the final MS

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

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Příloha č. 4

Schäfer, N., Matoušek, J., Rebl, A., **Stejskal, V.,** Brunner, R.M, Goldammer, T., Verleih, M., Korytář, T. 2021. Effects of hypoxic challenge with additional intraperitoneal stimulation on the immune status of pikeperch (*Sander lucioperca* L. 1758). Biology 10: 649. (IF 2021 – 5.079; SCI 2022 = 0; Q2)

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Effects of Chronic Hypoxia on the Immune Status of Pikeperch (*Sander lucioperca* Linnaeus, 1758)

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Simple Summary: Inadequate oxygen saturation, or hypoxia, belongs to one of the critical stress factors in intensive aquaculture. Exposure of fish to low oxygen levels over prolonged periods substantially affects their well-being and immune competence, resulting in increased disease susceptibility and consequent economic losses. In this interdisciplinary research, we aimed to provide a deeper understanding of the effect of chronic low oxygen saturation on pikeperch farmed in recirculating aquaculture systems. The obtained data offer unprecedented insights into the changes in the immunocompetence of studied fish and suggest high robustness of this new aquaculture species to the stress factors of intensive aquaculture.

Abstract: Inadequate oxygen saturation can induce stress responses in fish and further affect their immunity. Pikeperch, recently introduced in intensive aquaculture, is suggested to be reared at nearly 100% DO (dissolved oxygen), yet this recommendation can be compromised by several factors including the water temperature, stocking densities or low circulation. Herein, we aimed to investigate the effect of low oxygen saturation of 40% DO (±3.2 mg/L) over 28 days on pikeperch farmed in recirculating aquaculture systems. The obtained data suggest that—although the standard blood and health parameters did not reveal any significant differences at any timepoint—the flow cytometric analysis identified a slightly decreased proportion of lymphocytes in the HK (head kidney) of fish exposed to hypoxia. This has been complemented by marginally downregulated expression of investigated immune and stress genes in HK and liver (including *FTH1*, *HIF1A* and *NR3C1*). Additionally, in the model of acute peritoneal inflammation induced with inactivated *Aeromonas hydrophila*, we observed a striking dichotomy in the sensitivity to the low DO between innate and adaptive immunity. Thus, while the mobilization of myeloid cells from HK to blood, spleen and peritoneal cavity, underlined by changes in the expression of key proinflammatory cytokines (including *MPO*, *IL1B* and *TNF*) was not influenced by the low DO, hypoxia impaired the influx of lymphocytes to the peritoneal niche in the later phases of the immune reaction. Taken together, our data suggest high robustness of pikeperch towards the low oxygen saturation and further encourage its introduction to the intensive aquaculture systems.

Keywords: pikeperch; hypoxia; intraperitoneal stimulation; immune response; stress response; *Aeromonas hydrophila*

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1. Introduction

Low dissolved oxygen (DO) levels induce primary, secondary and tertiary stress responses in fish [1]. Optimal oxygen saturation is a vital parameter for animals and, therefore, tightly controlled in intensive aquaculture facilities. Hypoxia is defined as depletion of oxygen concentration, which can substantially affect the fish's well-being and immune status, resulting in increased susceptibility to stressors and reduced resistance to pathogens [2]. Bregnballe (2015) defined oxygen saturations below 40% (equivalent to ~3–4 mg/L at 20–25 °C) as inadequate for aquaculture facilities in general [3]. The required concentration of dissolved oxygen is dependent on the fish species and the corresponding water parameters. Furthermore, the animal-specific sensitivity to low oxygen saturation and the duration and the intensity of hypoxic conditions influence the outcome of the triggered response [4,5]. There are several reasons for low DO levels in aquaculture systems, including inadequate water circulation [6], high water temperatures [7] and high stocking densities [8]. Percids seem to be relatively tolerant to low DO levels [9,10]. For instance, feeding rate of yellow perch (*Perca flavescens*) consumption is negatively regulated only with oxygen levels of 3.5 mg/L and lower [11]. The growth of walleye (*Sander vitreus*) and yellow perch is affected by oxygen levels only below 2 mg/L [12–14]. Nevertheless, levels below 5 mg/L DO can induce significant stress responses in Eurasian perch (*Perca fluviatilis*) [15].

The pikeperch (*Sander lucioperca*) is native to fresh and brackish waters of the northern hemisphere [16], and due to its high-quality flesh and high market acceptance, it became a significant food fish for regional aquaculture in Europe [17]. Within its natural habitat, the DO levels range from 5.5 to 12.9 mg/L [18,19]. Thus far, nearly 100% DO (equivalent to ~7–9 mg/L DO at 20–25 °C) has been suggested as the optimal oxygen saturation for pikeperch aquaculture [20–22]. However, the lower limit of the pikeperch's tolerance to low DO levels under intensive farming, and its impact on physiology and immunity remains vague. Previously, Stejskal et al. (2012) observed that a 50–60% oxygen saturation (equivalent to ~4–6 mg/L DO at 20–25 °C) correlates with lower feed intake and a reduced growth rate in intensive pikeperch farming [23], while in independent study similar oxygen saturation for 36 days led to a significant increase in the complement activity of the sera [24]. Nevertheless, a more detailed understanding of the effect of chronic low oxygen saturation on immune system of pikeperch is absent. In this study, we aimed to fill this gap and elucidate how low oxygen saturation of 40% (±3.2 mg/L DO) for 28 days influences the standard health and immune system parameters and expression of selected genes involved in the regulation of immune and stress responses. Furthermore, to gain further insights into the capacity of the immune system to induce inflammatory responses under low DO, we employed a previously established model of acute peritonitis with *Aeromonas hydrophila*, Gram-negative bacteria associated with mortality of fish kept under adverse environmental conditions [25,26].

2. Materials and Methods

2.1. Fish Rearing and Experimental Design

Ninety-eight juvenile pikeperch (obtained from Anapartners, s.r.o., Prague, Czech Republic), with an average length of 29.51 ± 0.47 cm and body weight of 219.39 ± 9.79 g, were reared in a recirculating aquaculture system (RAS) at the Institute of Aquaculture and Protection of Waters (IAPW; České Budějovice, Czech Republic) from June to August 2018. Fish were randomly assigned in six identical black plastic 300 L tanks and acclimated for two weeks at 23.1 ± 1.0 °C with 12:12 h day/night light period and a light intensity of 20–35 Lx. Water was pre-treated by drum and moving bed filters in complement with UV disinfection and aeration. In all tanks, the inflow-outflow rate was 6 L/min, generated through mixing towers, and in-tank oxygen saturation was monitored online using the controller HACH SC 1000 (HACH Lange, Düsseldorf, Germany). Feeding was performed with a commercial extruded diet (EFICO Sigma 970, 3 mm, BioMar A/S, Brande, Denmark) by automatic feeders (EHEIM Twins, Deizisau, Germany; 6 meals per day) and one hand

feeding. Fish were fed ad libitum. The experimental design is illustrated in Figure 1. The experiment was performed in triplicate tanks for up to 28 days, with ±3.2 mg/L DO levels (40% oxygen saturation) for "low DO group" (total n = 49; 12–24 per tank) and ±8.3 mg/L DO levels (>95% oxygen saturation) for the control group (total n = 49; 12–24 per tank). Low DO conditions were established by additional nitrogen administration according to the oxygen depletion system generated by Pichavant et al. (2000) [27], including individual modifications. Water quality parameters (NH_3, NH^{4+}, NO^{2-} and NO^{3-}) were monitored throughout the experiment in the two-day interval using commercial kits (LCK 304, LCK 341, LCK 339 and HACH Lange, Düsseldorf, Germany) and spectrophotometric analyses (DR 3900 and HACH Lange, Düsseldorf, Germany). The concentration of ammonia, nitrite, and nitrate was 0.24 ± 0.11, 0.21 ± 0.13 and 4.54 ± 4.18, respectively. Temperature and pH were monitored daily with HACH HQ40 multimeter and reached 23.5 ± 0.7 °C and 7.38 ± 0.25, respectively.

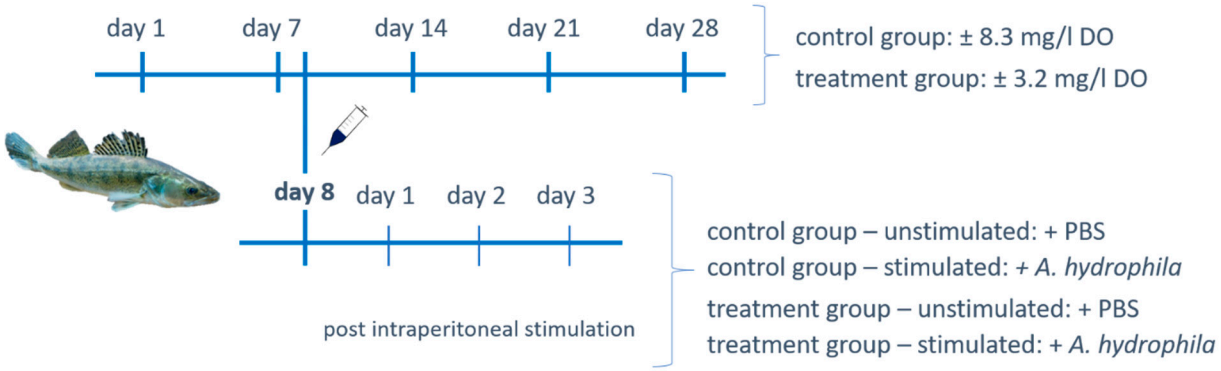

Figure 1. Outline of the experimental design. The 98 adult pikeperch were kept either under normoxic water conditions (±8.3 mg/L dissolved oxygen (DO) level) or low DO saturation (±3.2 mg/L DO level) for up to 28 days. Peripheral blood, head kidney (HK) and liver tissues were sampled from five fish of both groups. Additionally, at day 8 of the experiment, 48 fish were intraperitoneally injected with either 1×10^7 inactivated *Aeromonas hydrophila* cells in 100 µL sterile phosphate-buffered saline solution (PBS) or exclusively 100 µL PBS. At three following days, peritoneal leukocytes, peripheral blood, HK and spleen were collected from four fish per group.

Before sampling, fish were anesthetized with 30 µL/L clove oil and stunned in compliance with the terms of the Czech legislation (Section 29 of Act No.246/1992 Coll., on Protection of animals against cruelty, as amended by Act No. 77/2004 Coll.). All animal experiments have been approved by the Ministry of Education, approval ID: MSMT-18301/2018-2.

On days one, seven, fourteen, twenty-one and twenty-eight of the treatment, we sampled peripheral blood, head kidney (HK) and liver of five fish per control and low DO group. Additionally, to elucidate the impact of hypoxia on acute inflammation, we performed peritoneal stimulation described below. Upon induction of peritoneal inflammation, peripheral blood, HK, spleen and the peritoneal leukocytes were sampled at day one, two and three post-stimulation at days nine, ten and eleven of the hypoxia experiment. Peripheral blood was collected from the caudal vein with heparinized (Sigma-Aldrich, Taufkirchen, Germany) syringes. Parts of the HK, liver, and spleen were snap-frozen in liquid nitrogen and stored at −80 °C until RNA isolation. The peritoneal leukocytes were obtained from peritoneal lavage as described previously [28].

2.2. Intraperitoneal Stimulation with Aeromonas hydrophila

To evaluate the impact of low DO on the acute inflammatory response, fish were stimulated with 1.5% paraformaldehyde (PFA) inactivated *A. hydrophila*. To this end, 48 pikeperch (24 each per control and low DO group, 208.85 ± 6.58 g) were intraperitoneally injected at day eight of the experiment (Figure 1) with either a total of 1×10^7 *A. hydrophila* in 100 µL sterile phosphate-buffered saline solution (PBS) or exclusively with 100 µL PBS.

2.3. Cell Isolation

The blood samples were diluted 1:150 in DMEM (Dulbecco's modified eagle medium; Gibco/Life Technologies, Carlsbad, CA, USA) on ice. The remaining HK and spleen were homogenized by cell strainer (100 µm; Corning Inc., Corning, NY, USA) and resuspended in 4 mL DMEM (Gibco) on ice. Collected cells were further 1:1 diluted in DMEM (Gibco) on ice, layered onto an isotonic Percoll™ (Ge Healthcare, Uppsala, Sweden) gradient (34% plus 51%; r = 1.075 g/mL) on ice and centrifuged at 800× *g* for 15 min at 8 °C. We collected the HK leukocytes at the interphase of the different Percoll™ concentrations, washed the cell suspension and, finally, resuspended it in 500 µL DMEM (Gibco) on ice. Cells were further 1:2 diluted in DMEM (Gibco) on ice and applied for flow cytometry.

2.4. Flow Cytometry

To investigate the cell composition of peripheral blood, HK and spleen samples we applied flow cytometry analysis using the BD FACSCanto™ II system (BD Biosciences, Prague, Czech Republic) in medium flow rate (1 µL/s). Briefly, the leukocytes from HK and spleen were diluted to a concentration 1×10^6/mL and 100 µL of the cell suspension was used for the measurement. Furthermore, 2 µL of whole blood were diluted in 200 µL of DMEM containing the DIOC6 dye and recorded for 20 s at a constant flow of 1 µL/second. To determine the total number of peritoneal leukocytes, we employed the flow cytometry protocol described previously [28]. The cell morphology was evaluated using the FSC-SSC parameters, with lymphocytes being FSC^{lo}-SSC^{lo} and myeloid cells being defined as FCS^{hi}-SSC^{hi}.

2.5. Health Parameters

We measured traditional health indicators, including total length, weight, spleno- (SSI) and hepato-somatic indices (HSI), as well as concentrations of glucose, lactate and plasma cortisol levels. The SSI was calculated by the formula: spleen weight (g)/body weight (g) × 100; the HSI was calculated by the formula: liver weight (g)/body weight (g) × 100. Whole blood glucose and lactate levels were analyzed using the Accutrend® Plus device (Cobas; Roche Diagnostics, Mannheim, Germany). Plasma cortisol levels were measured by the Cortisol ELISA assay (DRG Instruments, Marburg, Germany) according to the manufacturer's instructions.

2.6. Gene Selection and Primer Design

To evaluate the transcriptional response to low DO saturation and the capacity of fish to induce inflammatory responses under stress, we established a screening panel with 15 genes involved in stress and immune response (listed in Table 1).

The genes, elongation factor 1 alpha (*EEF1A1*), ribosomal protein L32 (*RPL32*) and ribosomal protein S5 (*RPS5*) were applied as reference according to Swirplies et al. (2019) [29]. For the candidate genes interleukin 8 (*CXCL8*), hypoxia-inducible factor 1 subunit alpha (*HIF1A*), heme oxygenase 1 (*HMOX1*), heat shock transcription factor 1 (*HSF1*), heat shock transcription factor 2 (*HSF2*), heat shock protein 90 alpha family class A member 1 (*HSP90AA1*), interleukin 1 beta (*IL1B*), nuclear receptor subfamily 3 group c member 1 (*NR3C1*) and tumor necrosis factor (*TNF*) pikeperch-specific oligonucleotide sequences were already available from our former studies [29,30]. Using our recently published pikeperch genome (RefSeq NCBI: GCA_008315115.1) [31], we identified the remaining orthologs for *S. lucioperca*. The Pyrosequencing Assay Design software (version 1.0.6; Biotage, Uppsala, Sweden) was applied to derive optimal oligonucleotide primers. We purchased all primers from Sigma-Aldrich, Taufkirchen, Germany) and validated them by sequencing their PCR products (Applied Biosystems 3130 Genetic Analyzer; Life Technologies, Carlsbad, CA, USA).

Table 1. Gene-specific primers used in this study.

Gene Symbol	Official Names	Sense Primer (5′-3′)	Antisense Primer (5′-3′)	Primer Efficiency [%]	Fragment Length [bp]
	Reference genes:				
EEF1A1	Elongation factor 1 alpha	ATGGACAGACCCGTGAGCATG	TTCTTGATGTAGGTGCTCACTTC	105	151
RPL32	Ribosomal protein L32	GGCGTAAACCCAGAGGTATTGA	ACCTCGAGCTCCTTGACATTGT	105	157
RPS5	Ribosomal protein S5	GCAGGATTACATTGCTGTGAAAG	TCATCAGCTTCTTGCCATTGTTG	101	161
	Target genes:				
Stress response					
EPAS1	Endothelial PAS domain protein1	AGTGCAGAGGACGCACAGATG	TCATGTTCACCTGCGTGAGCC	100	139
HIF1A	Hypoxia inducible factor 1 subunit alpha	CCAGTCGAATCCCTTGAGAGTT	CTGTGGGGTCCTCTTAGCAAC	97	156
HMOX1	Heme oxygenase 1	GCTCGCTGTATGAGGTCTACC	TCTCTCCAGTCCTGGCCATAG	101	154
HSP90AA1	Heat shock protein 90 alpha family class A member 1	AGATACTACACCTCGGCTTCTG	TCACCAGTGATGTAGTAGATGTG	100	101
HSF1	Heat shock transcription factor 1	TGTGTCTTGTGCAGAGTGGAAC	GCTGGCCATGTTGTTGTGTTTG	111	101
HSF2	Heat shock transcription factor 2	AGCCGTCCCGCAGCTCCCT	CGGGACTCAGTTCGCACAGG	91	93
NR3C1	Nuclear receptor subfamily 3 group c member 1	CCAGTCCTGCATGGATTCACTT	AGGTCCATAGTGTTGTCACTGAA	100	180
Immune response					
CSF2 *	Colony stimulating factor 2	CCAGCAGGAATACACAGAAATCT	CGCAGATAGAGACAATGATGAAG	95	164
CXCL8 *	Interleukin 8	AACAGGGATGAGTCTGAGAAGC	GCTTGGAAATGAAGTCTTACATGA	98	158
FTH1	Ferritin heavy chain 1	AGAACTGGCAGACTGGGTGAC	CTGCTTTCTTTGCCCAGGGTG	99	102
IL1B *	Interleukin 1 beta	TCGACCTACTTGCACCCTACA	TCTGCCTCCACAACCTGAA	101	137
MHC II alpha *	Major histocompatibility complex II alpha	TGGACCAACCACTGACCAGAAT	CATCATCAGTCCCAGCCAATCA	99	168
MPO *	Myeloperoxidase	GTTTGATCGGCCGTCCTGCTA	ATTACCAGCCAAGCCATGGTCA	98	152
RAG1 *	Recombination activating 1	CTCAGGCTTCAGTGTCATGATC	AACCTCTTTCTCCTCCTCGTCT	95	157
TNF *	Tumor necrosis factor	GTCTTTGGAACCAGGCTATTTAC	TTTATGCCTCAGGCTTGACTGG	89	157

* Genes applied exclusively for stimulation experiment.

2.7. RNA/cDNA Preparation

Total RNA was extracted from collected samples by homogenizing tissues (HK, liver and spleen) separately within 1 mL TRIzol Reagent (Invitrogen/Thermo Fisher Scientific, Karlsruhe, Germany), as stated in the manufacturer's instructions. Subsequently, we purified all samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany), including DNAse treatment. For isolated HK leukocytes, 3.5 µL of 2-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany) was added, and samples were purified with the ISOLATE II RNA Mini Kit (Bioline/Meridian Bioscience, London, UK). Applying gel electrophoresis and spectrophotometry analysis in repeated measurements (ND 1000; NanoDrop Technologies/Thermo Fisher Scientific, Waltham, MA, USA), the quantity and quality of the isolated nucleic acids were determined. Collected RNA was stored at −80 °C until further application.

Synthesis of cDNA was performed from 1.0–1.5 µg of total RNA using the SuperScript II Reverse Transcriptase Kit (Thermo Fisher Scientific, Karlsruhe, Germany) according to the manufacturer's protocol, and cDNA was stored at −20 °C.

2.8. Real-TIME Quantitative PCR (rt-qPCR)

The gene expression during low DO levels was evaluated by real-time quantitative PCR (rt-qPCR). Therefore, we implemented the SensiFAST™ SYBR No-ROX Kit (Bioline, Luckenwalde, Germany) and the LightCycler96 system (Roche, Basel, Switzerland). PCR conditions were as follows: the initial denaturation step (95 °C, 5 min) was followed by 40 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 10 s), elongation (72 °C, 20 s) and a fluorescence measurement step for 10 s (75 °C). For the copy-number calculation by linear regression analysis ($R^2 > 0.999$), standard curves based on Cq values of tenfold dilutions of the generated fragments (1×10^3–1×10^8 copies) were generated. Cq values > 35 were considered as not detectable. For confirmation of the quality of all PCR products, we conducted melting curve analysis and gel electrophoresis.

Three reference genes (*EEF1A1*, *RPL32* and *RPS5*) for *S. lucioperca* [29] were evaluated for each sample and applied for data normalization.

2.9. Statistics

Rt-qPCR data were evaluated with the LightCycler 96 software v. 4.0.1. Flow cytometry data were analyzed by using the BD FACSDiva software FlowJow10. Statistically significant differences in blood parameters, gene expression and cell composition between control and low DO group were determined per day using the multiple *t*-test (Holm–Šídák corrected, $\alpha = 0.05$). For the additional stimulation experiment data, statistical significances were calculated with one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

3. Results

3.1. Reduced Oxygen Saturation Induces Marginal Changes in the Process of Adaptation

3.1.1. Blood and Health Parameters of Challenged Pikeperch

To evaluate the physiological responses of the fish challenged with the low DO levels, we recorded standard health parameters including blood glucose, lactate and plasma cortisol levels, as well as SSI and LSI throughout the experiment. Notably, these parameters did not reveal any significant differences between the "control" (±8.3 mg/L DO) and "low DO group" (±3.2 mg/L DO) at any time point of the experiment (Supplementary Table S1).

3.1.2. Composition of HK and Peripheral Blood upon Low DO Exposure

We hypothesized that inadequate oxygen saturation would affect the immune status of the fish, reflected by a change in the proportion of immune cells. Therefore, we employed flow cytometry to analyze the ratio between myeloid and lymphoid cells in HK and peripheral blood. (Figure 2).

Throughout the experiment, the average cell composition of the peripheral blood leukocytes remained almost unchanged between the control and the low DO group, with a proportion of approximately 95% lymphocytes to 5% myeloid cells (Figure 2A). Conversely,

the composition of HK exhibited notable differences between both groups. Thus, while the ratio between myeloid cells and lymphocytes in the control group underwent only mild fluctuations ranging from 63% to 79% of lymphocytes and 21% to 37% of myeloid cells, we observed more substantial changes in the group exposed to low DO. Particularly at the early time points (day one, seven and fourteen), we witnessed an increase in the proportion of myeloid cells, reaching to 46%, reflected by a 1.4× decrease in lymphocyte proportion (Figure 2B).

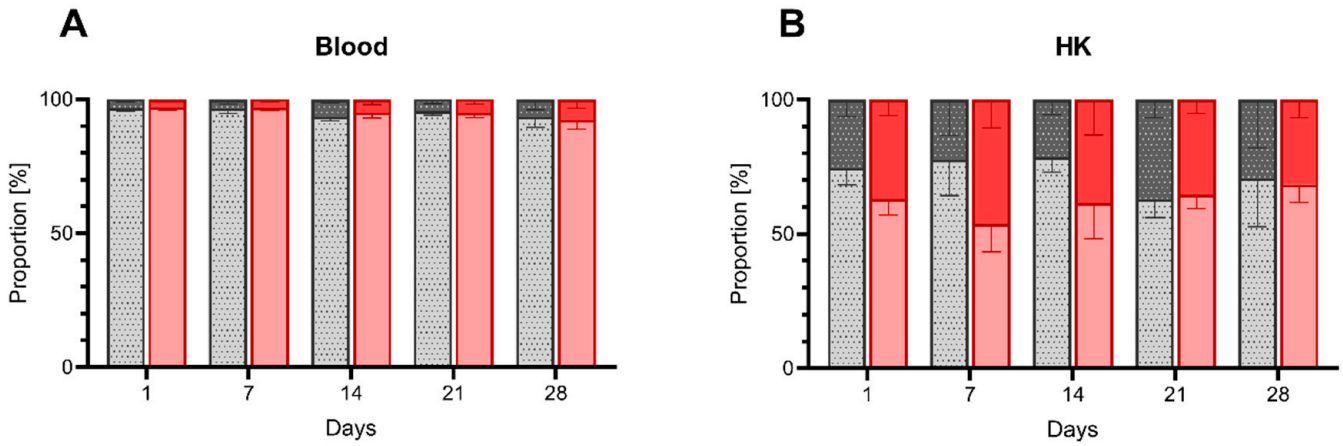

Figure 2. Proportion of myeloid and lymphoid cells within blood and HK of pikeperch challenged with low DO conditions. Proportion (%) of lymphoid (bright color) and myeloid cells (dark color) in collected blood (**A**) and head kidney (HK; (**B**)) samples of pikeperch. HK was additionally purified by Percoll-gradient. Columns represent mean of five individual samples (+SEM) from control (grey/patterned) and hypoxia group (red) after one, seven, fourteen, twenty-one and twenty-eight days of the experiments. Statistical significance per cell type per each day was determined using the multiple t-test (Holm–Šídák corrected), with alpha = 0.05.

3.1.3. Gene Expression Analysis in HK and Liver of Challenged Pikeperch

To provide deeper insights into the molecular mechanisms underlying the changes induced by low DO levels, we evaluated the expression of eight selected genes involved in response to hypoxia. The transcript numbers ranged from approximately 2×10^1 (*HSP90AA1*) to 2.5×10^6 (*HSF2*) copies per 100 ng RNA in HK and liver of individual fish (Figures 3A–C and 4A–C). We detected transcript numbers only below 2×10^3 transcripts/100 ng RNA for the genes *HMOX1* and *HSP90AA1* in HK and liver (Figures 3A and 4A). The highest copy numbers with 1.5×10^5 transcripts/100 ng RNA and above were observed for the genes *EPAS1*, *FTH1* and *HSF1* in HK and liver (Figures 3C and 4C).

In general, few genes were lower expressed under low DO levels in liver and HK than in the control group. Three of the analyzed genes (*EPAS1* in HK; *FTH1* and *NR3C1* in the liver) shared similar transcript patterns. Here, the expression levels of both groups are similar at the beginning and the end of the experiment, but at days seven and fourteen the low DO group showed lower transcript levels.

Four genes showed statistically different copy numbers between the control and the low DO group. For *HSP90AA1*, a change from significantly lower to significantly higher copy numbers during the treatment was observed in HK. *HIF1A* showed higher transcript levels during the low DO exclusively at day 2 in the liver, but lower copy numbers from day 14 till day 28 in HK. Another two genes (*FTH1* and *NR3C1*: HK and liver) showed lower transcript levels during the low DO challenge.

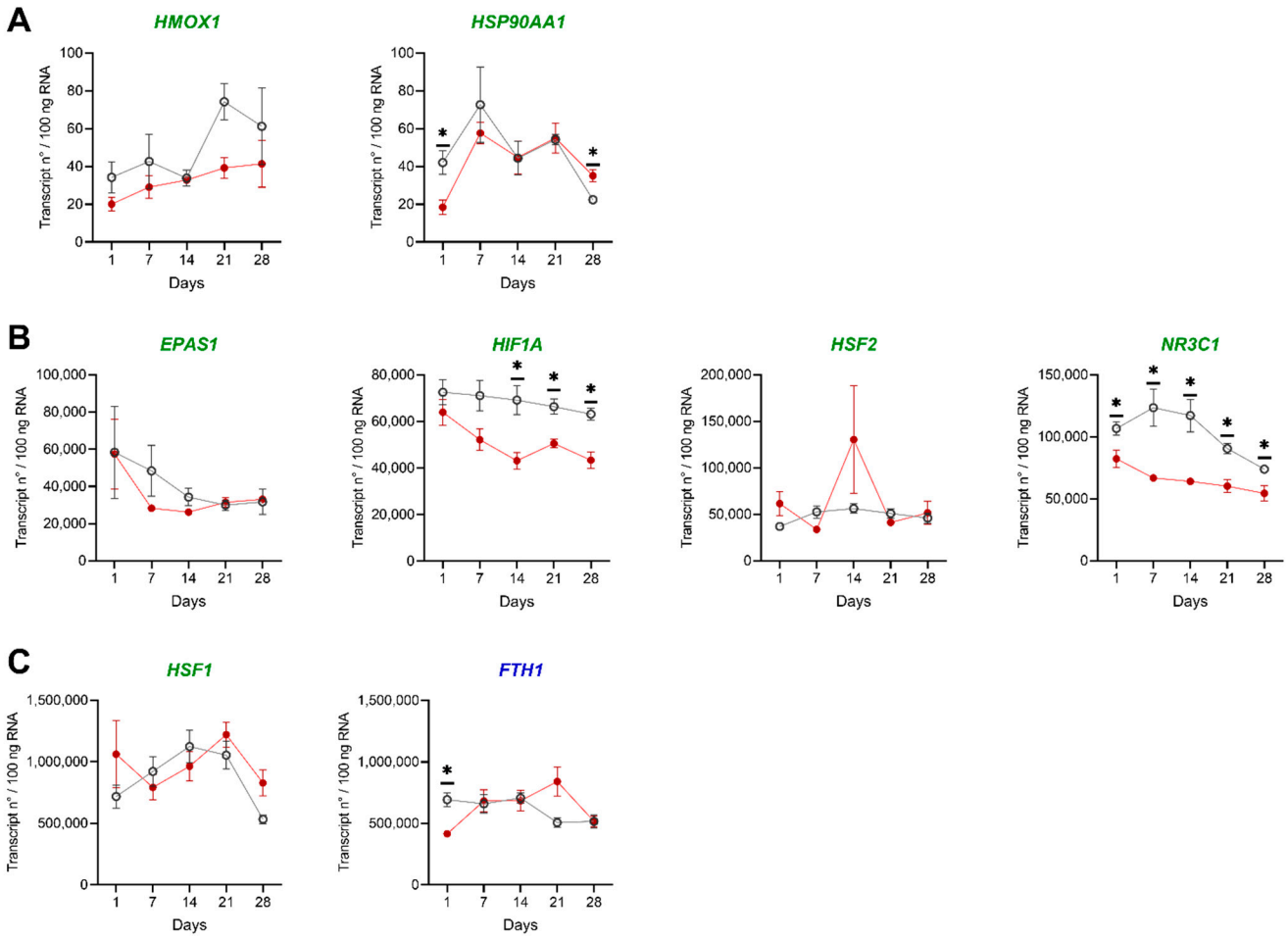

Figure 3. Stress and immune marker expression in HK of pikeperch exposed to low oxygen saturation. Gene expression of candidate genes in collected head kidney (HK) samples of pikeperch. Normoxic (grey/empty circles) and low DO (red/filled circles) groups after one, seven, fourteen, twenty-one and twenty-eight days of the experiment. Genes involved in stress (green) or immune response (blue) were either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent the mean of five individual samples (+SEM), calculated per 100 ng of total RNA. Statistical significance was determined between groups for each day using the multiple t-test (Holm–Šídák corrected), with alpha = 0.05; * < 0.05.

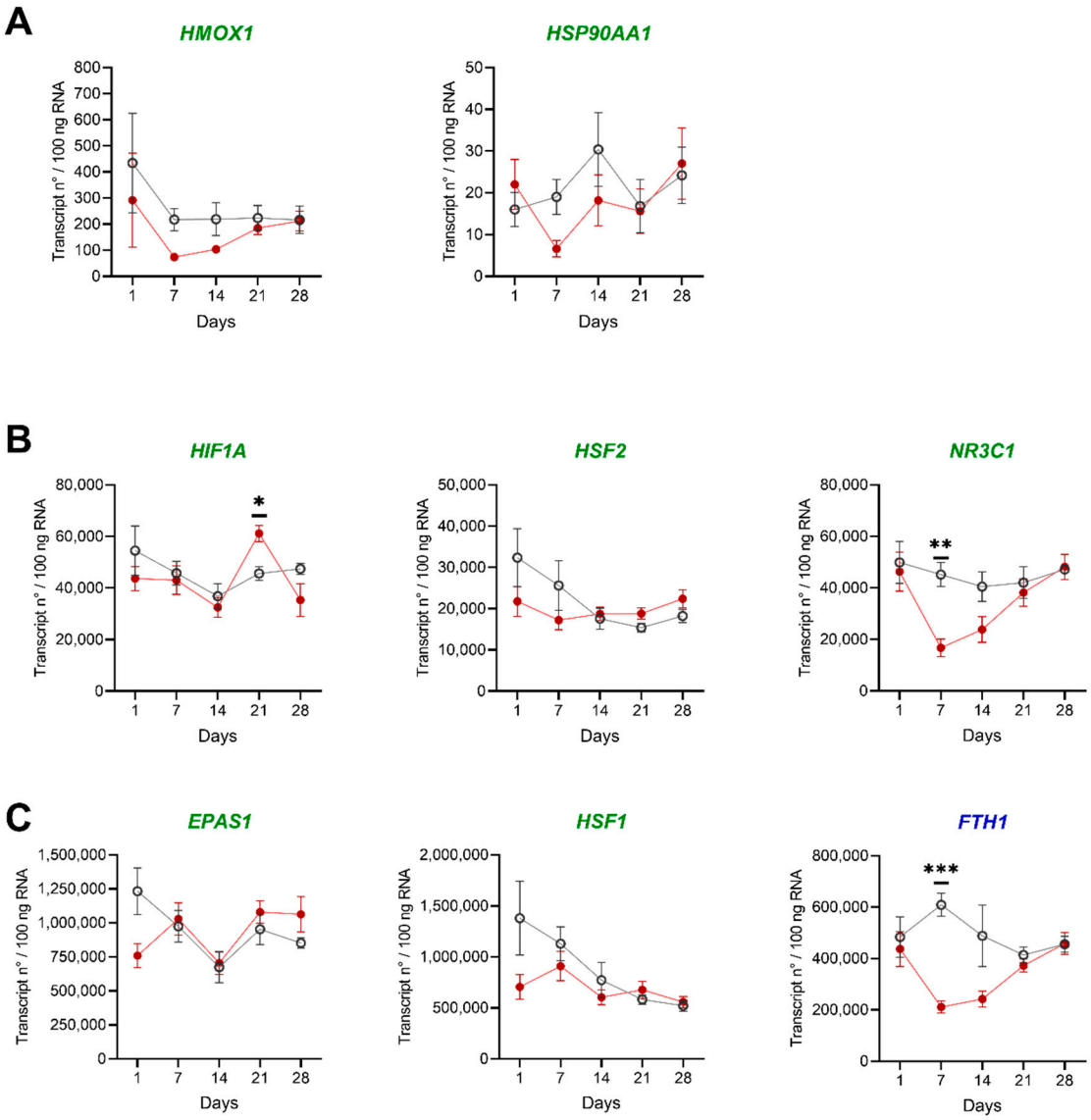

Figure 4. Stress and immune marker genes in liver of pikeperch challenged with low oxygen saturation. Gene expression of candidate genes in collected liver samples of pikeperch. Normoxic (grey/empty circles) and low DO (red/filled circles) group after one, seven, fourteen, twenty-one and twenty-eight days of the experiment. Genes involved in stress (green) or immune response (blue) are either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent the mean of five individual samples (+SEM), calculated per 100 ng of total RNA. Statistical significance was determined between groups for each day using the multiple t-test (Holm–Šídák corrected), with alpha = 0.05; * < 0.05, ** < 0.01 and *** < 0.001.

3.2. Induction of Peritoneal Inflammation under Low DO Levels

3.2.1. SSI after Intraperitoneal Stimulation

To elucidate to which extent is the immune response of the host compromised by the reduced levels of DO, on day eight (Figure 1), we employed an adapted model of peritoneal inflammation established previously [28]. Upon the stimulation, we determined the SSI in all tested groups, the control group and low DO group, both either unstimulated (PBS as control) or stimulated with inactivated *A. hydrophila* (Figure 5).

The average spleno-somatic indices ranged from 0.040 to 0.077. The injection of inactivated bacteria resulted in an increase of the SSI in both the control and the low DO group at day one to three post-stimulation, with the most prominent and significant changes seen between unstimulated and stimulated fish of the low DO group at day

one. Furthermore, we noticed a slight, albeit nonsignificant, decrease in the SSI in the PBS-injected fish in the low DO group compared to normoxia.

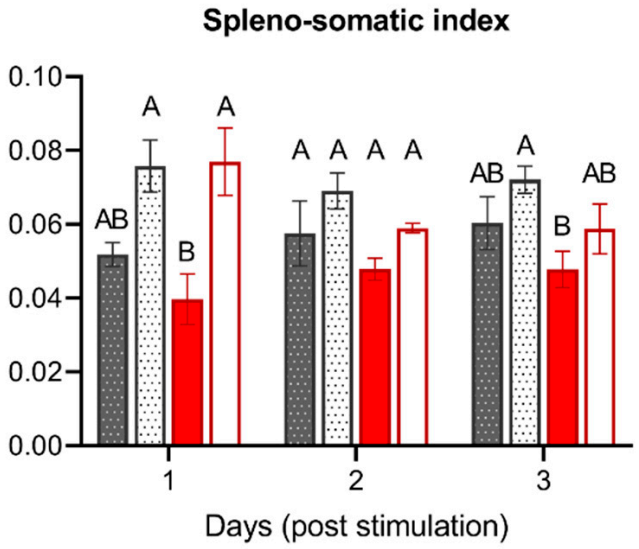

Figure 5. Spleno-somatic index of pikeperch during acute peritoneal inflammation. Spleno-somatic indices of pikeperch after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. The graph shows differences between normoxic control groups, either unstimulated (dark grey/patterned) or stimulated (bright grey/patterned), and low DO groups, either unstimulated (dark red) or stimulated (bright red). Columns represent mean of four individual samples (+SEM). Statistical significance per each day was determined using the one-way ANOVA followed by Tukey´s multiple comparison test ($p = 0.05$); different letters (A, B) represent significant changes between different control and low DO groups.

3.2.2. Leukocyte Migration upon Intraperitoneal Stimulation

To further evaluate the impact of low DO on acute inflammation, we analyzed the cell composition in the peritoneal cavity, blood, spleen and head kidney upon intraperitoneal injection with *A. hydrophila* (Figure 6A–C and Figure 7A–C).

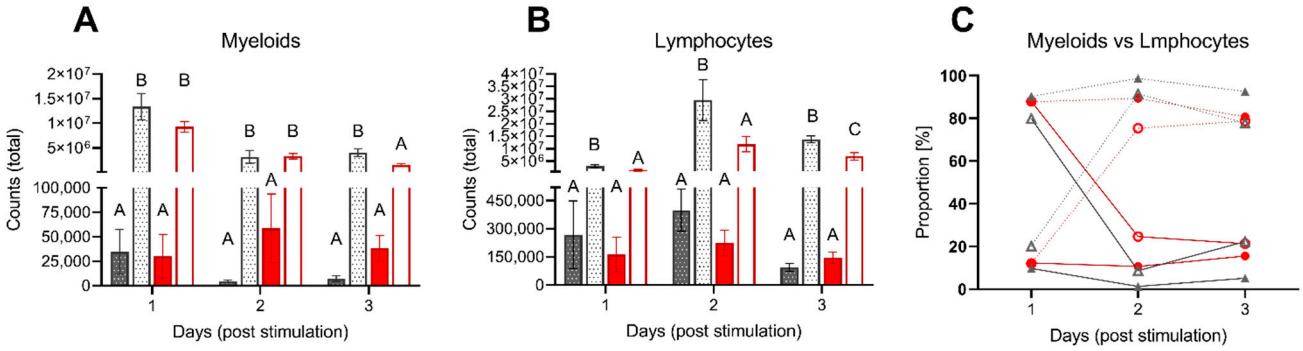

Figure 6. Kinetics of peritoneal leukocytes upon acute peritoneal inflammation. Total counts of myeloid cells (**A**), lymphocytes (**B**) and proportion of both cell types (**C**) after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. Graphs A and B show differences between normoxic control groups, either unstimulated (dark grey/patterned) or stimulated (bright grey/patterned), and low DO groups, either unstimulated (dark red) or stimulated (bright red). Columns represent mean of four individual samples (+SEM). Statistical significance per each day was determined using the one-way ANOVA followed by Tukey's multiple comparison test ($p = 0.05$); different letters (A–C) represent significant changes between different groups. Graph C shows differences between control groups, either unstimulated (grey, filled triangle) or stimulated (grey, open triangles), and the low DO groups, either unstimulated (red, filled circles) or stimulated (red, open circles). Dotted lines represent lymphocytes, filled lines represent myeloid cells.

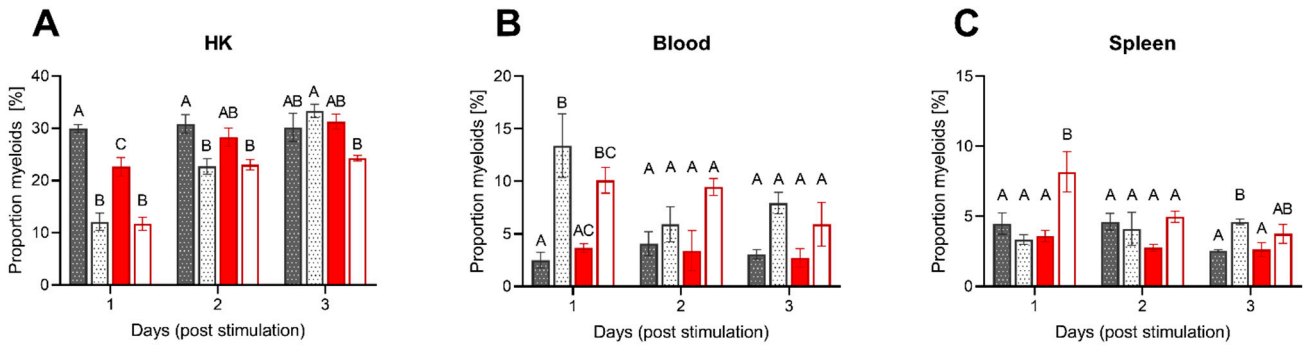

Figure 7. Proportion of myeloid cells within HK, peripheral blood and spleen of pikeperch upon intraperitoneal stimulation. Proportion (%) of myeloid cells in head kidney (HK; **A**), peripheral blood (**B**) and spleen (**C**) samples of pikeperch after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. The graph shows differences between normoxic control groups, either unstimulated (dark grey/patterned) or stimulated (bright grey/patterned), and low DO groups, either unstimulated (dark red) or stimulated (bright red). Columns represent mean of four individual samples (+SEM). Statistical significance per each day was determined using the one-way ANOVA followed by Tukey´s multiple comparison test ($p = 0.05$); different letters (A–C) represent significant changes between different groups.

While the PBS-injected fish did not undergo any remarkable changes in the number of cells in the peritoneal cavity and retained their original composition with 80–90% of lymphocytes and 10–20% of myeloid cells throughout the experiment, the injection of inactivated *A. hydrophila* led to rapid recruitment of myeloid cells to the peritoneal cavity, resulting in a complete change in its profile (Figure 6A–C). As soon as one day after the injection, the myeloid cells comprised over 80–90% of all peritoneal leukocytes (Figure 6C), reaching the total number of 1.4×10^7 and 9×10^6 in control and DO low groups, respectively (Figure 6A). In the following 24 h, the peritoneal niche underwent the second change in its composition, and the growing number of lymphocytes substituted the peak of myeloid cells. Their total number reached approximately 3×10^7 cells in the control group (Figure 6B), representing over 80% of all peritoneal leukocytes (Figure 6C). Notably, the recruitment of lymphocytes to the peritoneum of low DO fish was three times lower than in fish kept at normal oxygen saturation. On the third day, we observed a resolution of inflammation in both stimulated groups.

The recruitment of leukocytes to the peritoneal cavity was mirrored by the changes in the composition of blood and both systemic lymphoid organs. The changes were most prominent on the first two days when the stimulation with inactivated *A. hydrophila* decreased the proportion of myeloid cells in the HK from 30% observed in PBS injected controls to ~12% and ~23%, respectively (Figure 7A). In addition, we observed a significant decrease in the myeloid proportion of the low DO group (~23%) after the first day (without additional stimulation) compared to the control group (~30%) (Figure 7A). This decrease was complemented by the increased mobilization of myeloid cells to the peripheral blood, which increased from ~3% in PBS injected groups up to 13% in stimulated fish 24 h post-injection (Figure 7B). This has been further reflected by the increased ratio of myeloid cells in the spleen of low DO fish but not in the fish kept at standard oxygen saturation (Figure 7C). With the ensuing resolution of the inflammation in the peritoneal cavity, the proportion of myeloid cells in the blood decreased gradually to ~8%.

3.2.3. Gene Profiling in HK and Spleen during Acute Inflammation

We further aimed to evaluate the transcriptomic changes orchestrating the acute inflammation using established rt-qPCR analysis (Figure 8A–C and Figure 9A–C). Ten genes (*CSF2*, *EPAS1*, *FTH1*, *HIF1A*, *HMOX1*, *HSF1*, *HSF2*, *HSP90AA1*, *NR3C1* and *RAG1*) were exclusively determined in HK with additional five genes (*CXCL8*, *IL1B*, *MHC II alpha*, *MPO* and *TNF*) in both tissues. *TNF* was exclusively detectable in the spleen, with numbers only below 10 transcripts per 100 ng RNA in HK (data not shown).

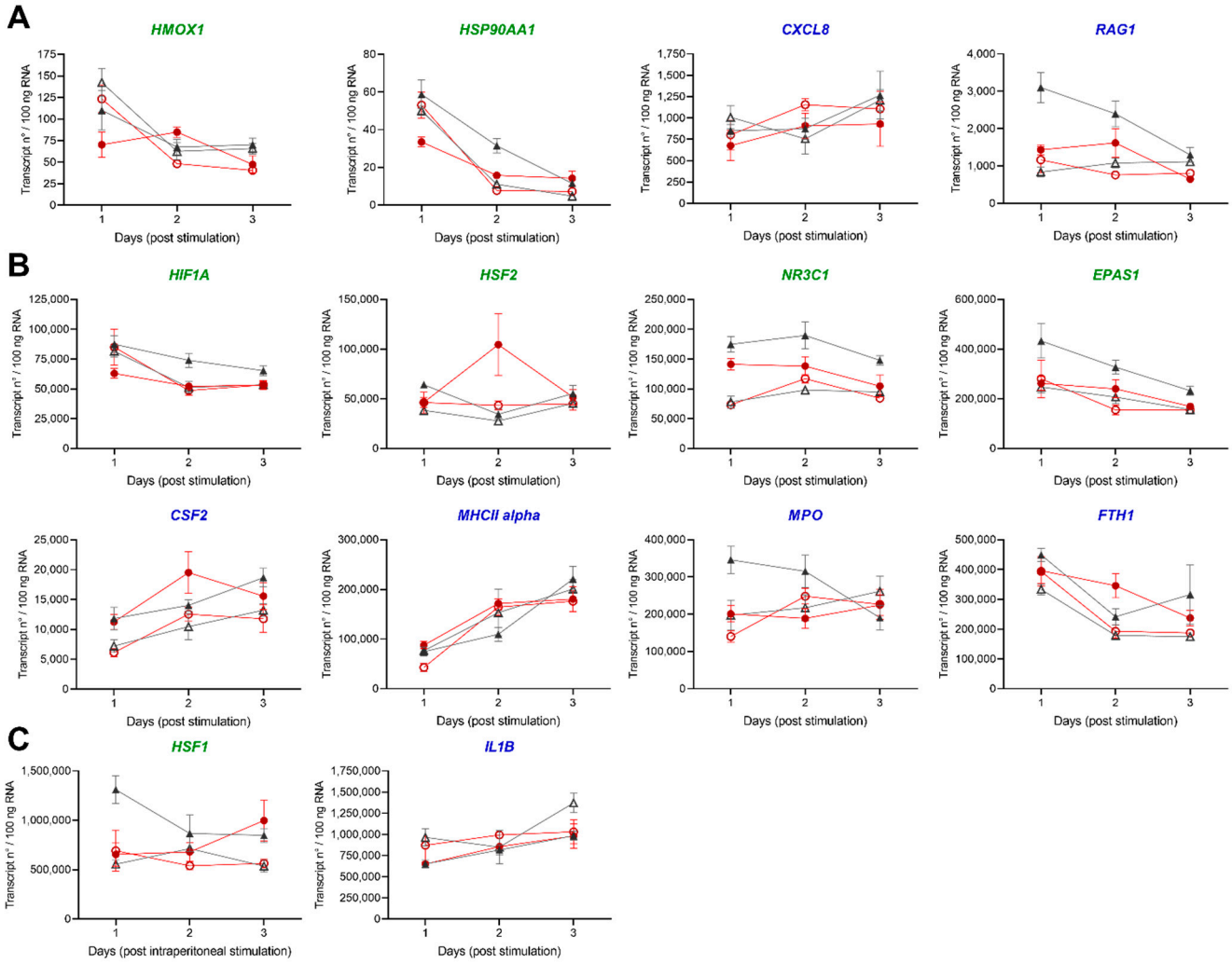

Figure 8. Expression of stress and immune markers in HK of pikeperch upon acute peritoneal inflammation. Expression of candidate genes in head kidney (HK) of pikeperch. Normoxic control group (grey), either unstimulated (grey/filled triangles) or stimulated (grey/empty triangles), and low DO treatment groups, either unstimulated (red/filled circles) or stimulated (red/empty circles) after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. Genes involved in stress (green) or immune response (blue) are either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent mean of four individual samples (+SEM), calculated per 100 ng of total RNA. Statistically significant differences were calculated via one-way ANOVA followed by Tukey's multiple comparison test ($p = 0.05$).

We detected the lowest copy numbers with transcripts only below 4×10^3 transcripts/100 ng RNA for five genes (*CXCL8*, *TNF*, *HMOX1*, *HSP90AA1* and *RAG1*) in HK and spleen of the individual fish (Figures 8A and 9A). For the three genes *HSF1*, *IL1B* and *MHCII alpha*, we determined copy numbers above 5×10^5 per 100 ng RNA in HK and spleen (Figures 8C and 9C).

In HK, we observed highly dynamic expression profiles of the selected genes. An intraperitoneal stimulation decreased the transcript levels of *FTH1*, *HMOX1*, *HIF1A* and *HSP90AA1* after two days of the experiment in the control and low DO group. *EPAS1*, *NR3C1* and *RAG1* showed lower copy numbers in stimulated control and low DO fish than the unstimulated control group on any day of the experiment. *CXCL8* transcript numbers decreased after stimulation in the control group and increased in the low DO group after two days. The transcript numbers of *MHCII alpha* increased two days post-stimulation in both groups (control and low DO).

In the spleen, the intraperitoneal stimulation led to a remarkable increase in expression of the genes coding for the inflammatory cytokines CXCL8 and IL1B, which reduced in

comparison to the levels seen in unstimulated fish two days post-stimulation. In contrast, *MHCII alpha*, *MPO* and *TNF* transcript levels increased in both groups (control and low DO) two days post-stimulation.

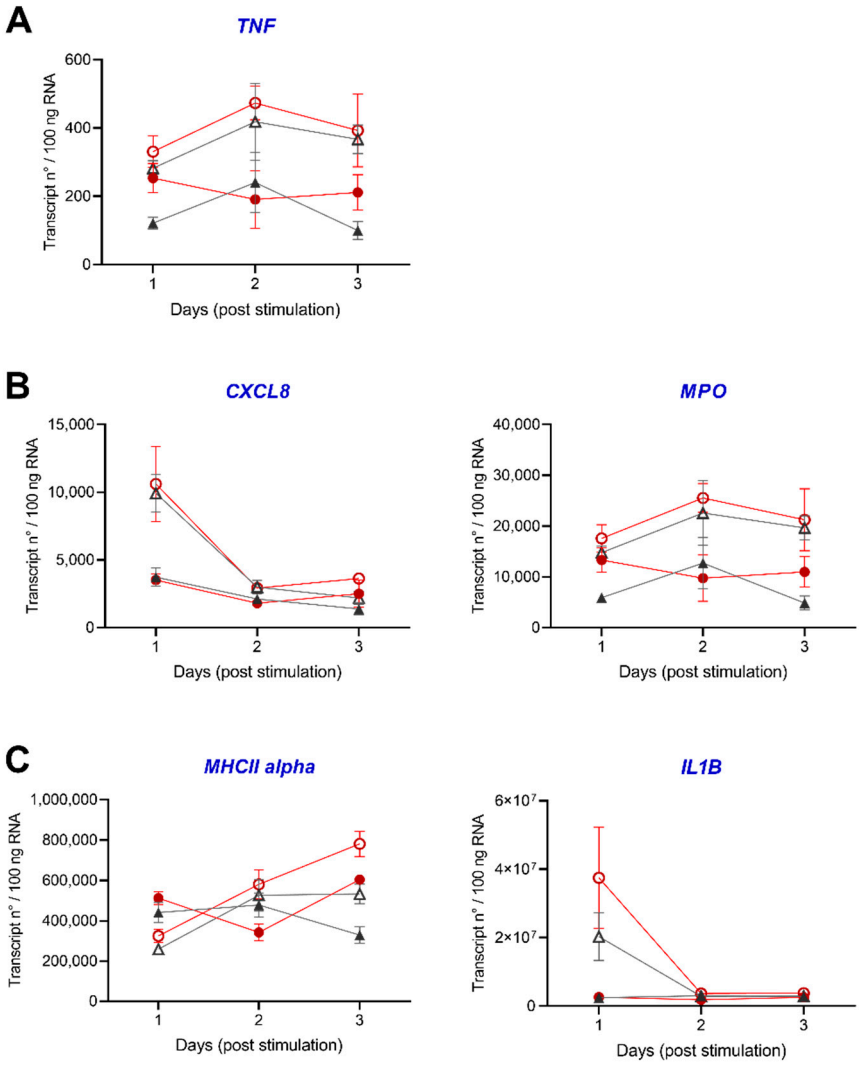

Figure 9. Expression of stress and immune markers in spleen of pikeperch upon acute peritoneal inflammation. Relative expression of candidate genes in spleen of pikeperch. Normoxic control group (grey), either unstimulated (grey/filled triangles) or stimulated (grey/empty triangles), and low DO groups, either unstimulated (red/filled circles) or stimulated (red/empty circles) after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. Genes involved in stress (green) or immune response (blue) are either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent mean of four individual samples (+SEM), calculated per 100 ng of total RNA. Statistically significant differences were calculated via one-way ANOVA followed by Tukey's multiple comparison test ($p = 0.05$).

4. Discussion

4.1. The Hypoxic Challenge of 40% DO Does Not Induce Substantial Changes in Major Health Parameters

Exposure to stress stimuli initiates a cascade of physiological mechanisms, allowing the mobilization of energy to cope with stressors and restore homeostasis. In teleost fish, like in other vertebrates, several markers, including blood parameters and health indices, are frequently applied as surrogates of the adaptation response. Apart from the spleno-somatic index, reflecting the immune status, or hepato-somatic index, representing the

organism's metabolic rate, the blood cortisol serves as the primary indicator of the ongoing stress response. Its increasing levels regulate a vast array of processes directing the energy metabolism toward the mobilization of hepatic glycogen and an increased availability of glucose to facilitate the successful adaptation to the stressor [32–35]. Unexpectedly, none of the examined parameters showed levels outside the physiological range or significant differences between the low DO group and the control group in the current study. Cortisol is an excellent marker for acute stress, including low DO levels, but its reliability under chronic conditions is uncertain due to its fast release and clearance [36–39].

The levels of free glucose are in line with former studies in pikeperch, whereas observed lactate levels were lower [40,41]. Both physiological parameters have been shown to increase after an acute short-term low DO event [42]. However, our findings are concordant with the study of O'Connor et al. (2011), in which different populations of three-spined stickleback (*Gasterosteus aculeatus*) showed no significant changes in whole-body cortisol glucose and lactate levels after a week of low oxygen conditions (2.2 mg/L DO) [43]. Similarly, Douxfils et al. (2014) reported a fast return to basal cortisol and glucose levels after a response to low oxygen saturation in juvenile Eurasian perch [44]. In common carp (*Cyprinus carpio*), unaltered HSI was detected after long-lasting low DO levels [45]. Overall, the absence of significant changes in the studied health parameters indicate relatively high tolerance of pikeperch to low levels of DO and high pace of adaptation responses preserving the homeostasis even at ±3.2 mg/L DO.

4.2. Effects of Low DO on Cell Distribution and Gene Expression

Previous studies have shown that inadequately low oxygen saturation results in modifications of the innate and adaptive immunity in fish and alters the cell composition in main lymphoid organs [46–52]. In the presented study, we employed flow cytometry to elucidate the changes induced by the chronic exposure to low DO in blood and head kidney. Interestingly, our results revealed an increased proportion of myeloid cells, both in circulation and in the lymphoid organs of low DO fish, reflecting a potentially higher mobilization of the immune system. This observation belongs to one of the hallmarks of the conserved transcriptional response to adversity and is in accord with an increased rate of circulating myeloid cells in the blood of maraena whitefish (*Coregonus maraena*) exposed to crowding stress [53], higher mobilization of myeloid cells to the blood of gilthead seabream (*Sparus aurata*) after exposure to short-term stress [54] or increased number of circulating myeloid cells observed in mammalian models of stress responses [55,56]. Induction of erythropoiesis at low DO levels, which increases oxygen transport in the blood, has been observed previously in teleosts [57]. Nevertheless, we did not observe any substantial increase in the number of circulating erythrocytes throughout the experiment (data not shown). Therefore, oxygen levels of ±3.2 mg/L DO may be classified as a hypoxic condition for pikeperch, but not as a situation of severe hypoxia.

The detected transcript patterns for the eight examined stress- and immune-relevant genes further illustrate the weak response to low DO. Although the examined genes were previously demonstrated to be responsive to hypoxic conditions or belong to downstream targets of hypoxia-inducible factor 1 alpha (HIF1A), which regulates the hypoxia response pathway, our analysis revealed only slight downregulation of their expression. More specifically, HIF1A is essential for the response of hypoxic fish with complex physiological and biochemical modifications involving the immune system [58,59]. In large yellow croaker (*Larimichthys crocea*), severe hypoxic conditions (1.6 ± 0.2 mg/L DO) for two days resulted in an upregulation of most immune genes, as well as *HIF1A*, in HK [60]. According to these investigations, we expected significant changes within the transcription in hypoxic challenged pikeperch, with increased expression of *HIF1A* and other stress marker genes such as *NR3C1* and *HSP90AA1*. However, we detected a prominent down-regulation of *HIF1A* transcript numbers in the head kidney of pikeperch, with significant differences at days 14 to 28 of the experiment, but relatively stable transcript levels in the liver. In European bass (*Dicentrarchus labrax*), acute hypoxic conditions of 1.9 mg/L DO for 4 h, and

chronic conditions of 4.3 mg/L DO for 15 days cause an elevated *HIF1A* transcription in the liver [61]. The hypoxia-sensitive percid species Eurasian perch showed up-regulated HIF1A transcription in the liver after an acute hypoxic oxygen saturation of 0.4 ± 0.1 mg/L DO for 1 h, but not after 15 days of 2.8 ± 0.3 mg/L DO [62]. Mohindra et al. (2013) observed in the hypoxia-tolerant Indian catfish (*Clarias batrachus*) a significantly up-regulated expression of HIF1A in the liver and down-regulation in the head kidney after 1 h of 0.98 mg DO per liter. Whereas, after another 5 h HIF1A was significantly up-regulated in the head kidney [63]. A down-regulation of HIF1A transcription in response to hypoxia stress was suggested to be the outcome of a hypoxia shock [64,65]. They concluded that the transcriptional regulation of HIF1A is a complex and tissue- and species-dependent process. This further suggest that the range of tolerance of pikeperch reared in intensive aquaculture is hardly impacted at DO saturations of 40%. It did not establish a severe stress response or severe immune suppression within 28 days. Collected data rather indicate an ongoing adaptation process already after 24 h lasting till day 21. Nevertheless, the obtained gene expression data are based on preselected candidate genes and global gene expression analyses, such as RNAseq or microarray-based analyses, might uncover yet not considered but regulated genes and pathways after DO decrease that influence or could affect homeostasis and fish welfare.

4.3. Acute Inflammation Is Moderately Influenced by Low DO

Previously, several observations suggested a negative impact of hypoxic conditions on the fish immune system [48,66,67]. To provide a deeper understanding of this phenomenon, we used a previously established model of acute peritoneal inflammation to evaluate how, and to what extent, acute inflammation is impaired by low DO. Generally, the processes driving the acute inflammation followed a pattern described previously in other fish species [28,68]. The injection of inactivated *A. hydrophila* induced a rapid mobilization of myeloid cells from head kidney and their release into the circulation [69,70]. Consequently, within 24 h post-injection, we observed increased SSI in both stimulated groups and a considerably increased expression of the myeloperoxidase (MPO), a key marker of granulocytes in the spleen [50]. In spleen, the detected increase of MPO lasted till the end of the experiment at 72 h. In the head kidney, a stimulation resulted in reduced transcript levels of MPO in both groups reflecting the efflux of granulocytes into the circulation. A depletion of neutrophils in head kidney after peritoneal inflammation has been detected in the goldfish (*Crassius auratus*) by Bielek et al. (1999) [71]. Simultaneously, we observed a dramatic increase in the number of myeloid cells in the peritoneal niche. Notably, in line with the aforementioned results, only marginal differences in the number of recruited cells were seen between the normal and low DO fish. On a molecular level, the rapid recruitment of myeloid cells into the peritoneal cavity was complemented by the increased production of proinflammatory cytokines (particularly CXCL8 and IL1B) in the spleen and head kidney of studied fish [72,73]. For both cytokines, the increase in gene expression was more pronounced in the stimulated spleen. Within HK the production of the major pro-inflammatory cytokine TNF was independent of the treatment marginally low (data not shown) [74]. While in spleen, higher expression was detectable for both groups one day post-injection and this increase persisted until the third day of the stimulation. The primary source of TNF is activated macrophages which, therefore, might also be involved in the detectable increased SSI [72].

Within the following 24 h, we witnessed a resolution of the acute inflammation, manifested by decreasing expression of inflammatory cytokines, reduced presence of myeloid cells in the circulation and an influx of lymphocytes into the peritoneal cavity. Strikingly, although we did not observe a strong influence of hypoxia on the recruitment of myeloid cells, the number of lymphocytes differed considerably between both groups, reaching almost three times lower numbers in the low DO group.

Taken together, these findings support the notion of dichotomy in the effect of hypoxia on the innate and adaptive arm of immunity, suggested by previous findings from mammalian models. Thus, while the innate immune cells, and granulocytes in particular,

are better equipped to maintain viability and functionality under hypoxic conditions, the lymphocytes require high energy metabolism coupled with sufficient oxygen availability for their survival and effective development of effector functions [75,76]. Similarly, in the present study, the recruitment, and the effector functions of the myeloid during the acute inflammation were comparable between both groups, while the influx of lymphocytes was impaired by the low DO, suggesting high evolutionary conservation of these processes in the tree of life.

5. Conclusions

In their natural habitats, fishes would avoid low oxygen levels by simply escaping the current situation. However, this option is not available in rearing tanks of intensive aquaculture facilities. In the present study, we evaluated the effect of hypoxic conditions (±3.2 mg/L DO) on the health and immune status of pikeperch reared in RAS. We defined stable blood parameters, slightly downregulated gene expression (*FTH1*, *HIF1A* and *NR3C1*) and a functional acute inflammatory response towards bacterial stimulation. Our results confirmed that pikeperch do not develop severe responses or immunosuppression at hypoxic conditions and together with our previous study investigating the challenge of rising water temperatures in pikeperch [29], indicates that pikeperch in aquaculture may not be as sensitive to common environmental stressors as previously thought.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10070649/s1, Table S1: Individual values of blood and health parameters measured in this study.

Author Contributions: A.R.: Methodology, Validation, Writing—Review & Editing, Supervision. J.M.: Investigation, Resources. M.V.: Methodology, Investigation, Validation, Writing—Review & Editing, Supervision. N.S.: Conceptualization, Project administration, Methodology, Funding acquisition, Investigation, Formal analysis, Validation, Writing—Original, Writing—Draft, Review & Editing, Visualization. R.M.B.: Resources. T.G.: Conceptualization, Funding acquisition, Writing—Review & Editing, Supervision. T.K.: Conceptualization, Project administration, Methodology, Investigation, Formal analysis, Validation, Writing—Review & Editing, Supervision, Resources. V.S.: Funding acquisition, Writing—Review & Editing, Resources. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the terms of the principles of laboratory animal care and national laws act 246/1992 of the Czech Code of Law (Ref. no. 22761/2009-17210).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this manuscript and its supplementary materials and are available on request from the corresponding authors.

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Příloha č. 5

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Effects of Garlic *Allium sativum* Powder on Nutrient Digestibility, Haematology, and Immune and Stress Responses in Eurasian Perch *Perca fluviatilis* Juveniles

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Simple Summary: Herbal medicine feed supplements are used as growth promoters, immune system stimulants, and to combat stress. We evaluated the effects of garlic powder in the diet of European perch. The inclusion of garlic powder was shown to improve whole body composition, feed digestibility, and biochemical and immunohematological effects, and increased resistance against overcrowding stress.

Abstract: The supplementation of fish diets with phytogenics can increase growth performance and can modulate immune system response. European perch *Perca fluviatilis* (initial weight 25.0 ± 0.4 g) were fed a diet including 0 (Control), 10 (G10), 20 (G20), and 30 (G30) g kg^{-1} garlic powder. No significant difference in the growth parameters and somatic indices were observed. Significantly higher fat digestibility was observed in G10 and G30 diets compared to in the control and G20 diets($p < 0.05$). Significantly greater red blood cell and white blood cell counts were observed with the G10 diet ($p < 0.05$). Garlic significantly decreased serum cholesterol in all of the experimental groups. Serum albumin was significantly higher in the G10 and G20 diets ($p < 0.05$). Immediately after the overcrowding stress challenge, the garlic groups showed significantly higher cortisol levels than the control group, while no significant difference was observed in the glucose concentration among groups. At 1 h post-stress, all of the groups that had been fed a garlic-supplemented diet showed lower cortisol levels than the control group, and this trend was maintained at 6 and 24 h post stress ($p < 0.05$), and glucose level in all garlic groups was significantly lower than control ($p < 0.05$). Garlic at 10 g kg^{-1} in feed can improve apparent fat digestibility and selected blood parameters and can enhance resistance against high-density and net handling stress in Eurasian perch.

Keywords: aquaculture; cortisol; fish; haematology; immunology; myeloid cells; stress

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1. Introduction

Commercial production of fish, shellfish, and seafood is projected to increase by approximately 62% by 2030 [1]. Thus, the aquaculture rearing system is changing from an extensive system to intensive and semi-intensive systems [2], which might increase the chance of infectious disease outbreaks occurring [3]. Botanical derivatives and extracts, also known as phytogenics, have been used in fish diets as natural growth promoters and as immune stimulants. Currently, many plant extracts are considered as safe and cost-effective additives to aquafeed [4]. Antibiotics that can control pathogens on fish farms present concerns with respect to consumer health, animal welfare, and environmental pollution [5].

Garlic *Allium sativum* belongs to the *Liliaceae* family [6]. It has long been used as a herbal medicine and may be relevant to aquaculture because of its immunostimulant properties [7]. Garlic contains alliin, which has sulfur compounds including gamma-

glutamyl-s-allyl-cysteine and S-allyl-L-cysteins sulfoxides. Moreover, garlic contains allicin (diallyl thiosulfate), which is responsible for garlic's typical pungent smell and its medicinal properties [6], and other bioactive compounds, including vitamins (ascorbic acid, thiamine and riboflavin), minerals (potassium, phosphorus, calcium, magnesium, sodium, iron, selenium, and germanium), flavonoids (phenolic acids) [8], and amino acids [9]. Garlic powder has been reported to promote growth in Japanese seabass (*Lateolabrax japonicus*) [10] and rainbow trout *Oncorhynchus mykiss* [11] and to improve body composition in brown trout (*Salmo caspius*) [12]. However, garlic powder was not shown to improve growth performance in Asian sea bass (*Lates calarifer*) [13] or rohu (*Labeo rohita*) [14]. In addition, it has been reported to increase apparent nutrient digestion in rainbow trout [15] and Nile tilapia (*Oreochromis niloticus*) [16]. Dietary garlic powder has shown a favourable effect on blood total protein, albumin, and phagocytic activity in rainbow trout [17,18]; this plant increased fish resistance to ammonia stress [19]. Garlic powder increased immunoglobulins in European seabass (*Dicentrarchus labrax*) [20]. Garlic microencapsulated extract improved growth performance, body proximate composition, and biochemical and immunohematological parameters in rainbow trout (*Oncorhynchus mykiss*) [21]. These studies, regardless of the form of garlic presentation within diets, suggest that garlic may be used as an alternative to antibiotics [22].

Eurasian perch (*Perca fluviatilis*) is a carnivorous percid fish inhabiting northern Eurasia [23]. It is a domesticated species with a wide habitat range, and can be found in brackish water, estuaries, and rivers in recent decades [24], showing potential for European inland culture [23]. Eurasian perch can be a suitable species for recirculation aquaculture system (RAS) production and intensive culture [25], but handling through counting, sorting, and tank cleaning as well as high density stocking, may potentially increase energy consumption and decrease feed intake and growth [26].

Regarding to use of Eurasian perch in RAS culture in recent decades [25], the aim of the present study was to investigate the effects of garlic powder in feed on growth performance, body proximate composition, apparent nutrient digestibility, selected blood and immune parameters, and resistance to high-density and net handling stress in Eurasian perch juveniles.

2. Materials and Methods

2.1. Ethics Approval

The experimental procedures were performed under guidelines of the European Communities Directive (No. 2010/63/EU) on the protection of animals used for scientific purposes and have been approved by the Czech Ministry of Health (MSMT–6744/2018–2).

2.2. Preparation of Garlic Powder and Feed

Garlic powder was purchased from EQUISERVIS, Prague, in Czech Republic. Garlic powder was produced by Pommier Nutrition, Thymerais—France. (Accessed: 4 June 2019) (www.pommier-nutrition.com). Experimental feeds (Table 1) were extruded at Exot Hobby s.r.o. (Černá v Pošumaví, Czech Republic), packed in plastic vacuum bags, and stored at 4 °C until use. In the present study, corn meal was replaced by 10, 20, and 30 g garlic powder per kilogram of diet feed. The proximate composition of the basal diet, including dry matter, crude protein, crude fat, and ash, was 93.48%, 47.20%, 16.33%, and 8.91%, respectively (Table 1).

2.3. Experimental Design

Eurasian perch juveniles with an initial weight of 25.0 ± 0.4 g were obtained from Anapartners s.r.o fish farm (Prague, Czech Republic). Fish were transferred to the aquaria at the Institute of Aquaculture and Protection of Waters (České Budějovice, Czech Republic) and were fed a basal diet formulation (Table 1) without garlic powder for a 14-day acclimation period before the feeding trial [12]. After adaptation, 1320 fish were randomly distributed into twelve 150 L tanks (110 fish per tank) with a water flow rate 10 L min^{-1} in RAS [25].

Each diet was tested with three replicates. Fish were fed manually to apparent satiation at 08:00, 12:00, and 16:00 for 87 days, and uneaten feed was collected at maximum of 30 min after each meal. Water temperature, pH, and dissolved oxygen (DO) were measured daily by an HQ40D portable multi-meter (Hach Lange GmbH, Düsseldorf, Germany) and were maintained at 22.1 ± 0.5 °C, pH 7.14 ± 1.61, and DO 8.16 ± 0.42 mg L^{-1}, respectively. The photoperiod was 12L:12D [27].

Table 1. Proximate composition of experimental diets with and without garlic powder.

Ingredients (g kg^{-1})	Control	G10	G20	G30
Fish meal	271	271	271	271
Soybean concentrate	290	290	290	290
Corn meal	97	87	77	67
Soybean meal	128.5	128.5	128.5	128.5
Garlic powder [a]	0	10	20	30
Fish oil	77	77	77	77
Rapeseed oil	58	58	58	58
Methionine [b]	8	8	8	8
Lysine [c]	5	5	5	5
Valine [d]	2	2	2	2
L-Threonine [e]	0.5	0.5	0.5	0.5
Vitamins & minerals [f]	8	8	8	8
Binder [g]	50	50	50	50
Yttrium oxide (Y_2O_3) [h]	5	5	5	5
Proximate composition analysis				
Dry matter%	93.48	93.54	93.71	93.17
Crude protein%	47.20	46.84	46.59	46.33
Fat%	16.33	16.51	15.98	16.13
Ash%	8.91	8.78	8.78	8.82
Fiber%	1.82	3.87	3.98	2.42
Nitrogen-free extract (NFE) [f,i]	19.22	17.54	18.38	19.47
Gross energy (Kj g^{-1}) [g,j]	17.90	18.24	17.99	17.72

[a] Garlic powder was purchased from EQUISERVIS, Prague, in Czech Republic. [b] Adisseo, Shanghai, China; [c] Inner Mongolia Eppen Biotech Co., Ltd., Ningxia, China.; [d] Ajinomoto Animal Nutrition Europe; [e] Ningxia Eppen Biotech, China; [f] Aminovitan Sak, Trouw Nutrition Biofaktory s.r.o, Prague, Czech Republic; [g] binder (NutriBind, Adisseo, Shanghai, China) (3.0%); [h] yttrium oxide (Y_2O_3), Sigma, Ronkonkoma, NY, USA; [i] nitrogen-free extracts (NFE) = dry matter—(crude protein + crude lipid + ash + fiber) [15]; [j] gross energy was calculated according to following formula: gross energy (MJ/kg) = (protein × 23.6 kJ g^{-1}) + (fat × 39.5 kJ g^{-1}) + (carbohydrates × 17.2 kJ g^{-1}) [15]; control: without garlic supplement; G10: 10 g garlic powder per 1000 g diet; G20: 20 g garlic powder per 1000 g diet; G30: 30 g garlic powder per 1000 g diet.

2.4. Growth Performance

At the end of the feeding trial, feed was withheld for 24 h. The fish were anesthetized by tricaine methane sulphonate (MS-222) at 200 mg L^{-1} water [21] and counted, and individual length and weight were measured. Growth performance and survival rate were calculated [21]. Final body weight (FBW), feed intake (FI), weight gain (WG), weight gain percentage (WG%), feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), survival rate (SR%), condition factor (CF%), hepatosomatic index (HSI), and viscerosomatic index (VSI) were calculated as follows:

FBW (g) = Final body weight
WG (g) = [Final body weight (g) − Initial body weight (g)]
WG% = [Final body weight (g) − Initial body weight (g)]/initial body weight (g) × 100
FI (g/fish) = dry feed consumed/number of fish
PER = Weight gain (g)/total protein intake (g)
FCR = dry feed intake (g)/WG (g)
SGR (% day^{-1}) = 100 × [ln final body weight (g) − ln initial body weight (g)]/time (days)
SR% = (number of fish after test/initial number of stocked fish) × 100
CF% = [fish body weight (g)/(fish length)^3(cm)] × 100
HSI% = [liver weight (g)/body weight (g)] × 100

VSI% = [viscera weight (g)/body weight (g)] × 100

2.5. Whole Body Proximate Composition

At the end of experiment, two fish per each tank (n = 6 per group) were randomly selected. Fish were anesthetized with MS-222 at 200 mg L^{-1} water [21] and were killed by a sharp blow to the head. The entire fish body was ground, packed individually into plastic bags, and stored at −20 °C for whole body proximate composition analysis. Both body and feed proximate composition analyses were conducted according to the methods of the Association of Official Analytical Chemists (AOAC) [28]. Crude fat was analyzed by the extraction method using hexane–isopropanol (3:2) according to Hara and Radin, [29] with slight modifications [30]. Crude protein was measured using the Kjeldahl method (BUCHI Labortechnik AG, type K-360, Königswinter, Germany) [12]. Dry matter was analyzed by drying in a NÜVE type FN 400P oven (NÜVE, Ankara, Turkey) at 105 °C to a stable weight [21]. Ash was analyzed in a muffle furnace L 40/11 BO (Nabertherm GmbH, Lilienthal, Germany)) at 550 °C for 4 h [12].

2.6. Apparent Digestibility Coefficients

Diets contained 5 g kg^{-1} yttrium oxide (Y_2O_3) as an inert marker (Table 1). Two hours after the final daily meal, the tanks were brushed and cleaned, and the remaining faeces were discarded. After cleaning, the faeces were collected overnight by sieving every two hours from outlets until the first daily feeding in the morning. The faeces from each tank were stored separately at −20 °C after centrifugation at 3000 rpm for 15 min for analysis [31]. The Y_2O_3 in the diet and faeces was measured using inductively coupled plasma emission spectrometry following digestion with nitric acid at 180 °C for 48 h. The apparent digestibility coefficients (ADC) of the dry matter, protein, and fat were calculated with the following formula [15]:

% digestibility = 100 × 100 [(yttrium in feed/yttrium in faeces) × (nutrient in faeces/nutrient in feed)].

2.7. Haematology and Biochemistry

After 24 h starvation to induce the post-absorptive condition, two fish from each tank (n = 6 per group) were randomly netted and anesthetized by tricaine methane sulphonate (MS-222) at 200 mg L^{-1} water [21]. Duplicate blood samples were drawn from the caudal vein into heparinized and non-heparinized sterile syringes. The heparinized blood samples were transferred to heparinized Eppendorf tubes and were placed on ice for haematological analysis. The non-heparinized blood samples were transferred to non-heparinized Eppendorf tubes, and samples were left on ice for 2 h to clot [32]. The serum was subsequently separated by centrifugation (Heraeus Megafuge 16 R Centrifuge) at 4200 rpm for 15 min at 4 °C and was stored at −80 °C until analysis [33]. Red blood cells (RBC), white blood cells (WBC), and subpopulations were counted according to a modified method of Korytář et al. [34]. The heparinized blood (10 µL) was diluted in 200 µL RPMI medium on ice for cell composition evaluation by a FACS Canto flow cytometer (Heidelberg, Germany). The biochemical parameters of the blood were assessed using an Abbott Architect c8000 clinical chemistry analyzer (Abbott, Chicago, IL, USA) and assay kits [19] according to manufacturer's instructions, as follows: serum total cholesterol, B7D6C7 G3-5321/R02 (Abbott, Chicago, IL, USA); triglycerides B7D7E7 G3-9334/R03 (Abbott, USA); alanine aminotransferase (ALT), B8L9x7 G5-4432/R05 (Abbott, USA); aspartate aminotransferase, G8-1502/R06 B8LY7 (Abbott, USA); albumin, 7D53-2030-3927/R6 (Abbott, USA); total protein, G6-6667/R04 B7D7D7 (Abbott, USA); and glucose, B3L8X7 G3-5375/R02) (Abbott, USA). Cortisol levels were analyzed with a cortisol assay kit (L2KCO2) using an immunochemistry analyzer Immulite 2000Xpi Siemens (Siemens Healthcare GmbH, Erlangen, Germany) at the Stafila laboratory, České Budějovice, Czech Republic.

2.8. Respiratory Burst and Phagocytic Activity

Two fish per tank (n = 6 per group) were anesthetized with MS-222 (200 mg L^{-1} water) [21]. The head kidney was removed, and the leukocytes were separated by pushing them through a nylon sieve with RPMI-1640 medium, according to the method by Biswas et al. [35]. A respiratory burst activity assay was conducted using nitro blue tetrazolium with minor modifications according to Zaineldin et al. [36]. Briefly, the leukocyte suspension was transferred into 96-well plates, and an equivalent volume of 0.2% nitro blue tetrazolium solution (Sigma, Ronkonkoma, NY, USA) was added to each well and was incubated for 30 min at room temperature. After incubation, N-dimethylformamide (Sigma, USA) was added and centrifuged for 5 min at 3000 rpm. The respiratory burst activity was reported as the mean fluorescence intensity.

Phagocytic activity was assessed using a modified method from Morimoto et al. [37]. The leukocytes of head kidney were separated by washing with PBS (5×10^5 cells mL^{-1}) and were incubated with latex beads at 25 °C for 2 h, after which cell-related fluorescence was evaluated, and the samples were transferred into 96-well plates and assessed with a FACS Canto flow cytometer (Heidelberg, Germany) to detect the fluorescence of the beads engulfed by the phagocytic cells.

2.9. High-Density and Net Handling Stress Challenge

At the end of the feeding period, working with one tank at a time, the volume of water was decreased to leave the fish in a high-density condition (0.67 kg/L) for one minute with adequate aeration to avoid additional stress [38]. The fish were netted and removed from the water for 30 s [27] and were then returned to the tank, where the water level of the tank was increased back to the original volume, and the density was reduced [38]. Immediately after, two fish per tank were randomly selected (n = 6 per group), and the tank was refilled. The fish were anaesthetized with MS-222, and 1 mL of blood was drawn from the caudal vein with non-heparinized sterile syringes. All of these fish were killed after sampling. Sampling was conducted prior to the stress challenge, immediately after stress, and 1, 6, and 24 h post-stress [27]. Blood sampling in each tank was completed within 5 min.

2.10. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics v. 22 (Armonk, NY, USA). Data normality and homogeneity were checked using the Kolmogorov–Smirnov test. Data were analyzed by one-way ANOVA. Significant differences among the mean values was set at $p < 0.05$ using the Duncan test. In addition, to determine if the effect was linear and/or quadratic, a follow-up trend analysis using orthogonal polynomial contrasts was performed. The results are presented as mean ± SD (standard deviation of the mean).

3. Results

3.1. Growth Performance and Feed Utilization

No significant differences in growth performance and feed utilization, including final weight, weight gain, weight gain percent, specific growth rate, feed intake, feed conversion ratio, protein efficiency ratio, or survival rate, were observed among the groups ($p > 0.05$). The condition factor was significantly lower in the G30 diet group compared to the other groups ($p < 0.05$). In particular, the condition factor significantly linearly decreased with the increasing dietary garlic powder levels ($p = 0.04$). In addition, no significant differences in the viscerosomatic or hepatosomatic index were found ($p > 0.05$). (Table 2).

Table 2. Growth performance and feed utilization of juvenile Eurasian perch consuming feed supplemented with garlic powder.

							Linear Trend		Quadratic Trend	
Parameters	**Control**	**G10**	**G20**	**G30**	**ANOVA**	η_p^2	***P*-Value**	**R^2**	***P*-Value**	**R^2**
Initial weight (g)	24.75 ± 0.47	25.37 ± 0.41	24.77 ± 0.13	25.18 ± 0.18	0.13					
Final weight (g)	66.29 ± 1.99	67.39 ± 1.89	64.89 ± 1.53	65.52 ± 2.93	0.55					
Feed intake (g fish^{-1})	68.49 ± 1.82	68.41 ± 1.06	68.00 ± 0.80	67.83 ± 1.76	0.92					
Weight gain (g)	41.53 ± 2.02	42.02 ± 1.51	40.12 ± 1.40	40.34 ± 3.09	0.65					
Weight gain%	167.82 ± 9.25	165.55 ± 3.67	161.97 ± 4.83	160.29 ± 13.38	0.71					
Feed conversion ratio	1.68 ± 0.15	1.64 ± 0.07	1.73 ± 0.08	1.71 ± 0.16	0.85					
Specific growth rate (% day^{-1})	1.12 ± 0.04	1.11 ± 0.01	1.10 ± 0.02	1.09 ± 0.05	0.76					
Protein efficiency ratio	1.25 ± 0.09	1.29 ± 0.03	1.24 ± 0.07	1.26 ± 0.13	0.90					
Survival rate (%)	96.36 ± 2.40	98.18 ± 0.91	96.96 ± 1.89	97.57 ± 2.78	0.75					
Condition factor%	1.25 ± 0.05 ^{b}	1.24 ± 0.02 ^{b}	1.18 ± 0.03 ^{b}	1.10 ± 0.03 ^{a}	0.00	0.78	0.04	0.33	0.05	
Hepatosomatic index%	1.59 ± 0.36	1.56 ± 0.28	1.53 ± 0.37	1.38 ± 0.30	0.44					
Viscerosomatic index%	12.06 ± 2.28	13.13 ± 2.17	12.88 ± 1.56	13.19 ± 1.85	0.49					

Values are presented as (mean ± SD; n = 110). Mean values with different superscripts within a row vary significantly according to one-way ANOVA ($p < 0.05$). R^2 = R squared. η_p^2 = partial eta squared. Control: without garlic supplement; G10: 10 g garlic powder per 1000 g diet; G20: 20 g garlic powder per 1000 g diet; G30: 30 g garlic powder per 1000 g diet.

3.2. Body Proximate Composition

No significant differences were observed the among groups in terms of whole body dry matter, fat, or ash ($p > 0.05$). The level of protein in fish consuming the G30 diet was significantly higher than the G1 group ($p < 0.05$), but there were no significant differences among the controls, G10, and G20 groups ($p > 0.05$) or among the controls, G20, and G30 groups ($p > 0.05$). There was a significant linear ($p = 0.01$) and quadratic ($p = 0.04$) trend regarding the dietary garlic powder level for body protein content, where body protein content decreased with the inclusion of garlic powder at G10 and then increased with the inclusion of garlic powder at G20 and G30. (Table 3).

Table 3. Body proximate composition of Eurasian perch consuming feed supplemented with garlic powder.

							Linear Trend		Quadratic Trend	
Parameters	**Control**	**G10**	**G20**	**G30**	**ANOVA**	η_p^2	***P*-Value**	**R^2**	***P*-Value**	**R^2**
Dry matter%	32.08 ± 1.15	31.75 ± 1.33	32.10 ± 1.71	32.26 ± 1.09	0.92					
Fat%	11.16 ± 1.50	10.96 ± 1.44	10.78 ± 1.13	9.76 ± 1.24	0.29					
Protein%	17.42 ± 0.60 ^{ab}	16.98 ± 0.69 ^{a}	17.39 ± 0.98 ^{ab}	18.31 ± 0.67 ^{b}	0.04	0.33	0.01	0.23	0.04	0.25
Ash%	3.38 ± 0.43	3.01 ± 0.60	3.16 ± 0.19	3.53 ± 0.29	0.16					

Values are presented as (mean ± SD; n = 6). Mean values with different superscripts within a row vary significantly according to one-way ANOVA ($p < 0.05$). R^2 = R square. η_p^2 = partial eta squared. Control: without garlic supplement; G10: 10 g garlic powder per 1000 g diet; G20: 20 g garlic powder per 1000 g diet; G30: 30 g garlic powder per 1000 g diet.

3.3. Apparent Digestibility Coefficient (ADC%)

Significantly higher dry matter digestibility was observed in all of the garlic-supplemented groups compared to the controls ($p < 0.05$). Moreover, significantly higher fat digestibility was found in the G10 and G30 groups compared to the control and G20 groups ($p < 0.05$). No differences in protein digestibility were observed among the groups ($p > 0.05$). A positive linear ($p = 0.00$) and quadratic ($p = 0.00$) trend was found for dietary garlic powder levels and protein digestibility, where protein digestibility increased with the inclusion of garlic powder at G10 and then decreased with the inclusion of garlic powder at G20 and G30. (Table 4).

Table 4. Apparent digestibility coefficient for dry matter, fat, and protein in Eurasian perch provided feed supplemented with garlic powder.

							Linear Trend		Quadratic Trend	
Parameters	**Control**	**G10**	**G20**	**G30**	**ANOVA**	η_p^2	***P*-Value**	**R^2**	***P*-Value**	**R^2**
ADCd%	77.53 ± 0.59 [a]	80.78 ± 0.50 [b]	79.60 ± 0.87 [b]	81.12 ± 1.83 [b]	0.01	0.71	0.69	-	0.27	-
ADCf%	78.29 ± 0.46 [a]	79.89 ± 0.68 [b]	78.35 ± 0.72 [a]	80.16 ± 1.08 [b]	0.03	0.65	0.94	-	0.09	-
ADCp%	92.41 ± 0.30	93.33 ± 0.28	92.66 ± 0.32	92.49 ± 0.55	0.06					

Values are presented as (mean ± SD; $n = 6$). Mean values with different superscripts within a row vary significantly according to one-way ANOVA ($p < 0.05$). R^2 = R squared. η_p^2 = partial eta squared. Control: without garlic supplement; G10: 10 g garlic powder per 1000 g diet; G20: 20 g garlic powder per 1000 g diet; G30: 30 g garlic powder per 1000 g diet; ADCd: Apparent digestibility coefficient of dry matter. ADCf: Apparent digestibility coefficient of fat. ADCp: Apparent digestibility coefficient of protein.

3.4. Haematology and Serum Biochemistry

The number of RBCs and WBCs in G10 were significantly higher than those observed in the other groups ($p < 0.05$). The WBCs had positive quadratic trend ($p = 0.01$) to the dietary garlic powder and reached a peak in the G10 group. The RBCs had positive quadratic trend to the dietary G10 group ($p = 0.01$). (Table 5).

Table 5. Haematological parameters of Eurasian perch fed with feeds supplemented with garlic powder.

							Linear Trend		Quadratic Trend	
Parameters	**Control**	**G10**	**G20**	**G30**	**ANOVA**	η_p^2	***P*-Value**	**R^2**	***P*-Value**	**R^2**
Red blood cells ($n \times 10^6$ μL^{-1})	283,896 ± 77,236 [ab]	464,543 ± 78,157 [c]	256,285 ± 16,266 [a]	352,395 ± 46,442 [b]	0.00	0.68	0.13	-	0.01	0.34
White blood cells ($n \times 10^6$ μL^{-1})	19,711 ± 5397	30,589 ± 7884 [b]	18,520 ± 4312 [a]	21,245 ± 5152 [a]	0.00	0.44	0.03	0.19	0.01	0.33
Lymphocytes (%)	91.84 ± 3.51	89.77 ± 4.56	93.91 ± 1.84	94.11 ± 2.87	0.11					
Myeloid cells (%)	8.15 ± 3.51	10.22 ± 4.56	6.08 ± 1.84	5.88 ± 2.87	0.11					

Values are presented as (mean ± SD; $n = 6$). Mean values with different superscripts within a row vary significantly according to one-way ANOVA ($p < 0.05$). R^2 = R square. η_p^2 = partial eta squared. Control: without garlic supplement; G10: 10 g garlic powder per 1000 g diet; G20: 20 g garlic powder per 1000 g diet; G30: 30 g garlic powder per 1000 g diet.

No significant differences in blood serum ALT and AST activity, triglycerides, or total protein were observed among the groups ($p > 0.05$). At all levels, garlic powder was associated with significantly lower levels of cholesterol ($p < 0.05$). Significantly higher levels of albumin were detected in the G10 and G20 groups compared to in the other groups ($p < 0.05$). A significant linear trend ($p = 0.00$) was observed regarding the dietary garlic powder level for albumin, where albumin increased with the inclusion of garlic powder at G10 and then decreased with the inclusion of garlic powder at G20 and G30. (Table 6).

Table 6. Serum biochemistry of Eurasian perch provided feed supplemented with garlic powder.

							Linear Trend		Quadratic Trend	
Parameters	**Control**	**G10**	**G20**	**G30**	**ANOVA**	η_p^2	***P*-Value**	**R^2**	***P*-Value**	**R^2**
Alanine aminotransferase (ukat L^{-1})	0.28 ± 0.10	0.27 ± 0.07	0.28 ± 0.08	0.19 ± 0.09	0.27					
Aspartate aminotransferase (ukat L^{-1})	1.39 ± 0.89	1.92 ± 1.26	1.83 ± 1.42	0.75 ± 0.40	0.24					
Cholesterol (mmol L^{-1})	8.28 ± 1.43 [b]	6.02 ± 1.13 [a]	7.01 ± 0.61 [a]	6.27 ± 0.44 [a]	0.00					
Triglycerides (mmol L^{-1})	9.28 ± 1.94	9.79 ± 6.20	14.59 ± 4.41	11.30 ± 3.63	0.17					
Albumin (g L^{-1})	11.16 ± 0.77 [b]	13.10 ± 1.28 [c]	12.83 ± 1.47 [c]	9.53 ± 1.28 [a]	0.00	0.61	0.00	0.55	0.00	0.55
Total protein (g L^{-1})	43.18 ± 3.52	42.86 ± 2.26	43.46 ± 1.94	41.51 ± 1.35	0.52					

Values are presented as (mean ± SD; $n = 6$). Mean values with different superscripts within a row vary significantly according to one-way ANOVA ($p < 0.05$). R^2 = R square. η_p^2 = partial eta squared. Control: without garlic supplement; G10: 10 g garlic powder per 1000 g diet; G20: 20 g garlic powder per 1000 g diet; G30: 30 g garlic powder per 1000 g diet.

3.5. Respiratory Burst and Phagocyte Activity

Garlic powder inclusion did not affect respiratory burst activity ($p > 0.05$) or lymphocyte and myeloid cell phagocytic activity and index ($p > 0.05$) (Table 7).

Table 7. Immunological parameters of Eurasian perch provided feed supplemented with garlic powder.

							Linear Trend		Quadratic Trend	
Parameters	**Control**	**G10**	**G20**	**G30**	**ANOVA**	η_p^2	***P*-Value**	**R^2**	***P*-Value**	**R^2**
Respiratory burst activity (MFI)	9290.33 ± 1185.85	7873.66 ± 1267.97	7688.00 ± 1675.28	10,838.00 ± 4639.88	0.60					
Lymphocytes phagocytic activity%	42.10 ± 8.51	46.81 ± 7.86	40.25 ± 10.62	41.87 ± 6.24	0.57					
Lymphocytes phagocytic index	14.45 ± 2.70	14.43 ± 4.86	14.14 ± 5.16	14.47 ± 5.25	0.99					
Myeloid phagocytic activity%	52.95 ± 7.69	48.03 ± 8.25	54.08 ± 10.61	52.20 ± 6.78	0.63					
Myeloid phagocytic index	16.15 ± 2.50	19.41 ± 7.48	17.37 ± 7.43	17.91 ± 6.97	0.85					

Values are presented as (mean ± SD; $n = 6$). R^2 = R square. η_p^2 = partial eta squared. Control: without garlic supplement; G10: 10 g garlic powder per 1000 g diet; G20: 20 g garlic powder per 1000 g diet; G30: 30 g garlic powder per 1000 g diet. MFI: Mean fluorescence intensity.

3.6. High-Density and Net Handling Stress Challenge

No significant differences in the level of serum cortisol and glucose were observed among the groups before stress ($p > 0.05$). Immediately after stress, all garlic diet groups showed significantly higher levels of cortisol compared to the control group ($p < 0.05$). No significant differences in glucose levels were observed among the groups ($p > 0.05$). At 1 h, significantly higher cortisol was observed in the controls, and there was a significantly lower level in the G30 group compared to in the other groups ($p < 0.05$). At 1 h, a positive quadratic trend was found between the increasing levels of garlic powder and serum cortisol ($p = 0.00$), where serum cortisol decreased with the inclusion of garlic powder at G10, increased with the inclusion of garlic powder at G20, and then decreased with the inclusion of garlic powder at G30. At 1 h, the control and G10 groups showed significantly higher glucose compared to in the G20 and G30 groups, while the glucose level of the G20 group was significantly lower than that of the other groups ($p < 0.05$). At 6 h, significantly higher and lower levels of cortisol were observed in the control and G20 groups, respectively, compared to in the other groups ($p < 0.05$), where the highest level of glucose was found in the control and G10 groups, and the lowest level of glucose was found in the G30 group at significant levels ($p < 0.05$). At 6 h, a significant linear trend ($p = 0.00$) regarding the dietary garlic powder level was observed for serum glucose. With increasing levels of garlic powder, the serum glucose decreased linearly. A positive quadratic trend was observed between the increasing levels of garlic powder and the serum glucose, where the serum glucose increased with the inclusion of garlic powder at G10 and then decreased with the inclusion of garlic powder at G20 and G30. At 24 h, significantly higher and lower cortisol levels were detected in the control and G30 groups, respectively ($p < 0.05$), while glucose was significantly higher in the control group than in the garlic-fed groups ($p < 0.05$) (Figures 1 and 2).

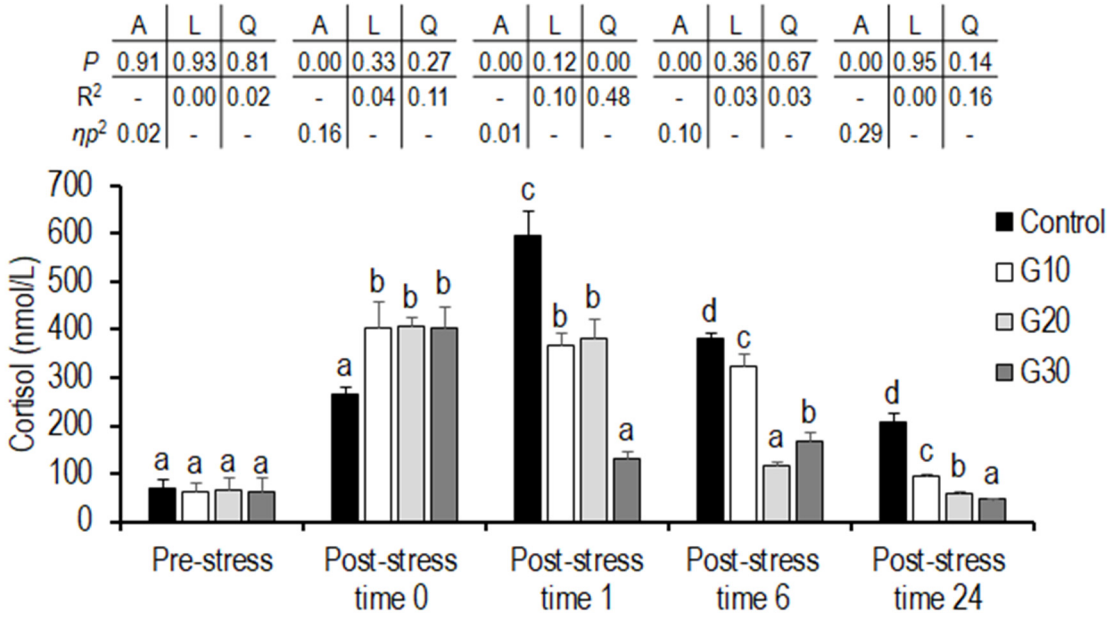

Figure 1. Serum cortisol of Eurasian perch provided feed supplemented with garlic powder under high-density and net handling stress. Values are presented as (mean ± SD; $n = 6$). Mean values with different superscripts within each time vary significantly according to one-way ANOVA ($p < 0.05$). A = the variance analysed by one-way ANOVA; L = the linear trend analysed by orthogonal polynomial contrasts; Q = the quadratic trend analysed by orthogonal polynomial contrasts. R^2 = R square. η_p^2 = partial eta squared. Control: without garlic supplement; G1: 10 g garlic powder per 1000 g diet; G2: 20 g garlic powder per 1000 g diet; G3: 30 g garlic powder per 1000 g diet. Pre-stress: before stress; Post-stress time 0: immediately after stress; Post-stress time 1: one hour after stress; Post-stress time 6: 6 h after stress; Post-stress time 24: 24 h after stress.

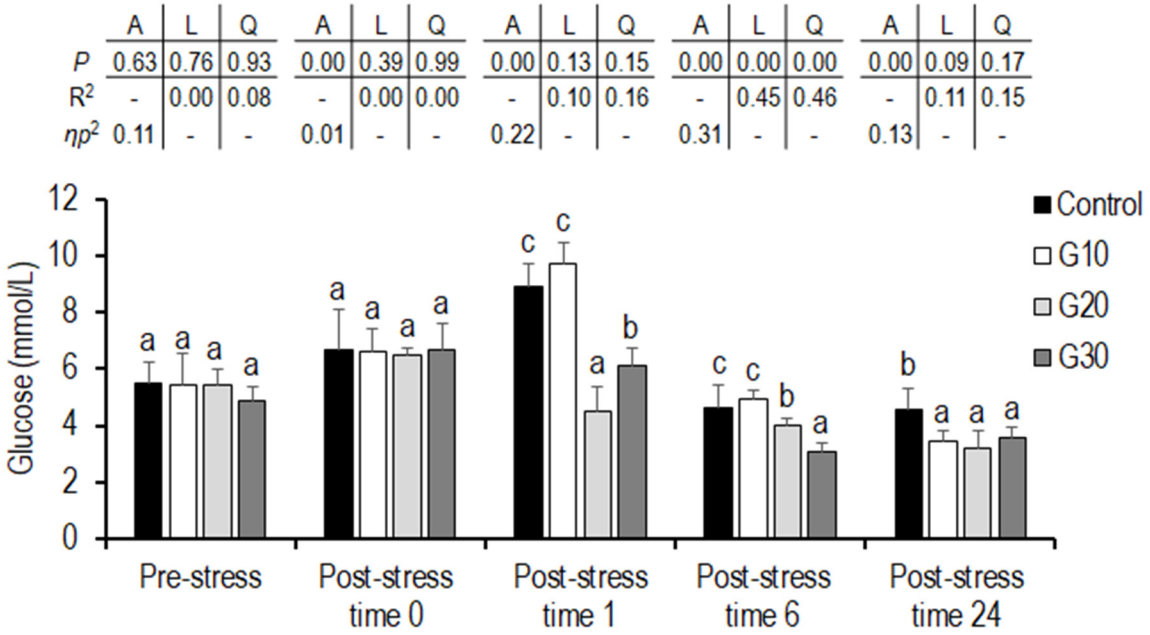

Figure 2. Serum glucose of Eurasian perch provided feed supplemented with garlic powder under high-density and net handling stress. Values are presented as (mean ± SD; $n = 6$). Mean values with different superscripts within each time vary significantly according to one-way ANOVA ($p < 0.05$). A = the variance analysed by one-way ANOVA; L = the linear trend analysed by orthogonal polynomial contrasts; Q = the quadratic trend analysed by orthogonal polynomial contrasts; R^2 = R square. η_p^2 = partial eta squared. Control: without garlic supplement; G1: 10 g garlic powder per 1000 g diet; G2: 20 g garlic powder per 1000 g diet; G3: 30 g garlic powder per 1000 g diet. Pre-stress: before stress; Post-stress time 0: immediately after stress; Post-stress time 1: one hour after stress; Post-stress time 6: 6 h after stress; Post-stress time 24: 24 h after stress.

4. Discussion

In the present study, the inclusion of garlic powder in compound diets for European perch did not show significant effects on growth performance. This finding agrees with Sahu et al. [14], who reported that garlic powder in the diet of rohu at 1, 5, and 10 g kg^{-1} feed did not significantly improve SGR or FCR. Another report documented that the use of garlic powder at the level of 40 g kg^{-1} in European sea bass did not have a significant effect on final weight, while 60 g kg^{-1} significantly decreased final weight, specific growth rate, and feed intake [39]. In contrast, garlic powder improved growth performance in Japanese sea bass at 25 g kg^{-1} [10], in brown trout at 20 and 30 g kg^{-1} [12], and in European sea bass at 10 g kg^{-1} [40]. Enhanced growth performance can be attributed to garlic bioactive compounds, including alliin, allicin, and organosulfur compounds, especially thiosulfinates [8], which increase digestion, nutrient uptake, and growth [16]. Differences among the results can be related to differences in the experimental design, fish species [10,12,40], fish size [39,40], garlic supplement type (powder or extract), and its purity [41,42] and garlic supplement level in the diet [18,39].

The liver is active in fish metabolisms, and HSI can be a marker of the harmful effects from the environment or diet [43]. In our research, the HSI and VSI indices did not differ among groups. This agrees with Shalaby et al. [16], who reported no effect of garlic powder at 10, 20, 30, and 40 g kg^{-1} feed on HSI in Nile tilapia. In contrast, 30 g kg^{1} garlic powder in the diet of brown trout [12] and 32 g kg^{-1} in the diet of Nile tilapia [42] were associated with significantly decreased HSI. In contrast, the inclusion of 10 g kg^{-1} garlic powder in the diet of brown trout also significantly increased HSI and VSI [12]. Furthermore, Lee et al. [44] confirmed that 5 g kg^{-1} of garlic extract did not have an effect on HSI in sterlet (*Acipenser ruthenus*) after 5 weeks, but 5 g kg^{-1} of garlic extract increased the somatic index (HSI) in sterlet after 10 weeks. Moreover, the use of garlic powder at levels 5, 10, 15, 20, and 30 g kg^{-1} in the sterlet diet significantly decreased HSI after a 12-week feeding trial in all garlic groups [45]. These reports showed that feeding trial duration has a strong effect on the hepatosomotic index. In contrast, our results confirm that no significant difference in HSI and VSI among groups can be significantly related to non-accumulation fat in the whole body and liver [40,46] or reduced fat accumulation in the whole body and liver in the garlic groups [21,42].

The biological characteristics of fish along with environmental parameters, feeding protocols, and parasitic infections, affect the fish condition factor [47]. In recent studies, the addition of garlic powder to brown trout feed [12] did not increase the condition factor. In the present study, the condition factor in the G30 group was significantly reduced. Lower levels—10 g kg^{-1} garlic powder in Japanese sea bass [10] and 20 g kg^{-1} in sterlet [45] feed—significantly increased the condition factor, suggesting increased diet palatability [10,45]. In contrast, garlic powder at levels of 10, 20, and 30 g kg^{-1} significantly decreased the condition factor in Indian major carp, which is in line with our results [48]. In our study, the decrease in the condition factor can be attributed to the pungent odour of garlic in G30, which may have reduced feed palatability [49] and feed intake [39]. Moreover, previous reports proved that use of garlic powder in levels of 25 g kg^{-1} in the diet of Japanese seabass [10] and 60 g kg^{-1} [39] and 20 g kg^{-1} of garlic powder in European sea bass feed [40] decreased feed intake. In the present study, feed intake decreased in the G30 groups and subsequently decreased the condition factor [48] for Eurasian perch.

The whole-body proximate composition of perch fed garlic powder did not show significant differences in dry matter, fat, or ash, while the G30 diet significantly increased body proximate protein. These results are comparable to those with 30 g kg^{-1} garlic powder in brown trout [12] and 30 g kg^{-1} in monosex redbelly tilapia (*Tilapia zilli*) [50], which improved body proximate protein composition. The inclusion of garlic powder in the diet of European seabass [40] and Nile tilapia [16,42] improved body proximate composition. Studies have shown that garlic supplementation can increase body proximate protein. Increasing protein and decreasing fat can be attributed to the organosulfur compounds found in garlic such as allicin, S-allyl cysteine, and diallyl-di-sulfide, which reduce fat

aggregation in the body [42] due to the increasing bile acids in the garlic treatments [51]. Bile acids are considered to be regulatory molecules, and they have been considered to stimulate specific nuclear receptors in cells in the liver and gastrointestinal tract [52]. Increased protein can be interpreted as a result of the essential amino acids contained in garlic [9], increasing free amino acids in the muscle and resulting in protein synthesis [40].

Plant ingredients in fish diets can balance some micronutrients or bioactive compounds [53]. The evaluation of the digestibility coefficients of feed ingredients specify the nutrient utilization for different fish species [54]. At our lowest test level, garlic powder significantly improved dry matter and fat digestibility. Esmaeili et al. [15] observed higher dry matter, fat, and protein digestibility in rainbow trout fed with 30 g kg^{-1} of garlic powder in feed. Shalaby et al. [16] demonstrated that 30 g kg^{-1} of garlic powder increased protein and fat digestibility in Nile tilapia, similar to our results in perch. Other studies have confirmed that garlic powder improved the digestibility of nutrients and SGR and decreased FCR in European seabass at 20 and 30 g kg^{-1} [40], in Nile tilapia at 32 g kg^{-1} [42], and in rainbow trout at 0.5, 1, 5, and 10 g kg^{-1} [18]. Moreover, we found some studies showing that the use of 10 g kg^{-1} of garlic powder in the diet of sobaity sea bream (*Sparidentex hasta*) [55] and 5, 10, 15, and 20 g kg^{-1} of garlic powder in the diet of Asian sea bass significantly improved nutrient digestibility, SGR, and FCR [13]. Furthermore, the administration of microencapsulated garlic extract in rainbow trout at a level of 10 g kg^{-1} improved nutrient digestibility, SGR, and FCR as well [21]. These reports reveal that the administration of garlic as either a powder or an extract in different fish species increases growth performance [21,55] and nutrient digestibility due to the bioactive compounds found in garlic, such as allicin, which improved growth performance and nutrient digestibility in Nile tilapia [16,42] and European sea bass [40].

Red blood cell and withe blood cell counts are good key indices for evaluating fish physiology and pathology [56]. In our research, the administration of garlic at 10 g kg^{-1} increased RBC and WBC numbers compared to the other groups. Garlic powder has shown similar results in rainbow trout at 0.5, 1, 5, and 10 g kg^{-1} [18] and in rohu at 10 g kg^{-1} [14]. Nya and Austin [18] reported that 10 g kg^{-1} of garlic powder increased the WBCs in rainbow trout but did not affect RBC numbers. In contrast, the administration of 10 g kg^{-1} of garlic extract (allicin) in the diet of rainbow trout increased RBC numbers, but significantly decreased WBCs [41]. The use of garlic powder did not alter RBC and WBC numbers in brown trout at 10, 20, or 30 g kg^{-1} [12] or in beluga (*Huso huso*) at 10 g kg^{-1} [57], and it had no effect on RBC numbers in European sea bass at 10, 20, or 30 g kg^{-1}, while 30 g kg^{-1} of garlic powder increased the WBCs in sea bass [40]. The higher number of WBCs found in perch in our study may be related to the immunostimulatory effect of garlic compounds on the kidney, spleen, and thymus [58], as reported in previous studies [13,18]. RBCs play important roles in oxygen transfer, decreasing hypoxia stress, and contributing to fish health [59]. Our findings of higher RBC counts can be attributed to the effect of garlic compounds such as allicin [41] on the head kidney as the main erythropoietic site in teleost fish [60]. In our study, diets containing garlic powder did not increase concentrations of blood lymphocytes or myeloid cells. This result is similar to the inclusion of 5, 10, 15, and 20 g kg^{-1} in the diet of Asian sea bass [13]. Nya et al. [41] reported that 10 g kg^{-1} of allicin in the diet of rainbow trout increased neutrophil concentration but showed no effect on lymphocyte and monocyte percentage. WBCs, including lymphocytes [61] and myeloid cells [62], have key functions against pathogens as a first line of defence [63]. Myeloid cells include neutrophils and eosinophils (granulocytes) along with monocytes (macrophages) in fish [62].

Fish health can be evaluated by blood serum biochemical parameters [33], specifically the levels of ALT and AST [21,55], which are affected by diet, environment, and stress [64]. The level of ALT and AST activity is considered an indicator of liver health [33]. The levels of blood serum ALT and AST can be affected by stocking density [65]; water parameters [66]; and fish species [55,57], age, and sex [67]. In the present study, garlic powder did not show significant effects on serum ALT and AST activity. In agreement with our results, garlic

powder in the 40 g kg^{-1} diet did not show significant effect on ALT and AST activity in Asian sea bass (*Lates calcarifer*) [68]. Furthermore, a mixture of cumin seeds (*Nigella sativa*) and turmeric (*Curcuma longa* Linn.) powder at the levels of 5 and 10 g kg^{-1} feed (1:1 w/w) did not show significant difference in the levels of ALT and AST in the Asian sea bass (*L. Calcarifer*), which is the same as in our study [69]. Other studies showed no effect on ALT activity in sobaity sea bream [55] or beluga at 10 g kg^{-1} feed [57]. Serum AST activity significantly increased in sobaity sea bream with 10 g kg^{-1} of garlic [55] and decreased in beluga [57]. Garlic powder at 32 g kg^{-1} [42] and 30 and 40 g kg^{-1} significantly decreased blood serum ALT and AST activity in Nile tilapia [16]. Moreover, garlic powder at the levels 5, 10, and 15 g kg^{-1} in feed decreased the level of blood serum ALT and AST significantly in common carp (*Cyprinus carpio*) [70]. In contrast, the inclusion of 40 and 50 g kg^{-1} of garlic powder significantly increased blood serum ALT and AST activity in rainbow trout [33]. The present study showed that the levels of ALT and AST can at least be related to fish species and to herbal medicine level and species [68,70] in the diet, similar to previous studies [55,69]. Moreover, no significant difference in the level of blood serum ALT and AST in our experimental fish, indicating that 10, 20, and 30 g kg^{-1} of garlic powder in perch diet were safe doses, as they did not disturb liver finction, as confirmed in the previous studies [68,69]. The reduction of ALT and AST activity in the blood serum can be attributed to the antioxidant compounds found in garlic, including S-allyl cysteine and diallyl-di-sulfide [71] and the flavonoids rutin, tangeretin, and nobiletin [72]. These antioxidant compounds hinder fat peroxidation in the cell membrane and prevent ALT and AST secretion into the blood [55].

Triglyceride and cholesterol were measured as blood serum biochemical parameters [55]. We observed no significant differences in the triglyceride levels among groups, while cholesterol was significantly lower in the garlic-fed groups. Garlic powder at 5, 10, 15, and 20 g kg^{-1} feed reduced cholesterol and triglycerides in Asian sea bass [13] as well as in rainbow trout at 20, 30, and 50 g kg^{-1} [33]. In contrast, 10 g kg^{-1} garlic powder in feed increased cholesterol and triglyceride levels in sobaity sea bream [5]. Apparently, garlic sulphur compounds reduce triglyceride levels in the blood serum [42]. Allicin is a main bioactive compound found in garlic that is responsible for hypolipidemia and hypocholesterolemia [73] and inhibits cholesterol biosynthesis [74]. In this line, our result showed that garlic powder at the higher level of G30 (30 g garlic powder per kg feed) significantly decreased blood serum cholesterol levels in our experimental species. In line with our study, Shalaby et al. [16] confirmed that garlic powder improved nutrient digestibility, SGR%, and FCR and increased fat digestibilty. Moreover, garlic powder decreased whole body fat and blood plasma lipids in Nile tilapia (*O. niloticus*). In another research study that was of a similar design to ours, garlic powder improved SGR, FCR, and nutrient digestibility and decreased total blood serum lipids, triglycerides, and cholesterol in Asian sea bass [13]. Moreover, Adineh et al. [21] reported the use of microencapsulated garlic extract at the level of 10 g kg^{-1} feed in rainbow trout improved SGR%, FCR, and nutrient digestibility and decreased whole body fat, which is the same as our results. Another study showed that garlic oil (0.15 g kg^{-1} feed) and powder (32 g kg^{-1} feed) increased nutrient digestibility by improving SGR% and FCR and decreased fat accumulation in the whole body and in the blood serum triglycerides and cholesterol [42] like our study. Previous studies [13,16,21,42] confirm our results and have demonstrated that whole body fat accumulation, apparent fat digestibility, and levels of blood serum triglycerides and cholesterol are related. In fact, those studies confirmed that increasing fat digestibility decreases fat accumulation in the whole body and reduces blood serum triglycerides and cholesterol [16,42].

In the present study, blood serum albumin was significantly higher in the G10 and G20 groups. Albumin has a protein structure. Albumin is primarily produced in the liver and prevents blood from leaking out of blood vessels. Albumin also transfers medicines and other substances across the blood for tissue growth and is used for tissue growth and healing [75]. Garlic powder increased blood serum albumin in amur carp [76] and rainbow

trout [18]. The inclusion of garlic powder at levels of 10, 20, and 30 g kg^{-1} in brown trout feed did not significantly increase blood serum albumin [12], but an increase was seen in Asian sea bass at the levels of 5, 15, and 20 g kg^{-1} feed [13]. These differences in results can be related to the garlic dose and fish species as well as feed ingredient composition.

Blood serum protein parameters specifically show the status of fish as they react to internal and external factors [42]. Blood serum protein provides energy, creates new cells, reconstructs muscles, transports other nutrients such as messengers in the body, and supports the immune system [70]. We did not find blood serum total protein to differ among groups. This was also reported by Talpur and Ikhwanuddin [13], who administered garlic powder to Asian sea bass at the levels of 5, 10, 15, 20 g kg^{-1} feed, and by Nya and Austin [17], who used 5 and 10 g kg^{-1} in the feed of rainbow trout. In contrast, garlic powder at 10 g kg^{-1} in the diet of sobaity sea bream [55] and at 20 g kg^{-1} in brown trout [12] increased blood serum total protein. Total protein indicates immune system status [77]. Increased blood serum protein in the garlic groups can be interpreted as a higher amount of amino acids in the garlic groups as well as higher amounts of sulfur compounds including S-allyl cysteine sulfoxide [9] and and stimulate liver to synthesize blood serum proteins [42].

Phytogenics enhance the immune system of fish [78], but in our study, garlic in the diet of perch did not improve respiratory burst activity. This finding is in agreement with Mahfouz et al. [79], who reported that 20 g kg^{-1} of garlic powder in Nile tilapia feed did not increase respiratory burst activity, which may be related to fish species, culture, and feeding conditions. Respiratory burst is a latent metabolic route in the cells and is activated upon pathogen exposure. It destroys pathogens through the synthesis of powerful oxidizing compounds [80]. The use of 5 and 10 g kg^{-1} of garlic powder in rainbow trout increased respiratory burst reactive oxygen species [17] and 15 g kg^{-1} in Amur carp (*Cyprinus carpio haematopterus*) diets [76] was shown to increase respiratory burst activity. Increasing superoxide anion production elevates reactive oxygen species [14]. The administration of 10 g kg^{-1} garlic powder to Asian sea bass [13] and 0.5 and 1 g kg^{-1} to rainbow trout [18] increased superoxide anion production ($p < 0.05$).

Phagocytic activity is considered to be an indicator of fish immune system activity [81]. We did not find the inclusion of garlic powder in the diet of Eurasian perch to be associated with the phagocytic activity of lymphocytes or myeloid cells, unlike another reports that indicate that garlic powder increased phagocytic activity and the phagocytic index in Nile tilapia at 10 and 20 g kg^{-1} [82], Asian sea bass at 20 g kg^{-1} [13], and rainbow trout at 10 g kg^{-1} [18]. Garlic extract (allicin) increased phagocytic activity in rainbow trout at 5 and 10 g kg^{-1} feed [41]. Fish species and the level of garlic can determine its effect on the immune system. The phagocytic boost of garlic powder or garlic extract [18,41] can be attributed to the immunostimulatory effect of compounds such as allicin [41], germanium, and lectin [83]. However, the present study showed that garlic powder cannot boost phagocytic activity, at least in perch. Although we did not find a significant immune response in our experimental fish in our study, immune response may happen during a longer feeding trial, at higher levels of garlic powder [14], or with the use of garlic extract in the diet [41]. In light of this, Sahu et al. [14] mentioned that superoxide anion production, which elevates reactive oxygen species was significantly higher in garlic groups compared to in control groups after 20-, 40-, 60- and 70-day feeding trials. However, the level of superoxide anion production after 60 days was higher than it was at 20, 40, and 70 days. This result shows that immune response can at the very least be related to feeding trial duration.

A mixture of 200 ppm garlic and labiatae essential oils (Delacon, Austria) (PHYTO diet) did not reduce blood plasma cortisol or glucose in European sea bass [84]. Garlic powder at 10, 20, and 30 g kg^{-1} feed in brown trout [12] and at 1, 5, and 10 g kg^{-1} in rohu [14] showed no significant effect on serum glucose, while it decreased levels of blood serum glucose at 5, 10, 15, and 20 g kg^{-1} in the feed of Asian sea bass [13] and 40 g kg^{-1} in Nile tilapia feed [16]. Zaefarian et al. [12] suggested that the efficacy of garlic supplementation

intake can be related to culture conditions and fish species. The reduction of glucose in blood serum can be attributed to the effect of garlic organosulfur compounds such as alliin (S-allyl cysteine sulfoxide) [85] and diallyl trisulfide [86], which have been shown to stimulate insulin secretion in diabetic mice [85] and rats [86], respectively. Although increasing levels of amino acids elevate insulin secretion, especially in carnivorous fish [87], increasing blood glucose levels in fish also elevate insulin levels [88]. Garlic organosulfur compounds increase glycemic control through enhanced insulin secretion and increase insulin sensitivity [85].

Blood cortisol and glucose are considered primary and secondary stress indicators in fish [89]. Cortisol is the key circulating glucocorticoid in fish, and its level is indicated by its cytosolic receptor, which regulates the expression of genes involved in growth, metabolism, and immune function [90]. Cortisol, a common stress indicator increased blood glucose in response to stress [91].

In the present study, post-challenge, the observed blood serum cortisol was significantly higher in all of the garlic groups compared to the control group, while there was no difference in the serum levels ($p > 0.05$) among groups. Elevated blood serum glucose indicates a higher stress level, requiring fish to increase energy expenditure [92]. Along with serum cortisol, glucose increases in response to energy demands [93]. Under stress, catecholamines and cortisol exert an effect on hepatocytes and induce glycolysis and gluconeogenesis, leading to an increase serum glucose [94].

At 24 h post-stress, the G30 group showed lower blood serum cortisol and glucose compared to the other groups ($p > 0.05$). At 1, 6, and 24 h post-stress, blood serum cortisol was lower in all of the garlic groups compared to the levels in the control grpi. High-density stocking [95], handling [27], heat stress [96], and low water pH [66] have been reported to increase levels of cortisol and glucose in fish. The inclusion of 2 mg nano selenium and 2 ppm garlic extract reduced blood plasma cortisol and glucose in grass carp (*Ctenopharyngodon idella*) under stocking density stress [97], while 200 ppm of a mixture of garlic and labiatae essential oil (Delacon, Austria) (PHYTO diet) reduced blood serum cortisol after 2 h overcrowding stress but did not show any effect on blood glucose ($p > 0.05$) in European sea bass [84]. In the present study, lower cortisol and glucose may be attributed to the bioactive compounds found in garlic, including alliin and diallyl trisulfide [98], which were higher in the G30 diet compared to in the other diets [13,21,42].

5. Conclusions

Garlic powder at 10 g kg^{-1} diet shows beneficial effects on haematology, blood biochemical parameters, and the apparent digestibility of nutrients including fat. The inclusion of garlic at 30 g kg^{-1} improved whole-body protein composition and increased resistance against high-density and net handling stress in European perch.

Further research should include garlic *A. sativum* powder in the diets of European perch of different sizes and developmental stages to evaluate growth performance and haematological and immunological parameters, including digestive enzymes and liver antioxidant activity. We suggest further study to identify bioactive compounds in garlic that are effective in immune-related gene expression.

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Data Availability Statement: The data presented in this research is available on request from the corresponding author.

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REVIEW

REVIEWS IN Aquaculture

Environmental consequences of using insect meal as an ingredient in aquafeeds: A systematic view

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Abstract

We retrieved data from various studies to investigate the consequences of insect meal production and insect meal-based diets with respect to their environmental impact, including global warming potential, energy use, land use, water use, acidification, eutrophication as well as to economic fish-in fish-out ratio and solid waste output production. Analysis indicated that insect meals' production exerted positive effects on land use but was associated with greater energy use and a larger carbon footprint compared to conventional protein sources. Substitution of silkworm meal (*Bombyx mori*) meals for fishmeal in aquatic animal diets significantly reduced solid phosphorus waste compared to insect-free diets. In contrast, the inclusion of black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*), mealworm (*Tenebrio molitor*) and grasshopper (*Zonocerus variegatus*) has led, in comparison to insect-free diet, to greater solid nitrogen waste. Reducing the proportion of fishmeal and, to a lesser extent fish oil, by various insect meals in aquatic diet formulations significantly reduces economic fish-in fish-out, indicating less marine forage fish required per unit fish yield. The simulated data showed environmental benefit associated with land use of insect-containing aquafeeds compared to insect meal-free feeds, especially insect species of *M. domestica* and *T. molitor*. In all, this study suggested a trade-off of using insect meal as an aquafeed ingredient regarding environmental consequence. Since insect meal has excellent potential to supply protein for aquafeeds in the coming years, improvement in insect meal production systems and nutritional composition will be essential to make insect meal a sustainable aquafeed ingredient.

KEYWORDS
alternative protein, aquafeed, economic fish-in fish-out, environmental sustainability, insect meal, waste output

1 | INTRODUCTION

The contribution of aquaculture to seafood production has increased continuously over the past two decades, reaching 46% in 2016–2018, up from 25.7% in 2000, with an annual growth rate of 5.3% from 2001 to 2018, surpassing that of any other major food production system.[1] The trend is expected to continue in response to increasing world population and higher demand for seafood protein.[2] The rapid growth of the sector raises significant concerns regarding forage fish stock, natural resources, environmental issues and waste generation.[3–5] Aquafeed is the major factor driving these challenges.[2,6–10] Typically, aquafeeds rely largely on fishmeal/oil derived from marine forage fish and, to a lesser extent, from fishery/aquaculture by-products, as protein and lipid sources.[1] Aquaculture

has become the largest consumer of global fishmeal and fish oil production, accounting for 68% and 89% respectively.[11] Worldwide, wild fish production decreased by 26.5% from 2000 to 2018,[12] after peaking in 1994,[13] and will reach ecological limits in 2037.[14] The use of by-products from fisheries and aquaculture in aquafeeds has increased but will be insufficient for projected aquafeed demands by 2050.[14] As a result, the growing aquaculture industry will face a severe issue of limited fishmeal/fish oil supply, and fishmeal/fish oil replacement in aquafeeds is inevitable.

Efforts to reduce proportions of fishmeal and fish oil in aquafeeds over the past two decades[15–17] have led to the increasing inclusion of plant-derived ingredients.[18] However, inclusion of these ingredients in aquafeeds, with respect to the environment, places greater pressure on water and land resources,[18–21] and generates more waste[3,22] compared to fishmeal-based diets. Multiple alternative ingredients for aquafeeds have been investigated, among which insect meal and fisheries by-products show the greatest potential to meet protein required for aquafeeds in the coming decades.[11]

Insect meal draws increasing interest as an alternative to fishmeal in terrestrial and aquatic animal diets because of its favourable nutrition profile,[23,24] the feasibility of commercial-scale production and consumer acceptance.[11,25] Successful inclusion of insect meal in the preference to fishmeal in feed has been well reviewed for many aquatic species.[26–30] Partial replacement of fishmeal by black soldier fly (*Hermetia illucens*) in European perch (*Perca fluviatilis*) diet resulted in significant improvement of forage fish input relative to farmed fish production (fish-in fish-out ratio).[31–33]

Production of insect meal has been shown to consume less land and water resources than does soybean meal.[34,35] Meal of the common housefly (*Musca domestica*) as partial replacement for fishmeal in tilapia (*Oreochromis niloticus*) diets exhibited a positive effect on water environmental parameters compared to insect meal-free diets.[36] Reports of the environmental consequences of insect meal-based diets compared to those of fishmeal-based diets for aquatic animals are scarce.[37,38] Le Féon et al.[37] reported that inclusion of yellow mealworm (*Tenebrio molitor*) in rainbow trout (*Oncorhynchus mykiss*) feed reduced net primary production use (in kg C, quantifying the biotic resource that is not available for other systems anymore), and available water remaining (in m^3, considering the water availability in the studied area minus the water required by humans and aquatic ecosystems), but did not decrease land use, acidification, eutrophication, global warming potential and energy use compared to an insect meal-free diet. Conversely, an *H. illucens*-based diet in arctic char (*Salvelinus alpinus*) resulted in reduced environmental impacts, including abiotic depletion, acidification, eutrophication, global warming potential, human toxicity and marine ecotoxicity compared to an insect meal-free diet.[38] Dietary *H. illucens* in *P. fluviatilis* was reported to require considerably less water than a fishmeal-based diet, while increasing global warming potential, land demand and energy use.[33] A broader understanding of the environmental impact of insect meal and insect meal-based feeds in combination with their effects on fish production (e.g. nutritional properties, growth, meat quality) can inform the choice of insect meal as a protein source for the sustainability of future aquafeeds.

This review aimed to characterize the environmental consequences of insect meals as a nutrition source for aquatic animals. We retrieved life cycle assessment studies addressing the environmental impact of various insect meals and compare with conventional aquafeed ingredients. Peer-reviewed publications assessing insect meal as replacement for fishmeal in aquatic animal diets were synthesized to calculate economic FIFO ratio, solid waste output and environmental impact categories of insect-containing feed relative to fishmeal (insect-free) feed. We suggest areas to enhance the efficiency and sustainability of insect meals in aquafeeds.

2 | METHODS

2.1 | Database search and criteria

The relevant literature was searched using online databases Scopus, Web of Science and Google scholar in December 2020.

2.2 | Environmental impact of insect meal and other feed ingredients

Life cycle assessment analysis evaluates environmental impacts of products and systems throughout their life cycle.[39] This tool has been increasingly used in assessing the environmental sustainability of aquaculture systems,[8,39–41] aquafeeds,,[6,9,37,38,42–44] aquafeed ingredients.[45] The targeted literature reported for life cycle assessment of insect meal production was searched, using keywords such as *insect meal*, *LCA* or *Life Cycle Assessment*, *global warming potential*, *energy use*. A total of 13 published articles and one PhD thesis (from 2014 to 2020) were compiled (Table S1). Environmental impact categories based on life cycle assessment studies included global warming potential (kg CO_2 equivalent (eq.), energy use (MJ); land use (m^2a (arable land), water use (m^3), acidification (g SO_2 eq.) and eutrophication (g PO_4 eq.).

2.3 | Total solid waste and nitrogen and phosphorus waste

The combined keywords, for example *insect meal*, *fish diets* and *digestibility* were used to search publications relevant to insect meal as replacement for fishmeal in aquatic animal diets and apparent digestibility of dry matter, crude protein and phosphorus. The literature also contained information on feed utilization to calculate the following:

$$\text{Total solid waste (TSW)} = \left[\text{feed (DM) consumed} \times (1 - \text{ADC DM})\right] + \text{waste feed (DM)}$$

Solid nitrogen waste (SNW) or solid phosphorus waste (SPW) = [N or P consumed × (1 − ADC of N or P)] + (N or P of waste feed), where DM is dry matter, N, P are nitrogen and phosphorus, respectively, and ADC is apparent digestibility coefficient.

Data derived from 27 peer-reviewed publications (from 1990 to 2020) were compiled to investigate solid waste output trends corresponding to insect meal inclusion in aquafeeds (Table S2). Insect meal replacement levels for fishmeal ranged 3.52%–50.80% (IQR) with 45% experiments using *H. illucens*, followed by *T. monitor* (22%), silkworm (*Bombyx mori*), *M. domestica* (11%) and other insects (7%). Most insect meals were full-fat processing (68% of total observed insect meals), while defatted and partial defatted forms accounted for 21 and 11% respectively. The calculated solid waste output values were converted to response ratio r, representing the ratio of measured indices in experimental and control groups,[46] which was employed in the meta-analysis of insect meal inclusion on fish growth performance.[30] Our analyses were constrained to solid waste assessment only because of the insufficient number of studies reported dissolved waste.

2.4 | Economic fish-in fish-out ratio and environmental impact categories

To be included in these analyses, experimental studies needed to (i) perform on aquatic animals; (ii) include at least one insect meal level as partial or total replacement for fishmeal; (iii) provide sufficient information on feed formulation, the proportion of each constitution, feed conversion ratio. Studies that assessed the mixture of insect meals or insect meal with other components as replacement for fishmeal were not considered. Keywords such as *insect meal*, *fishmeal*, *replacement*, *fish*, *growth* were used in different combinations to get matches. Altogether 84 peer-reviewed articles (from 1990 to 2020) were compiled (Table S3).

2.4.1 | Economic fish-in fish-out

The ratio of forage fish input to farmed fish production (fish-in fish-out) is considered a measure of sustainability.[31] We adopted the term 'economic fish-in fish-out ratio' (eFIFO) from Kok et al.,[19] based on economic outcome and is commonly used in life cycle assessments. The calculation included data on the use of fish by-products, currently reported to comprise 25%–35% of global fishmeal and fish oil production,[1] a useful measure when establishing industry policy. The eFIFO differs from conventional fish-in fish-out,[31,32,47] which did not align with life cycle assessment, omitted fisheries by-products from the calculation,[19] and was recognized as an overestimation of wild fish used,[47] and a flawed concept.[17]

The eFIFO ratio was calculated by the formula: $\text{eFIFO} = \text{FCR} \times \sum (F_{i,j} \times \text{EF}_{i,j})$

where FCR is feed conversion ratio, *i* is fishmeal or fish oil, *j* is source of ingredient, F_i is proportion of fishmeal or fish oil in the diet (%).

EF_i, is embodied fish in fishmeal or fish oil, which is dependent on raw fish used, that is: fish species, size and capture season. eFIFO calculation in our study was based on EF_i database of Kok et al.[19] during 1995 and 2020.

Seven taxonomic groups contributing to eFIFO data were categorized according to Tacon and Metian[32] (Table S3). To consider whether the eFIFO of each taxon can meet global projections, we calculated eFIFO values predicted for 2025 and estimated the feasible fishmeal substitution level at which the predicted eFIFO is obtained. The projected eFIFO for 2025 of each taxon was calculated based on the above-mentioned formula, in which embodied fish in fishmeal and fish oil (EF_i) for 2025 was 3.54 and 4.06, respectively,[19] and FCR and the proportion of fishmeal, fish oil in the diet for each taxon by 2025 were retrieved from Tacon and Metian.[16,32]

2.4.2 | Environmental impact categories of insect meal-based and insect meal-free diets

Experimental diets extracted from publications (Table S3) were used to evaluate the environmental impacts of a set of six impact categories, including global warming potential (kg CO_2 eq.), acidification (g SO_2 eq.), eutrophication (g P eq.), land use (m^2a eq.), energy use (MJ) and water use (m^3) per kg of feed based on the environmental impact at the plant gate database of feed ingredients generated by the Global Feed Lifecycle Institute.[48] We limited our data to publications focusing on *H. illucens*, *T. molitor* and *M. domestica* because of unavailable life cycle assessment studies on other insect species. Since the environmental impact of ingredients in the GFLI database varies with location, average global values were used. The minerals, additives and vitamins used are classified as 'Total minerals, additives, vitamins, at plant/RER Mass S' in the GFLI database. Environmental impact values for each of the three insect meals were expressed as mean values for each insect group (Table S1). Due to unavailable data on water use for the production of one kg of *T. molitor* meal, we used the value of 4.3 m^3 required for one kg fresh mealworm[49] with an assumption that the drying process of mealworm did not require additional water.[50] The environmental impact categories were also converted to response ratio, as mentioned previously.

2.5 | Data analysis

The raw data on the environmental impact of insect meals were calculated for the interquartile range (IQR), from the first (Q1) to the third (Q3) quartile, using the 'summary' function. The relationship between fishmeal replacement with insect meal and waste output was tested using the generalized additive model ('gam') and the linear model ('lm') functions. Analyses of covariance (ANCOVA) were used to determine the variation in waste output parameters. Strong outlier values (Q1 > 3 × IQR or Q3 < 3 × IQR) were excluded from

the dataset to mitigate heterogeneity. The significant correlations of eFIFO relative to replacement levels of fishmeal with insect meal were tested with linear and gam models, and the 'ANOVA' function was used to compare regression models. All analyses were performed using the R statistical package (R Development Core Team 2009–2020, available at www.r-project.org/).

3 | RESULTS AND DISCUSSION

3.1 | Environmental impact of insect meal production

The environmental impact of three insect meals–*H. illucens*, *T. molitor* and *M. domestica*—which were extracted from the literature, and the interquartile range (IQR), mean values were summarized (Table S1). Regarding global warming potential and energy use, the production of the investigated insects was comparable, while *M. domestica* required less land use than did *T. molitor*.

Environmental impact categories of insect meals and other conventional and novel feed ingredients used in aquafeeds are depicted in Figure 1. The IQR baselines of bulked ingredients are also presented. Insect meal production, along with fishmeal and single-cell protein, appeared to be efficient in terms of land use. Land use of these ingredients was found to be lower than the bulked Q1. Recent research confirmed better land use efficiency of insect meals (e.g. *M. domestica*,[51] *H. illucens*,[34,35] and, to a lesser extent, *T. molitor*[52]) compared to soybean meal. This suggests that the preferable use of alternative aquafeed ingredients (e.g. insect meals, single-cell protein) to terrestrial crops concerning natural resource conservation.

Fishmeal appeared to have the lowest impact in all categories, except energy use, whereas soybean meal and plant protein were instead closed to the bulked Q1 of energy use (Figure 1). Silva et al.[45] also reported that soybean meal had lower energy use (fossil fuel) than fishmeal, which confirmed the reliability of the present compilation.

Six ingredients exhibited a good fit within the bulked IQR concerning greenhouse emissions, the exception was microalgae. Regarding energy use, insect meals, microalgae and single-cell protein ingredients showed an immense impact, falling beyond the bulked Q3. Several studies confirmed the negative impact of global warming potential and energy use associated with insect meal production versus that of fishmeal and soybean meals.[34,35,51,52] A similar pattern was also observed for insect meals in terms of water use and eutrophication. Water use of *H. illucens* meal was comparable with that of fishmeal and less than that of plant ingredients and microalgae, as reviewed by Smetana et al.[35] However, compiled data showed a contradictory pattern (Figure 1), which could be attributed

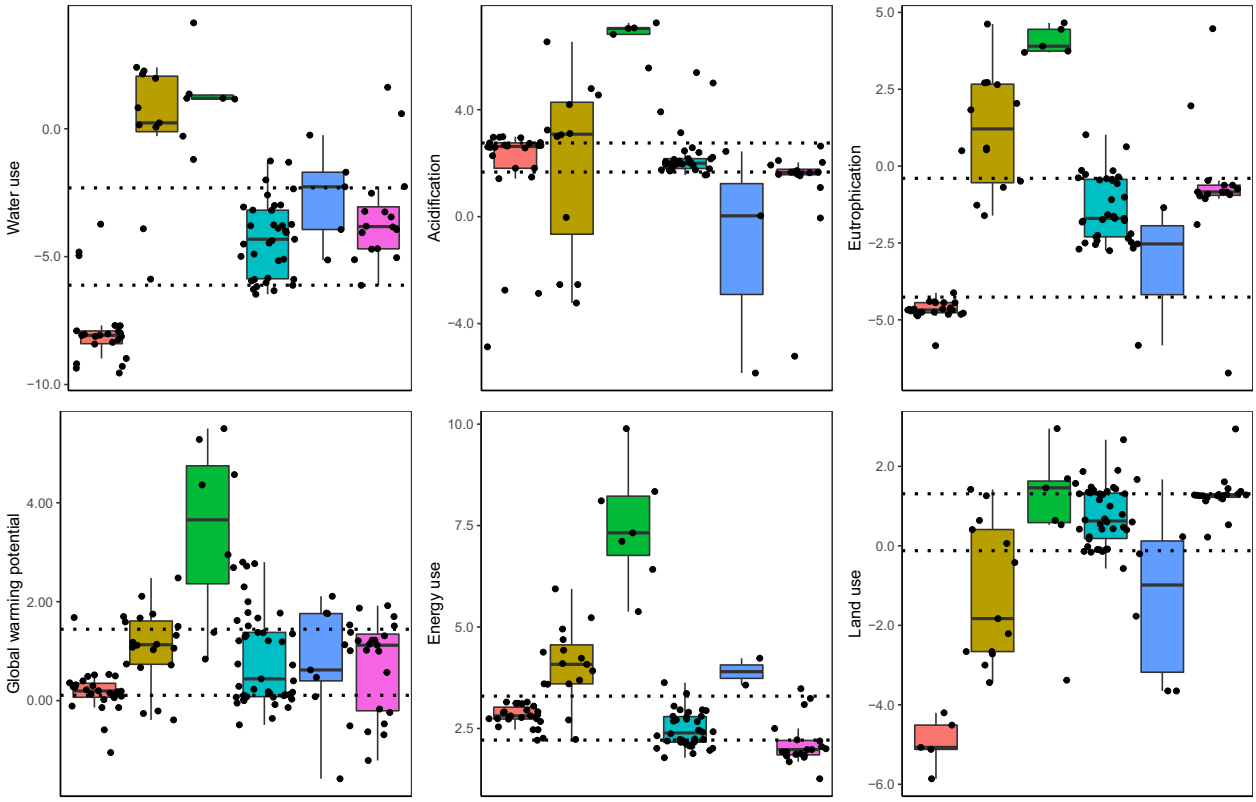

FIGURE 1 Log values of environmental impact categories of insect meals and other feed ingredients per kg. Black dots are observed data points. The lower and upper dashed lines represented the first quantile and third quantile of bulked ingredient data. Data sources: insect meals (Table S1), fishmeal[6,45,48,52,59,127–129] (number of observation, *n* = 27), soybean meal[34,45,48,50,52,126,129–135] (*n* = 27) and plant concentrate,[6,48,50,59,136–138] microalgae[50,134,139–141] (*n* = 9), single-cell proteiny[50,59,126,133,142–144] (*n* = 9)

to the high water use required by housefly production (Table S1). Roffeis et al.[53] reported the need for extra water for mixing substrates and killing flies (*M. domestica*). The high impact of housefly production could be explained by the geographical context of their study[54] because a high percentage of water was consumed to maintain facility hygiene.[55] Feed for insects was the most significant driver for water use,[56] especially for those derived from crop products[49] commonly used for insect rearing (Table S1). This could further explain the high impact of insect meal production. Given that most studies compiled in our review were performed under small-scale facilities, system improvement could improve water use and other environmental categories of insect meal products.[55–57] Insect meal production associated with acidification was highly heterogeneous and partly deemed in the bulked IQR. We also found excellent environmental performance associated with acidification in single-cell protein production (Figure 1).

Our review highlighted the high environmental impact of novel aquafeed protein sources, including insect meals, microalgae and single-cell protein, compared to conventional aquafeed ingredients, especially for global warming potential, energy and water use. This could be ascribed to the insufficiency of production technology and production scalability.[35,58,59] Insect production upscaling could reduce environmental impact and consequently compete with conventional ingredients.[35,57] Among those novel alternative aquafeed ingredients, insect meal has been suggested to be the best potential candidates for improving processing techniques, costs and scalability.[11] Moreover, feed for insect rearing was the largest contributor to environmental impact categories (*T. molitor*,[37,52] *M. domestica*[51,53] and *H. illucens*[34,35,60,61]). Therefore, sourcing suitable substrates to feed insects and expanding the efficiency of facilities will be critical to improving the environmental benefit of insect meals.

3.2 | Total solid waste, nitrogen and phosphorus waste

The total solid waste and phosphorus and nitrogen waste from aquaculture are considered primary eutrophication agents of aquatic ecosystems. Minimizing these outputs through diet formulation has been proposed as a long-term strategy to ensure environmentally friendly and sustainable aquaculture.[3,5]

There was no significant relationship between the fishmeal replacement level and total solid waste for all insect meals ($p = 0.597$) as well as for individual insect meals ($p > 0.05$). Dietary insect meals comprising *B. mori*, *H. illucens*, *M. domestica* and *T. molitor* significantly increased solid nitrogen waste ($p < 0.05$). A significant negative relationship was found between dietary *B. mori* and solid phosphorus waste ($p < 0.05$; Figure 2, Table S4). ANCOVA analysis showed a significant association between nutrient digestibility (dry matter, *F*-value =24.75, $p < 0.0001$; protein, *F*-value =4.80, $p = 0.032$; phosphorus, *F*-value =7.19, $p = 0.01$) and chitin (*F*-value =5.98, $p = 0.017$) with total solid waste. Chitin (*F*-value =6.78, $p = 0.02$), protein digestibility (*F*-value =13.57, $p = 0.002$), fish habitat (*F*-value =13.57, $p = 0.002$) and insect species (*F*-value =5.46, $p = 0.033$) imparted significant variations in nitrogen waste. Solid phosphorus waste was found to be significantly influenced by the phosphorus digestibility (*F*-value =11.56, $p = 0.011$), dietary phosphorus (*F*-value =8.49, $p = 0.002$) and insect species (*F*-value =4.71, $p = 0.045$).

A recent study by Weththasinghe et al.[62] on *O. mykiss* fed full-fat *H. illucens* meal confirmed our finding that there was a positive correlation between dietary insect meal and the faecal excretion of nitrogen. Nitrogen waste load is directly linked to the apparent protein digestibility of the diet. Digestibility of dietary protein was reported to be affected by the presence of chitin in insect meal.[63–67] Chitin is not digestible by monogastric animals, and it exhibits a high protein-binding capacity, which could impair protein digestion.[64] Chitin may interfere with leucine aminopeptidase activity—a brush border enzyme that breaks down peptides into amino acids in the proximal and middle intestine, where the majority of proteins are digested and absorbed.[68] Gasco et al.[65] reported that the assumed 1.7% chitin content of 36% fishmeal replacement by *T. molitor* meal improved protein digestibility compared to an insect-free diet in European sea bass. This could be associated with the chitinase that is present in some marine species that degrade chitin, consequently reducing the digestibility constraints of including insect meal.[69–72] A ratio of non-essential-to-essential amino acids of ≤1.0 in most insect meals[23] was known to negatively affect protein digestibility.

Weththasinghe et al.[62] evidenced reduction of faecal phosphorus output with increasing insect meal inclusion in diets of *O. mykiss*, which was confirmed in our compilation. This could result in a significantly lower phosphate concentration ($P - PO_4^{3-}$) in the rearing water of fish fed insect-based diets compared to insect-free diets, which was reported earlier for *O. niloticus*.[36] The ANCOVA analysis in the present study showed that the solid phosphorus load is mainly attributed to feed digestibility and phosphorus content, which is in agreement with previous work.[3] Some studies have confirmed a positive relationship between dietary insect meals and phosphorus digestibility in aquatic animals. For example Rahimnejad et al.[73] reported that the phosphorus digestibility of *L. vannamei* was significantly enhanced by increasing the inclusion level of *B. mori*. A similar finding was reported for bullfrogs (*Rana catesbeiana*) fed dietary *M. domestica* meal.[66] It is likely that a higher calcium-to-phosphorus ratio (Ca:P) and a higher phosphorus content in fishmeal compared to insect meal could impact phosphorus digestibility.[66,73] For instance *M. domestica* meal has a lower Ca:P (0.29) and phosphorus content (1.60%) than fishmeal (1.56% and 2.79% respectively).[24] In contrast, the major phosphorus forms, hydroxyapatite and tricalcium phosphate, in fishmeal are not well utilized by aquatic animals.[74] Our findings suggest that the use of silkworm (*B. mori*) in aquatic animal feeds could reduce the phosphorus load from aquaculture.

Accessed studies employed different techniques for producing experimental diets, for example meat grinder,[75] extruder,[66] resulting in variation in physicochemical composition, digestibility. The extrusion technique can significantly improve aquafeed nutrient digestibility and feed stability[17,76] and is an environmentally

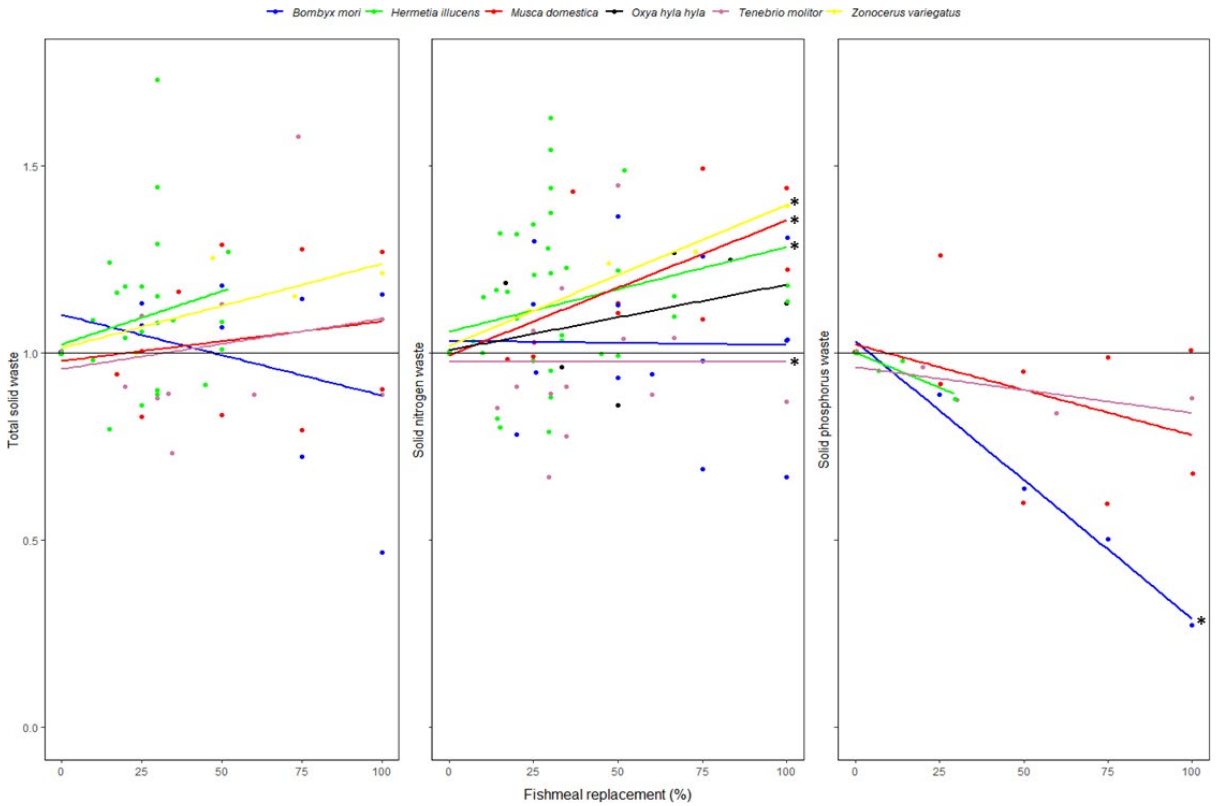

FIGURE 2 Effect of insect meals inclusion in aquatic animal diets on total solid waste, nitrogen waste and phosphorus waste compared to insect meal–free diets. Asterisks indicate a significant linear relationship between insect meal and waste output indies ($p < 0.05$)

friendly process.[77] The broader application in aquafeed production may reduce waste output and enhance the performance of the cultured animal. Further study is needed to confirm the benefits of dietary insect meals in aquafeeds on water quality. Manipulation of insect meal composition, for example chitin and nutrient imbalance, and improvement of feed manufacturing technology, feeding strategy could further benefit waste output from insect-based aquafeeds.

3.3 | Economic fish-in fish-out

Globally, approximately 70% of forage fish from capture fisheries is used in the production of animal feed, a large proportion of which goes into aquafeeds in the form of rendered fish oil and fishmeal.[1,14] Under the current aquaculture scenario, this finite resource will be close to its ecological limits by 2037,[14] which, in combination with the continuous rise in the market price of the rendered products, poses a challenge to the ever-growing aquaculture industry.[78] Reducing forage fish-derived fishmeal and fish oil in aquafeeds is an essential strategy for the long-term sustainability of fishery resources and aquaculture operations.[79] The eFIFO has been considered a novel and suitable proxy for quantifying fish demand for aquaculture production based on the economic allocation principle and suggests the importance of finite wild marine resources to meet marine aquafeed ingredients.[19]

Our compiled data comprised 13 insect species and found that replacing fishmeal with these insect meals in aquatic animal feeds steadily decreases eFIFO in all taxa (Figure 3). Comparing regression models using ANOVA indicated that linear regression models were the best description of the relationship between insect meal replacement and the eFIFO. The overall eFIFO of carp taxon felt lower than the Q1 bulked eFIFO of all taxa and remained low compared to that of other taxa (Figure 3).

Substantial or total replacement of fishmeal with insect meals could reduce eFIFO to <1.0 in all taxa, suggesting the potential for insect meals to turn the aquaculture industry from a net consumer to a net producer of fish. This is consistent with the current trend in forage fish use in aquaculture, which produces three or four times the number of fish it consumes.[19]

Reduction in the proportion of fishmeal and fish oil in aquafeeds by increasing insect meal to meet predicted eFIFO by 2025 seems feasible in all taxa. This requires a substitution of insect meals for fishmeal, from 65% (for salmon) to 93% (for shrimp and tilapia) (Figure 3). Shrimp, marine fish and salmon are reported to be the highest consumers of fishmeal and fish oil.[32] The predicted eFIFO in 2025 is 0.3, 0.6 and 0.7 for shrimp, marine fish and salmon, respectively, necessitating 93%, 83% and 65% fishmeal replacement by insect meals (Figure 3). Panini et al.[80] and Motte et al.[81]

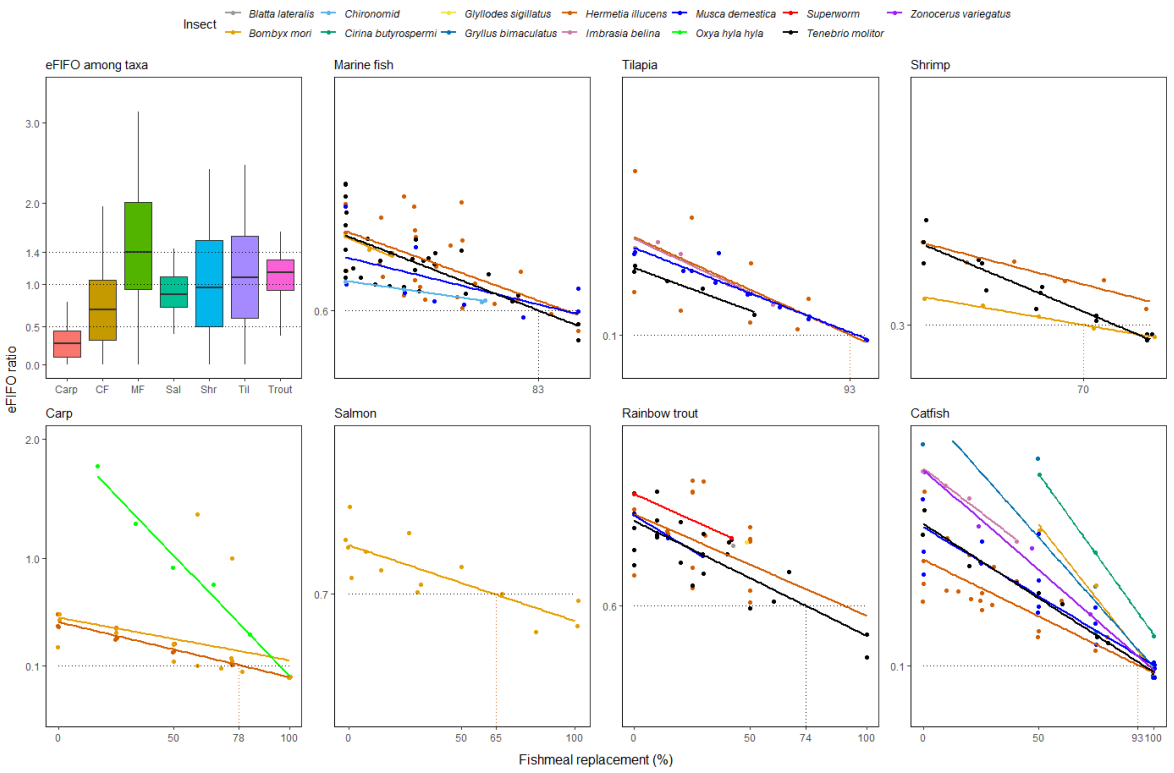

FIGURE 3 Relationship between eFIFO and fishmeal substitution by various insect meals in cultured fish species. The solid lines and dots represent the mean values and observed data points respectively. Horizontal dotted lines represent predicted eFIFO by 2025, and vertical dotted lines indicate the threshold of fishmeal substitution by insect meal at which predicted eFIFO would be reached. In the 'eFIFO among taxa' boxplot, the horizontal dashed line represents the mean of bulked eFiFO separating the interquartile range (the first and third quartile) of the bulked eFIFO. CF, catfish; MF, marine fish, Sal, salmon; Shr, shrimp; Til, tilapia

reported 100% fishmeal replacement by *T. molitor* without impairing the growth and feed utilization of shrimp. Similar patterns were confirmed in the marine fish, *P. major*[82] and *S.* salar.[83,84] The eFIFO in 2025 of carp, catfish and tilapia groups was predicted to be as low as 0.1 (Figure 3), meaning that diets for those species would include low levels of fishmeal/fish oil or no fish-derived components. Growth rate and feed conversion efficiency were not negatively affected with fishmeal/fish oil-free diets in common carp (*C. carpio*),[85,86] tilapia (*O. niloticus*)[87,88] and catfish (*C. gariepinus*).[89] Tacon and Metian[32] stated that fish oil has not been included in feeds for carp since 1995 or for tilapia since 2007, which will continue to 2030 as projected by Cottrell et al..[90] Herbivorous and/or omnivorous fish, such as carp and tilapia, are less sensitive to dietary fishmeal/fish oil reduction than carnivorous species.[91] Carp was the largest aquafeed consumer in 2017, which is a trend that is likely to continue in the coming years,[13] therefore, the simulation model[14] suggests that this sector has the highest potential to reduce forage fish use by 2050.

Globally, reducing forage fish demand for aquafeeds would be more effective by limiting fish oil than fishmeal[90] due to the low conversion rate from whole fish to fish oil (5%) than to fishmeal (22.5%).[32] The majority of global FO production goes into feed for salmonids, marine fish and shrimp.[16,92] In addition to providing protein, insect meal represents a potential source of fat for aquaculture feeds. Lipid content varies among *T. molitor* (16.6%–40.3%), *H. illucens* (11.3%–40.7%), *M. illucens* (7.1%–25.3%),[23] and is highly dependent on rearing substrate and processing.[30] Investigation of insect oil as a lipid source for aquatic animals is in its infancy and currently limited to carp, trout and salmon.[68,93–96] Insect fatty acid profiles comprise large proportions of saturated and monounsaturated FAs, oleic acid and negligible levels of long-chain polyunsaturated FAs.[23,24,97] The lack of long-chain polyunsaturated FAs could limit use of insect oil as lipid source or replacement for fish oil in nutrition of aquatic animals, especially high-value species.

Insect meals and oils could play an essential role in conserving finite forage fish resources while meeting the increasing demand for aquafeed protein/lipid sources. This requires nutritional fortification.[98] Blending insect meal or oil with other materials[96,99–101] or compensating for deficient components in insect-based diets, for example amino acids,[84,102,103] essential FAs from microalgae and supplementing digestive enzymes[65] could allow the inclusion of higher proportions of insect ingredients, thus reducing dependence on marine fish resources.

3.4 | Environmental impact of insect meal-based aquafeeds

Feed production is a key driver of the environmental impact of aquaculture,[10] and modification in feed ingredients is considered critical

to reducing that burden.[43] Since insect meal is recognized as a potential protein source in aquafeed[11,104] and aquafeed protein sources differ in environmental impact,[42] understanding the environmental consequences of insect meal- and fishmeal-based feeds is essential to increasing sustainability of aquafeeds.

The relationship between fishmeal replacement level with insect meal and environmental impact in aquafeeds is depicted in Figure 4 and Table S6. The generalized additive model demonstrated a significant increase in fishmeal replacement level with the increased environmental impact of global warming potential, energy use, water use, acidification and eutrophication. A similar pattern was observed for land use with linear models (Table S6), except for *M. domestica*-contained diets. The results suggested that the dietary housefly (*M. domestica*) linearly reduced the environmental impact associated with land use. Our synthesis also evidenced an eligible impact concerning land use of a *T. molitor*-based diet compared to a fishmeal diet, as illustrated by a relatively low model slope (0.002; Table S6). Further scrutiny of these insect meals in aquafeeds with respect to mitigating environmental consequences hints towards promising outcomes.

This finding primarily reflects the environmental properties of insect meal versus fishmeal, as presented in Figure 1, because most impacts were influenced by modifying the proportion of insect meal over fishmeal. In addition, the experimental diets in the compiled studies (Table S3) were formulated on an isonutrient basis, in which changes in the proportion of other ingredients were also made. Therefore, to some extent, the differences in environmental impacts between insect meal-containing diets and fishmeal diets could also be influenced by other components, such as plant ingredients, which were considered to have a similar environmental performance to fishmeal/fish oil.[45]

Reported environmental impacts associated with insect meal in aquafeeds are scarce, but include feed for perch (*P. fluviatilis*),[33] rainbow trout (*O. mykiss*)[37] and arctic char (*S. alpinus*).[38] Stejskal et al.[33] confirmed the reduction of water use associated with *H. illucens*- compared to fishmeal-based feed, while global warming potential, land use, energy use increased. However, Smárason et al.[38] compared *H. illucens*- and fishmeal-based feed associated with seven impact categories and reported benefits of *H. illucens* inclusion on abiotic depletion, acidification, eutrophication, global warming potential, human toxicity potential and marine aquatic ecotoxicity potential, but with a negative impact on energy use. Le Féon et al.[37] confirmed more enormous impacts of acidification, eutrophication, global warming potential, land use and energy use associated with *T. molitor*—compared to fishmeal-based feed. The discrepancy in those results could be attributed to data source and diet formula modification with a various share of insect meal, fishmeal and other ingredients. We synthesized environmental impact data for insect meals from up-to-date life cycle assessment studies (Table S1), which varied with respect to numerous factors, for example growth substrate, location and size of facility. Our study suggested environmental benefit associated with land use of *M. domestica*-, and to a lesser extent *T. molitor*-contained aquafeeds. Notably, aquafeeds are formulated from multiple components, and thus with regard to reducing environmental impacts, insect meal protein is probably not a holistic option for this purpose. Diet modification by combining insect meal and other environmentally efficient ingredients and further improving environmental performance associated with insect meal production could lower environmental impacts.

3.5 | Increasing environmental benefits of insect meals and insect meal-based diets

3.5.1 | Improving nutritional value

As mentioned, insect meal possesses properties associated with solid waste output and eFIFO that hinder its inclusion in aquafeeds. Limitations related to chitin content and inadequate nutritional profiles could be addressed by manipulating substrates and processing.[11,26,30]

Chitin, one of non-protein nitrogen compound found in the cuticle of most insects, is reported to exert a negative effect on diet digestibility and growth performance of fed organisms,[105] while a low proportion of chitin can induce immunological effects and microbiota modulation.[75,106–108] Manipulation of chitin content in insect meal products to a threshold level that ensures a positive response in fed species requires further research. Chitin can be easily removed by alkaline extraction,[109] but this may result in high cost as well as issues of chemical residue and pollutants.[110] Supplementation with chitinase/chitinolytic-producing bacteria could be a feasible approach to ensure cost-effective feed, reduce the environmental impact of chitin waste and induce an immune response to fed fish.[110]

Reduction of eFIFO in aquafeeds by inclusion of insect meal requires substantial fishmeal replacement without compromising fish growth, which is chiefly influenced by nutritional-balance of experimental diets.[30] Deficiency of amino acids and fatty acids of insect meal relative to fishmeal has been reported,[23,24] and manipulation of those components via rearing substrates remains a challenge.[111,112] Defatting could be an efficient means of improving amino acid content,[113] but this involves intensive energy use, which in turn increases environmental impact and costs.[26,34,52,114] It is more efficient to combine IM with complementary raw materials[11,30,115] or supplement IM-based diets with amino acids.[83]

Manufacturing techniques such as extrusion could be an effective means of enhancing nutrition utilization of aquafeeds by the fed organism.[17] The suitability of extruded insect-contained aquafeeds has recently been reported.[62,77,95] Feeding practices should also address minimizing feed waste and improving feed conversion ratio,[3,5] thereby improve environmental impacts.

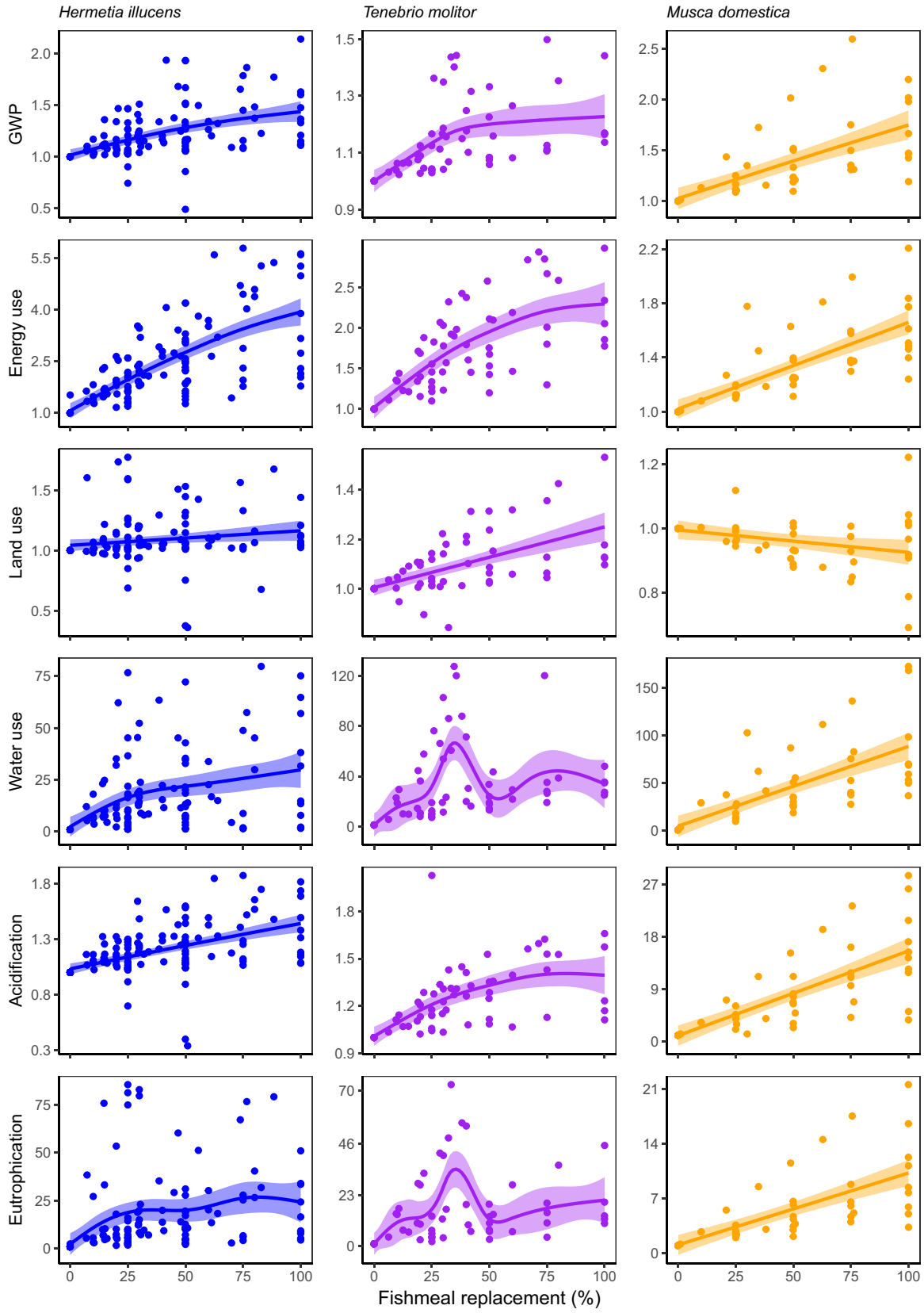

FIGURE 4 The relationship between response ratio of fishmeal replacement level and environmental impacts in aquafeeds. Coloured solid lines and shaded areas represent fitted models with the mean and 95% confidence interval respectively. Dots are observed data points. GWP, global warming potential

3.6 | Reducing the environmental impact of insect meals

Insect rearing facilities, including production of insect feed, rearing area and processing have been reviewed,[58,116] in this section, we focussed on reducing inefficiency of insect production with respect to environmental issues.

3.6.1 | Insect feeds

Feed production for insect is the most critical aspect of insect meal production with respect to the environment.[37,56] Investigating environmental aspects of substrates for insect rearing could enhance the benefits of insect meal use in aquafeeds. Some insect species have effectively transformed organic waste and manure into biomass,[117] which could partially address global waste concerns. From an environmental perspective, culture of *H. illucens* on cattle manure and municipal waste is superior to using traditional media such as chicken manure and beet pulp, but comparative to distiller's grains with solubles (DDGS).[118] Bava et al.[61] reported lower environmental impact of farming *H. illucens* on maize distiller compared to hen diet substrates. It was recommended that brewer spent substrates generated from sorghum and barley with supplementation by brewer's yeast or brewer's yeast plus molasses are more suitable for *H. illucens* rearing with respect to improving protein content and minerals.[119] Housefly (*M. domestica*) was found to thrive on manure,[56] with chicken manure more environmentally efficient than sheep manure.[53] Spent grain substrates (fermented or heat-dried) offer superior greenhouse gas emission levels compared to crop-derived feed for *H. illucens*.[120] At insect rearing on an industrial scale, Scala et al.[121] reported superior output of *H. illucens* reared on spent grains than with fruit substrates, suggesting potential of spent grain for more sustainable insect productive systems.

Feeding insects on waste food should be done with caution, as it may compete with the bioenergy industry in sourcing material, increasing the environmental impact.[51] The most efficient solution is to source surplus organic substrate and to use waste material from insect rearing as fertilizer or in the bioenergy sector.[122] Organic waste and manure substrates are not favourable for *T. molitor*[37,123] in term of growth production (Table S1), which could be attributed to low nutrient values and high starch content of these substrates.[123,124] However, *T. molitor* thrives on mixtures such as dried brewing by-products, derivatives of potato processing, DDGS, by-products of the biofuel industry, livestock feeds and plant-derived products.[37,56] Distiller's dried grains with solubles are not shown to be an ideal substrate for *T. molitor* culture and resulted in a higher environmental burden compared to others tested, while a mixture of wheat bran and animal feeds is preferable.[37]

In Europe, substrates used in insect culture are regulated by European Commission regulation 767/2009 and 999/2001, with non-authorized substrates including separated digestive tract content, manure, catering waste and processed animal protein, except fishmeal. Regulations 1069/2009, 142/2011 and 767/2009 list authorized plant-based substrates. Effort should focus on identifying the most suitable authorized materials to limit the environmental footprint of insect products. Distiller's dried grain with solubles appears to be the most promising candidate for both *H. illucens* and *M. domestica* production, while livestock feed is optimal for *T. molitor*. To confirm this, more research is needed to explore potential of those substrates on production output, nutritional composition of the resulting insect meal, economic feasibility and environmental impact.

It should be taken into consideration that these substrates are currently used as feed in the livestock sector, and their demand for insect production could lead to elevate the global price[57] and/or increase demand for alternative sources to fill the protein gap for other animals.[35] Therefore, criteria for insect diets should comply with current regulations and prioritize local sources to reduce cost of transport,[53] utilizing surplus production/side streams or ingredients that are not competitive with other farming sector.[116,121,125]

3.6.2 | Insect rearing facilities

It is necessary to design space-efficient insect rearing production facilities to optimize land use, which is species-specific. For instance the use of three-dimensional crawling space design for vertical crawling, jumping, flying insect is preferable to two-dimensional flat spaces which could yield higher productivity per unit area.[116] Expanding production vertically or increasing use of multilevel shelves or stackable boxes in insect construction facilities can further optimize land use.[37,51,116]

The largest portion of energy use for insect meal production is associated with providing heat for insect rearing[34,51,52] and drying.[126] It may be energy efficient to install insect production facilities in an equatorial climate.[34] It has been suggested that renewable energy sources could be a promising solution, potentially reducing the burden by approximately 25%.[35] The same finding was reported by Samuel-Fitwi et al.[42] who stated that aquafeed production showed lower environmental impact when using wind power compared to fossil fuel sources. Use of by-products of insect meal production for anaerobic digestion and fertilizer could contribute to environmental conservation.[51] The use of photovoltaic energy as an energy source for insect meal production is also a potential option. This was applied in Italy, resulting in a decrease of 14.2% in global warming potential, 19.2% in energy use and 1.8% in land use.[34] Adaptation to utilize residual heat from nearby facilities could considerably reduce impact, saving 1247 kg CO_2 eq. global warming potential, 23,949 MJ energy use and 1 m^2a land use per ton of insect meal.[51]

Insect farming is undergoing increasing production,[11,104] which could offer considerable environmental benefits in general[125] and energy use in particular[51] associated with insect meal products and insect meal-based aquafeeds. Further life cycle assessment studies should focus on broader perspectives of production facilities and location in combination with optimal insect rearing substrates.

4 | CONCLUSIONS

Our results provide insights into the environmental impact of insect meal production and its use in aquafeeds. Insect meals of *H. illucen*, *T. molitor* and *M. domestica* are the least land use among conventional and novel aquafeed ingredients, while together with alternative protein sources, exert an enormous impact on global warming potential, energy use, water use, acidification and eutrophication. Dietary silkworm (*B. mori*) significantly reduced faecal phosphorus waste, whereas inclusion of *H. illucens*, *M. domestica*, *T. molitor* and *Z. variegatus* elevated faecal nitrogen waste in comparison with insect-free diets. Substitution of fishmeal by insect meal also significantly reduced economic fish-in fish-out, the marine fish and whole fish demand for one unit of aquaculture fish produced. In addition, from a life cycle assessment perspective, insect meal shows promising in terms of mitigating the environmental impact of aquafeeds associated with land use, especially *T. molitor* and *M. domestica* insect species. Therefore, our study suggested the potential of insect meal for an aquaculture industry to thrive on the limited natural resources—agriculture land, and to grow with less phosphorus load. Intensifying industrial insect farming with standard and energy-efficient facilities and developing suitable insect-specific substrates to address nutritional composition and environmental aspects will be essential for insect meal as a future protein source supply for aquafeeds. We also suggest that insect meal is not the sole solution for lowering the environmental impacts of future aquafeeds. The combination of multiple alternative protein and lipid sources in aquafeeds will be strategic approach for environmental sustainability of aquafeeds, thus aquaculture sector.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Partially Defatted *Hermetia illucens* Larva Meal in Diet of Eurasian Perch (*Perca fluviatilis*) Juveniles

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Simple Summary: The replacement of fishmeal by insect meal is a promising strategy to obtain more sustainable fish feeds, a major goal in aquaculture. Black soldier fly *Hermetia illucens* larva meal has a high crude protein and fat content, essential for omnivorous and carnivorous fish. We used partially defatted *H. illucens* larva meal as a substitute for 20, 40 and 60% of the fishmeal in standard diets for Eurasian perch and measured its effect on growth performance, feed utilization, body indices, fish body composition and blood indices. We found no significant differences in survival, size heterogeneity, hematology indices; or in whole-body dry matter, crude protein and ether extract content. The 60% inclusion reduced final body weight, specific growth rate, feeding rate, protein efficiency ratio, condition factor and hepatosomatic index. The fish-in-fish-out index decreased proportionally with increased *H. illucens* meal inclusion. Partially defatted *H. illucens* larva meal seems to be a promising alternative to fishmeal for Eurasian perch nutrition at moderate inclusion level.

Abstract: Insect meal is gaining increased attention in aquafeed formulations due to high protein content and an essential amino acid profile similar to that of fishmeal. To investigate insect meal in feed for European perch *Perca fluviatilis*, a promising candidate for European intensive culture, we replaced standard fishmeal with partially defatted black soldier fly *Hermetia illucens* larva meal at rates of 0%, 20%, 40% and 60% (groups CON, H20, H40 and H60, respectively) and compared growth performance, somatic indices, hematological parameters, whole-body proximate composition and occurrence of spleen lipidosis. In addition, we assessed the economic and environmental sustainability of the tested feeds by calculating economic conversion ratio (ECR) and economic profit index (EPI). The tested groups did not differ in survival rate. Significant differences were documented in final body weight and specific growth rate, with the highest values in CON, H20 and H40. The proximate composition of fish whole-body at the end of the experiment did not differ in dry matter, crude protein or ether extract, while organic matter, ash and gross energy composition showed significant differences. The fatty acid content and n-3/n-6 ratio showed a decreasing trend with increasing H. *illucens* larva meal inclusion. No differences were found in hematological parameters among tested groups. The H. *illucens* larva meal inclusion significantly affected ECR and EPI, even at 20% inclusion level the cost of diets did not differ from the control fish meal based diet. Results suggested that 40% inclusion of H. *illucens* larva meal can be used successfully in standard diets for perch.

Keywords: alternative feed; insect meal; splenic lipidosis; economic and environmental sustainability

1. Introduction

Intensive culture of the carnivorous freshwater Eurasian perch (*Perca fluviatilis* L.) is increasing in recirculating aquaculture systems (RAS) and represents an expanding branch of commercial fish farming in Europe. Nevertheless, as a relatively new aquaculture species, production is low [1]. It is commonly reared on feed formulated primarily for salmonids or marine fish species [2]. Diets for carnivorous species contain high levels of protein, which have been obtained from marine fishmeal (FM), considered optimal because of its balanced nutritional composition [3,4]. Currently, with FM increased cost and unsustainability [5], plant protein sources, especially soybean meal, are being used in aquaculture to decrease the dependency on FM and reduce feed costs [4]. High levels of plant protein in feeds can reduce growth performance or induce fish health issues, chiefly due to imbalance in essential amino acid (EAA) content, low feed acceptance and the presence of anti-nutritional factors [3,6,7]. Processed animal proteins (PAP) such as poultry by-product meal, meat meal and meat and bone meal are valid proteins for aquaculture feeds but their use is limited by legislation. In the EU, PAP from poultry and swine have only recently been reintroduced into aquafeed (EC No. 56/2013) after more than 10 years of ban due to Bovine Spongiform Encephalopathy (EC No 999/2001), while in other parts of the world, its use is common practice [8].

Recently, interest has turned to PAPs from insects as a component of aquafeeds [9,10]. Insect larva meals are rich in proteins and their EAA profile is close to that of FM and considered superior to that of plant proteins [9]. The use of insect PAP has recently been sanctioned by the European Commission (Brussels, Belgium) (Regulation 2017/893/EC, 2017).

The black soldier fly *Hermetia illucens* belongs to the family *Stratiomyidae* and is among the most promising insect species for mass-rearing for animal feed [11]. Commercial *H. illucens* meal has an average protein content of 55% dry matter (DM) with lipid content ranging from 5% to 35% DM, depending on the defatting process applied during meal production. Research into its efficacy has thus far been contradictory: Similar or better growth performance to that of fish fed conventional protein sources (mainly FM or soybean meal) using commercial *H. illucens* meal at inclusion levels from 2.5% to 40% were obtained for Atlantic salmon (*Salmo salar)* [12–14], rainbow trout (*Oncorhynchus mykiss)* [15,16], European sea bass (*Dicentrarchus labrax)* [17], yellow catfish (*Pelteobagrus fulvidraco)* [18] and rice field eel (*Monopterus albus)* [19]. Conversely, other authors reported reduced acceptance and growth [20,21], with high levels of inclusion. Divergence in results is likely due to the differences among H. *illucens* meals and the level of inclusion in the diet and also suggest species differences in adaptation to insect meals. The use of *H. illucens* meal in perch diets has not been investigated.

The goal of this research was to determine the effects of partially defatted *H. illucens* meal as partial substitute for FM on growth performance, somatic indices, occurrence of splenic lipidosis, hematological parameters and proximate whole-body composition of juvenile *P. fluviatilis*. The research also aimed to provide new data on the economic and environmental sustainability of this novel protein source.

2. Materials and Methods

An 84-day growth trial was carried out at the Faculty of Fisheries and Water Protection of the University of South Bohemia (České Budějovice, Czech Republic). The trial was designed and carried out in accordance with the Czech and European Communities Directive (2010/63/EU) on the protection of animals used for scientific purposes, protocol number MSMT-6744/2018-2.

2.1. Experimental Diets

Four experimental diets were formulated to be isonitrogenous (crude protein, CP: ~54 g 100 g DM); isolipidic (ether extract, EE: ~13 g 100 g DM); and isoenergetic (gross energy, GE: ~23 MJ kg DM). An FM-based diet was used as control (CON) and three additional diets included FM replacement with 20% (H20), 40% (H40) and 60% (H60) partially defatted *H. illucens* larva meal obtained with a mechanical process performed using high pressure and without solvents was provided by Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany). In order to ensure that diets were isonitrogenous, isolipidic and isoenergetic, the proportion of wheat meal and fish oil was reduced with increase in *H. illucens*.

The experimental feeds were prepared at the Department of Agricultural, Forest and Food Sciences experimental facility. Finely ground ingredients and fish oil were thoroughly mixed with water and pelleted using a 2 mm meat grinder and dried at 50 °C for 48 h. Feeds were stored in dark bags at −20 °C until use. The ingredients of the experimental diets are reported in Table 1. An additional control group (BIO) was fed a commercial extruded diet (BioMar Inicio 2 mm, BioMar A/S, Brande, Denmark) containing fish meal, wheat gluten, wheat, pea protein, soybean concentrate, rapeseed oil, fish oil and yeast extract as main ingredients. Proximate composition (on a wet basis) according to manufacturer's label was CP 52%, crude lipid 23%, carbohydrates 12%, ash 8.7%, fiber 0.9%, total phosphorus (P) 1.2% and GE 23.5 MJ/kg.

Table 1. Ingredients and proximate composition of *Hermetia illucens* larva meal and experimental diets.

Ingredients (g/kg)	*H. illucens* Larva Meal	CON	H20	H40	H60
FM (Chile, super prime) [a]	-	720	570	420	270
H. illucens larva meal [b]		0	200	400	600
Wheat meal	-	120	90	60	30
Fish oil	-	60	40	20	0
Starch, D500	-	80	80	80	80
Mineral mixture [c]	-	10	10	10	10
Vitamin mixture [d]	-	10	10	10	10
Proximate composition[e]					
DM (g/100g)	94.18	88.74	90.76	90.59	90.51
CP (g/100g DM)	55.34	54.50	54.37	54.10	53.91
EE (g/100g DM)	17.97	11.92	11.95	11.62	11.64
Ash (g/100g DM)	7.12	14.77	13.70	12.44	11.41
Chitin (g/100g DM)	5.00	-	0.98	2.12	3.15
NFE (g/100g DM) [f]	14.57	18.81	19.02	19.72	19.89
Gross energy (MJ/kg DM) [g]		22.90	22.54	23.02	23.26

FM, fishmeal; DM, dry matter; CP, crude protein; EE, ether extract; NFE, nitrogen free extracts, groups CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of H. *illucens* meal, respectively. [a] Fishmeal was purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (% as-fed basis): 90.4 DM; 66.7 CP; 8.3 EE; 14.9 Ash. [b] *Hermetia illucens* larvae meal purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany). [c] Mineral mixture (g or mg/kg diet): bicalcium phosphate 500 g, calcium carbonate 215 g, sodium salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulphate 20 g, zinc sulphate 4 g, copper sulphate 3 g, potassium iodide 4 mg, cobalt sulphate 20 mg, manganese sulphate 3 g, sodium fluoride 1 g (Granda Zootecnica, Cuneo, Italy). [d] Vitamin mixture (IU or mg/kg diet): DL-tocopherolacetate, 60 IU; sodium menadione bisulphate, 5 mg; retinylacetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamine, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; vitamin B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnica, Cuneo, Italy). [e] Values are reported as mean of triplicate analyses. [f] Calculated as 100 − (CP + EE + Ash + Chitin). [g] Determined by bomb calorimetry.

2.2. Fish and Feeding Trial

Eurasian perch juveniles were obtained from pond-reared larvae and intensively reared juveniles in an RAS [22]. The RAS (4360 L total water volume) included fifteen 75 L rearing tanks, a mechanical drum filter AEM 15 (AEM-Products V.O.F., Lienden, The Netherlands), a 1620 L tank with a series of filtration sections, Bioakvacit PP10 (Jezírka Banát s.r.o., Hněvotín, Czech Republic), a moving bed biofilter (1620 L) with media BT10 (Ratz Aqua & Polymer Technik, Remscheid, Germany), UV treatment AquaForte 55 W (AquaForte, Veghel, The Netherlands) and an Eheim Jäger Thermocontrol 300 flow-through heater (Eheim GmbH & Co KG, Stuttgart, Germany) incorporated directly into the recirculation flow. The flow rate in the tanks was approximately 80 L h^{-1} with light aeration. Photoperiod was set at 12:12 h (dark: light) with light intensity of 500–700 Lx at the surface. Oxygen saturation (83.7 ± 6.2%), pH (6.83 ± 0.52) and water temperature (22.5 ± 0.7 °C) (HACH HQ 40, Germany) were measured daily at 08.00 and 16.00. Ammonia, nitrate and nitrite concentrations were analyzed at two-day intervals with kits (HACH, LCK 304, LCK 339, LCK 341), using a HACH DR2800 spectrophotometer. The concentration of nitrite-N, nitrate-N and ammonia-N were 0.62 ± 0.44 mg L^{-1}, 88.88 ± 57.31 mg L^{-1} and 2.07 ± 1.02 mg L^{-1}, respectively.

A total of 750 juvenile European perch were lightly anaesthetized (0.3 mL L^{-1} of clove oil), individually weighed (initial body weight (BWi) 21.9 ± 4.2 g) using a digital balance (Pioneer, Ohaus Corporation, Parsippany, NJ, USA, d = 0.01 g) and randomly allocated to one of the fifteen 75 L rectangular plastic tanks at a stocking density of 14.6 kg m^{-3}. The four experimental diet groups and the BIO group were randomly allocated to the fifteen tanks, with each diet tested in triplicate. Fish were fed manually to subjectively-judged satiation five times daily (09:00, 11:00, 13:00, 15:00 and 17:00 h). Care was taken to avoid feed waste and to ensure that all supplied feed was consumed. The feeding trial lasted 84 days.

2.3. Growth Performance

At the end of the trial, all fish were individually weighed and growth performance was calculated using following equations:

$$\text{Survival (S, \%)} = 100 \times \text{Nf (Ni} - \text{Ns)}^{-1}$$

$$\text{Initial coefficient of variation (ICV, \%)} = (\text{SD/BWi}) \times 100$$

$$\text{Final coefficient of variation (FCV, \%)} = (\text{SD/BWf}) \times 100$$

$$\text{Specific growth rate (SGR, \% day}-1) = ((\text{lnBWf} - \text{lnBWi})/\text{Nd}) \times 100$$

$$\text{Feed conversion ratio (FCR)} = (\text{TFS/WG})$$

$$\text{Protein efficiency ratio (PER)} = (\text{WG (g)/TPS total protein fed (g, DM)})$$

$$\text{Feeding rate (FR, \%/d)} = ((\text{TFS} \times 100/\text{Nd}))/(\text{e (lnBWf} + \text{lnBWi}) \times 0.5),$$

where Ni and Nf = initial and final number of fish per tank, Ns = number of sampled fish per tank, BWf = final body weight (g), BWi = initial body weight (g), Nd = number of feeding days, TFS = total feed supplied (g), TPS = total protein supplied (g, DM), SD = standard deviation of subsample BW, BWi = initial mean body weight, BWf = final mean body weight, lnBWf = natural logarithm of final body weight, lnBWi = natural logarithm of initial body weight, DM = dry matter, WG = weight gain.

2.4. Condition Factor, Somatic Indexes and Occurrence of Spleen Lipidosis

To calculate condition factor (K), at the end of the growth trial, fifty fish from each tank were anaesthetized (0.3 mL L^{-1} of clove oil) and individually weighed and measured for total length (TL, mm) and standard length (SL, mm) within 1 mm using a ruler.

K was calculated as:

$$K = (BWf/TL) \times 100,$$

where BWf = final body weight (g), TLf = final body length (cm).

At the end of the trial, 30 fish/tank were killed by overdosing of anesthesia with clove oil and wet weight of liver, spleen, viscera and perivisceral fat recorded (±0.01 g) for calculation of somatic indices using the equations:

$$\text{Hepatosomatic index (HSI)} = Wl \text{ (weight, g)} \times 100/BW \text{ (body weight, g)}$$

$$\text{Splenosomatic index (SSI)} = Ws \text{ (weight, g)} \times 100/BW \text{ (body weight, g)}$$

$$\text{Viscerosomatic index (VSI)} = Wv \text{ (weight, g)} \times 100/BW \text{ (body weight, g)}$$

$$\text{Perivisceral fat index (PFI)} = Wpf \text{ (weight, g)} \times 100/BW \text{ (body weight, g)},$$

where Wl = liver weight (g), Ws = spleen weight (g), Wv = viscera weight (g), Wpf perivisceral fat weight (g).

Frequency of occurrence splenic lipidosis [2], was calculated according to the equation:

$$SL = 100/Nt \times Nsl,$$

where Nt is total number of investigated fish and Nsl is number of fish with spleen lipidosis.

2.5. Chemical Analyses

The H. *illucens* larva meal chemical analysis was obtained from Renna et al. [15]. The proximate composition and energy of diets are reported in Table 1. Feed samples were finely ground (MLI 204; Bühler AG, Uzwil, Switzerland) and analyzed for DM (AOAC, n. 934.01), CP (AOAC, n. 984.13) and ash (AOAC, n. 942.05) content according to AOAC International [23]. The EE content (AOAC, n. 2003.05) was analyzed according to AOAC International [24]. The GE content was determined using an adiabatic bomb calorimeter (C7000; IKA, Staufen, Germany). Chitin content was determined following Finke [25], by correcting for the amino acid (AA) content of the acid fiber detergent (ADF) fraction and assuming the remainder of the ADF fraction to be chitin. The AA composition of H. *illucens* larva meal and FM used in the experimental diets is shown in Table 2. Amino acid quantification was conducted according to De Marco et al. [26]. After 22 h hydrolysis in 6N HCl at 112 °C under a nitrogen atmosphere, the AA content in the hydrolysate was assessed by HPLC after post-column derivatization. Performic acid oxidation occurred prior to acid hydrolysis for methionine and cystine. Tryptophan was not determined.

Table 2. Amino acid (AA) profile (% of protein) of *Hermetia illucens* larva meal and experimental diets.

	H. illucens	CON	H20	H40	H60
Essential AA					
Arginine	3.9	6.2	5.7	5.2	4.7
Histidine	2.2	2.4	2.4	2.3	2.3
Isoleucine	3.3	4.2	4.0	3.8	3.6
Leucine	5.2	7.3	6.8	6.4	5.9
Lysine	3.8	7.4	6.7	5.9	5.1
Methionine	2.1	2.7	2.5	2.2	2.0
Cysteine	0.1	0.9	0.7	0.5	0.4
Phenylalanine	3.0	4.0	3.7	3.5	3.3
Tyrosine	4.8	3.1	3.4	3.8	4.1
Threonine	3.1	4.1	3.9	3.7	3.5
Valine	4.9	4.9	4.9	4.9	4.9
Non-essential AA					
Alanine	6.2	6.1	6.1	6.1	4.9
Aspartic acid	6.7	8.8	8.4	7.9	7.5
Glycine	4.2	0.9	1.6	2.2	2.9
Glutamic acid	8.8	7.0	7.3	7.6	7.9
Proline	5.5	12.3	10.9	9.5	8.0
Serine	3.7	4.1	4.0	3.9	3.8

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of H. *illucens* larva meal, respectively.

At the end of the trial, whole-body homogenate of six fish from each group was analyzed for DM, CP, EE, organic matter (OM) and ash content according to the procedure used for feed analyses [23,24]. The DM content was measured according to AOAC (n. 934.01; [23]).

Fatty acid profiles were determined both in feed and fish whole-body homogenate (3 fish/tank, 9 fish/group), according to the method of Sampels et al. [27]. Initially, lipids were extracted by the hexan-isopropanol method according to Hara and Radin [28]. Fatty acid methyl esters (FAME) were prepared by the BF_3 method according to Appelqvist [29] and analyzed using FAME C 11:0 as an internal standard by a gas chromatograph (Trace Ultra FID; Thermo Scientific, Milan, Italy) equipped with a flame ionization detector, using a BPX 70 column (length 50 m, i.d. 0.22 mm, film thickness 0.25 μm) (SGE Inc., Austin, TX, USA). The peaks were identified using Thermo Xcalibur 3.0.63 (Thermo Fisher Scientific Inc., Waltham, MA, USA) software and quantification was achieved by comparing sample retention times and peak areas to retention times and peak area in 7 levels (1000 ug/mL–15 ug/mL) of the standard mixture Supelco 37 component FAME mix (Sigma-Aldrich, St. Louis, MO, USA). Fatty acid profiles for feed are shown in Table 3, analysis were performed in triplicate.

Table 3. Fatty acid profile of experimental diets for Eurasian perch. Data are expressed as percent of total FAs (mean ± SD, n = 3).

FA	CON	H2O	H4O	H6O
C12:0	2.54 ± 0.27	11.89 ± 0.17	24.47 ± 1.81	34.37 ± 1.23
C14:0	4.76 ± 0.04	6.54 ± 0.06	8.77 ± 0.11	10.74 ± 0.38
C14:1	0.05 ± 0.01	0.13 ± 0.01	0.24 ± 0.01	0.33 ± 0.01
C15:0	0.40 ± 0.01	0.35 ± 0.01	0.28 ± 0.01	0.23 ± 0.01
C16:0	15.38 ± 0.04	16.01 ± 0.05	16.58 ± 0.32	17.21 ± 0.69
C16:1	4.21 ± 0.01	4.49 ± 0.01	4.79 ± 0.10	5.08 ± 0.11
C18:0	3.77 ± 0.03	3.63 ± 0.04	3.22 ± 0.07	3.02 ± 0.13
C18:1n9trans	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
C18:1n9	26.60 ± 0.20	22.08 ± 0.02	16.20 ± 0.36	11.29 ± 0.27
C18:1n7	3.23 ± 0.02	2.57 ± 0.01	1.68 ± 0.04	0.94 ± 0.04
C18:2n6	9.18 ± 0.03	8.61 ± 0.01	7.79 ± 0.18	7.20 ± 0.14
C18:3n6	0.20 ± 0.01	0.16 ± 0.01	0.11 ± 0.01	0.07 ± 0.01
C18:3n3	3.15 ± 0.01	2.56 ± 0.01	1.80 ± 0.05	1.14 ± 0.03
C20:0	0.32 ± 0.01	0.27 ± 0.01	0.21 ± 0.01	0.15 ± 0.01
C20:1n9	2.55 ± 0.02	1.91 ± 0.01	1.05 ± 0.03	0.32 ± 0.03
C20:3n6	0.76 ± 0.01	0.56 ± 0.01	0.31 ± 0.01	0.09 ± 0.01
C20:3n3	0.71 ± 0.01	0.56 ± 0.01	0.38 ± 0.01	0.23 ± 0.01
C20:4n6	0.32 ± 0.01	0.24 ± 0.01	0.13 ± 0.01	0.03 ± 0.01
C22:0	0.16 ± 0.01	0.13 ± 0.01	0.08 ± 0.01	0.05 ± 0.01
C22:1n9	0.37 ± 0.01	0.27 ± 0.01	0.15 ± 0.01	0.04 ± 0.01
C20:5n3	6.80 ± 0.03	5.44 ± 0.01	3.72 ± 0.10	2.21 ± 0.01
C22:2	0.07 ± 0.01	0.05 ± 0.01	0.32 ± 0.50	0.65 ± 0.56
C24:0	0.17 ± 0.01	0.13 ± 0.01	0.10 ± 0.01	0.05 ± 0.03
C24:1n9	0.55 ± 0.01	0.44 ± 0.01	0.29 ± 0.01	0.16 ± 0.01
C22:5n3	1.44 ± 0.01	1.06 ± 0.02	0.69 ± 0.02	0.31 ± 0.02
C22:6n3	12.23 ± 0.18	9.85 ± 0.11	6.61 ± 0.15	4.03 ± 0.14
SFA	27.58 ± 0.34	39.00 ± 0.12	54.02 ± 1.06	66.47 ± 0.77
MUFA	37.63 ± 0.23	31.96 ± 0.03	24.45 ± 0.54	18.23 ± 0.40
PUFA	34.79 ± 0.21	29.04 ± 0.11	21.53 ± 0.51	15.30 ± 0.38
n-3	24.33 ± 0.22	19.47 ± 0.12	13.20 ± 0.32	7.90 ± 0.28
n-6	10.46 ± 0.03	9.57 ± 0.01	8.33 ± 0.19	7.39 ± 0.15
n-3/n-6	2.33 ± 0.02	2.03 ± 0.01	1.58 ± 0.01	1.07 ± 0.03

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively; SD, standard deviation, FA fatty acid, SFA saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.

2.6. Haematological Analyses

At the end of experiment, three fish per tank (nine fish from each group) were over-anaesthetized with clove oil and blood samples were taken for hematological analysis. Red blood cell count (RBCC), hematocrit (HCT), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were measured according to Svobodova et al. [30].

2.7. Economic Analysis and Environmental Sustainability of Feeds

To determine the relative efficacy and benefits of tested diets, economic conversion ratio (ECR) and economic profit index (EPI) for each tested group was calculated by the following equations:

$$\text{ECR (€ kg of fish}^{-1}\text{)} = \text{FCR} \times \text{DP}$$

$$\text{EPI (€ fish}^{-1}\text{)} = (\text{WG} \times \text{SP}) - (\text{WG} \times \text{DP}),$$

where FCR is feed conversion ratio (kg feed per kg fish); DP is cost per kg feed; WG is weight gain. The per kilogram cost in euros, excluding labor and taxes, of all components from commercial retailers was as follows: FM = € 1.48; H. *illucens* larva meal = € 3.5; wheat meal = € 0.61; fish oil = € 1.32; gelatinized starch = € 0.75; mineral mixture = € 0.49; vitamin mixture = € 3.85. This resulted in per kg feed cost of CON = € 1.31; H20 = € 1.75; H40 = 2.18; H60 = € 2.61; and BIO = € 2.53. Eurasian perch sale price (SP) was calculated at € 6.50 kg^{-1}.

Fish-in fish-out (FIFO) ratio was used as a practical measure of the quantity of live fish from capture fisheries required for each unit of farmed fish produced [31]. This indicator of environmental sustainability of feeds was calculated as follows:

$$\text{FIFO} = (\text{LFM} + \text{LFO})/(\text{YFMw} + \text{YFOw}) \times \text{FCR},$$

where LFM is level of fishmeal in the diet; LFO is level of fish oil in the diet; YFMw is yield of fishmeal from wild fish; YFOw is yield of fish oil from wild fish; FCR is feed conversion ratio.

We estimated the impact of FM substitution with *H. illucens* larva meal rapported to Metric Tons (MT) on freshwater demand (WD, m^3/MT), land demand (LD, ha/MT), energy use (EU, GJ/MT) and greenhouse gas production (GWP, kg CO_2–eq). Mean WD, LD and EU for FM, wheat, fish oil, starch and mineral and vitamin mixes were obtained from Chatvijitkul et al. [32]. Data of WD, LD, EU and GWP for *H. illucens* larva meal was retrieved from Roffeis et al. [33]. Finally, GWP for FM was sourced from Thevenot et al. [34] and GWP for wheat meal from Heusala et al. [35].

2.8. Statistical Analyses

All data were tested for homogeneity of variance using Cochran, Hartley and Bartlett tests. Normality of data was tested by Shapiro-Wilk test. Perivisceral fat index, splenosomatic index, some minor fatty acids, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and economic profit index were analyzed using Kruskal-Wallis non-parametric test as these data does not show normality. All other remaining parameter results were analyzed separately by one-way ANOVA. Differences were considered significant at $p \leq 0.05$ (post-hoc test: Tukey test). The data were expressed as mean ± SD and statistical analyses were performed using STATISTICA 12.0 (StatSoft CR, Prague, Czech Republic). As BIO was a completely different diet, not comparable with respect to composition, nutrient and energy contents, it was not included in the statistical analyses.

3. Results

3.1. Diet Composition

Diets were comparable in proximate composition, which reflected the calculated one. The amino acid profile of H. *illucens* larva meal and experimental diets is presented in Table 2. Leucine,

tyrosine and valine were the most common EAAs in the H. *illucens* larva meal, with the non-essential AAs glutamic and aspartic acid showing the highest content. *Hermetia illucens* larva meal showed similar values for histidine and lower values for arginine and lysine than observed in FM [36]. With increasing dietary H. *illucens* proportions, all EAAs decreased except valine and tyrosine, which remained constant and increased, respectively.

3.2. Growth Trial

Fish survival and growth performance are shown in Table 4. With all diets, fish tripled their initial body weight. Fish readily accepted the feeds and no rejection was recorded. At the end of the 84-day experiment, no significant differences in survival were observed among diets. There were no significant differences among experimental groups in BWi, ICV and FCV. On the other hand, BWf, SGR, PER and FR differed significantly with diet, with the H60 treatment showing lower values compared to other treatments.

Table 4. Survival and growth performance of Eurasian perch fed experimental diets and the commercial control diet (mean ± SD; n = 3).

Items	CON	H20	H40	H60	SEM	*p*-Value	BIO *
Survival, %	98.7 ± 2.3	98.7 ± 2.3	98.0 ± 1.2	99.3 ± 1.2	0.512	0.878	96.0 ± 4.0
BW_i, g	21.9 ± 0.1	22.0 ± 0.1	22.1 ± 0.1	22.0 ± 0.1	0.023	0.195	22.0 ± 0.1
BW_f, g	63.8 ± 1.2 ^{a}	67.1 ± 2.0 ^{a}	68.1 ± 1.8 ^{a}	58.0 ± 3.2 ^{b}	1.305	0.002	74.1 ± 6.0
WG, g	41.8 ± 1.0 ^{a}	45.1 ± 2.0 ^{a}	46.0 ± 1.7 ^{a}	36.0 ± 3.2 ^{b}	1.296	0.002	52.1 ± 5.9
ICV, %	19.4 ± 0.6	19.6 ± 0.8	19.5 ± 0.9	19.3 ± 0.9	0.205	0.981	19.0 ± 0.9
FCV, %	37.9 ± 1.6	32.9 ± 7.0	34.3 ± 7.1	38.3 ± 1.8	1.439	0.525	42.4 ± 9.1
SGR, %/d	1.25 ± 0.06 ^{a,b}	1.30 ± 0.03 ^{a}	1.30 ± 0.04 ^{a}	1.14 ± 0.03 ^{b}	1.331	0.000	1.39 ± 0.11
FCR	1.00 ± 0.07 ^{a,b}	0.91 ± 0.05 ^{b}	0.91 ± 0.04 ^{b}	1.12 ± 0.06 ^{a}	0.029	0.006	0.96 ± 0.13
PER	1.72 ± 0.12 ^{a,b}	1.91 ± 0.11 ^{a}	1.90 ± 0.08 ^{a}	1.55 ± 0.08 ^{b}	0.050	0.000	1.88 ± 0.23
FR, %/d	1.36 ± 0.03 ^{a,b}	1.30 ± 0.04 ^{a}	1.30 ± 0.01^{a}	1.39 ± 0.04 ^{b}	0.014	0.023	1.47 ± 0.06

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, SEM, standard error of the mean; BW_i, initial body weight; BW_f, final body weight; WG, weight gain; ICV, initial coefficient of variation of weight; FCV, final coefficient of variation of weight; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; FR, feeding rate. Different letters within a row indicate significant differences ($p \leq 0.05$). * Statistical analysis did not include BIO.

3.3. Condition Factor, Somatic Indices and Occurrence of Spleen Lipidosis

Fish fed H60 showed lower K and HSI compared to fish fed the CON diet, while no differences among treatments were recorded in any other parameter (Table 5). No splenic lipidosis was recorded in fish fed insect meal, while a high occurrence was recorded in fish fed the BIO diet.

Table 5. Condition factor (n = 45), somatic indices and occurrence of splenic lipidosis (n = 90) of Eurasian perch juveniles fed experimental diets and the commercial control diet (mean ± SD).

Items	CON	H20	H40	H60	SEM	*p*-Value	BIO *
K	1.20 ± 0.02 ^{a,b}	1.22 ± 0.02 ^{a}	1.19 ± 0.01 ^{a,b}	1.15 ± 0.01 ^{b}	0.008	0.020	1.28 ± 0.03
HSI	1.76 ± 0.20 ^{a}	1.41 ± 0.12 ^{a,b}	1.48 ± 0.10 ^{a,b}	1.21 ± 0.07 ^{b}	0.067	0.006	1.37 ± 0.04
SSI	0.12 ± 0.04	0.11 ± 0.04	0.10 ± 0.04	0.11 ± 0.05	0.010	0.964	0.13 ± 0.02
VSI	2.91 ± 0.38	2.79 ± 0.17	2.94 ± 0.19	3.06 ± 0.05	0.063	0.608	2.90 ± 0.19
PFI	6.19 ± 0.68	5.63 ± 0.19	6.06 ± 0.78	5.53 ± 0.25	0.157	0.826	9.43 ± 0.97
SL	3.9 ± 6.71	NF	NF	NF		-	19.5 ± 13.6

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively, BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, SEM, standard error of the mean; K, condition factor; HSI, hepatosomatic index; SSI, splenosomatic index; VSI, visceromatic index; PFI, perivisceral fat index; SL, splenic lipidosis; NF, not found.

Different letters within a row indicate significant difference ($p \leq 0.05$). * Statistical analysis did not include BIO. No statistical analysis was performed for SL, as some diet groups did not show lipidosis.

3.4. Proximate and Fatty Acid Composition of Whole Fish Homogenate

The proximate composition of the whole fish homogenates showed no significant differences in DM, CP and EE content (Table 6). On the other hand, OM and GE content showed a decreasing trend with increased the *H. illucens* meal in the feed, while the opposite was recorded for ash content.

Table 6. Proximate composition of whole-body homogenate of Eurasian perch fed experimental diets and the commercial control diet (mean ± SD, n = 6).

Items	CON	H20	H40	H60	SEM	*p*-Value	BIO*
DM (g/100 g)	33.3 ± 1.0	32.9 ± 0.6	32.5 ± 0.6	32.1 ± 0.5	0.186	0.142	36.6 ± 1.1
CP (g/100 g DM)	24.1 ± 3.1	21.8 ± 0.9	21.6 ± 0.6	20.7 ± 0.3	0.466	0.065	22.4 ± 0.5
EE (g/100 g DM)	10.1 ± 1.3	9.5 ± 0.2	8.7 ± 0.5	8.5 ± 0.8	0.232	0.052	13.5 ± 1.0
OM (g/100 g DM)	28.6 ± 1.0 ^{a}	27.9 ± 0.6 ^{a}	27.2 ± 0.6 ^{a}	26.4 ± 0.8 ^{b}	0.254	0.001	32.1 ± 0.9
Ash (g/100 g DM)	4.7 ± 0.3 ^{b}	5.0 ± 0.2 ^{b}	5.3 ± 0.2 ^{b}	5.6 ± 0.3 ^{a}	0.098	0.003	4.5 ± 0.4
GE (MJ/kg DM)	0.81 ± 0.04 ^{a}	0.78 ± 0.01 ^{a}	0.75 ± 0.02 ^{a}	0.74 ± 0.03 ^{b}	0.009	0.014	0.95 ± 0.04

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, SEM, standard error of the mean; DM, dry matter; CP, crude protein; EE, ether extract; OM, organic matter; GE, gross energy. Different letters within a row indicate significant difference ($p \leq 0.05$). * Statistical analysis did not include BIO.

The fatty acid composition of Eurasian perch was significantly affected by the feed (Table 7). In general, saturated fatty acids (SFA) content tended to increase with increased *H. illucens* larva meal proportions with exception of C15:0 and C20:0. A trend similar to SFA was observed for monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Significant differences were found both in omega-6 and omega-3 content that decreased among tested *H. illucens* larva meal diets and consequently, the n-3/n-6 ratio decreased with increasing *H. illucens* larva meal inclusion.

Table 7. Fatty acid (FA) profile of whole-body homogenate Eurasian perch fed experimental and commercial control diet. Data are expressed as percent of total FAs (mean ± SD, n = 9).

FA	CON	H20	H40	H60	SEM	*p*-Value	BIO *
C12:0	1.00 ± 1.56 ^{b}	4.76 ± 1.81 ^{b}	8.83 ± 1.24 ^{a,b}	12.03 ± 1.49 ^{a}	0.79	<0.001	0.20 ± 0.20
C14:0	4.67 ± 0.61 ^{d}	5.96 ± 0.64 ^{c}	7.41 ± 0.35 ^{b}	9.14 ± 0.72 ^{a}	0.315	<0.001	5.52 ± 0.18
C14:1	0.54 ± 0.07 ^{d}	0.63 ± 0.05 ^{c}	0.79 ± 0.03 ^{b}	0.96 ± 0.06 ^{a}	0.03	<0.001	0.66 ± 0.03
C15:0	0.42 ± 0.02 ^{a}	0.36 ± 0.03 ^{a,b}	0.34 ± 0.02 ^{b}	0.34 ± 0.05 ^{b}	0.008	<0.002	0.49 ± 0.02
C16:0	17.49 ± 1.04	18.45 ± 0.79	17.81 ± 0.67	18.3 ± 1.79	0.206	0.335	18.98 ± 1.03
C16:1	9.46 ± 0.57 ^{b}	9.9 ± 0.46 ^{a,b}	10.35 ± 0.42 ^{a}	10.44 ± 0.51 ^{a}	0.109	0.001	10.81 ± 0.41
C18:0	1.6 ± 0.07	1.20 ± 0.74	0.96 ± 0.80	1.70 ± 0.40	0.112	0.262	1.23 ± 0.08
C18:1n9trans	1.06 ± 0.10 ^{a}	0.84 ± 0.16 ^{b}	0.84 ± 0.12 ^{b}	0.74 ± 0.13 ^{b}	0.03	<0.001	2.57 ± 0.13
C18:1n9	27.34 ± 0.96 ^{a}	26.34 ± 0.79 ^{a}	23.99 ± 1.04 ^{b}	21.42 ± 0.68 ^{c}	0.436	<0.001	21.16 ± 0.36
C18:1n7	3.07 ± 0.20 ^{a}	2.00 ± 1.24 ^{a,b}	1.70 ± 1.05 ^{b}	2.07 ± 0.17 ^{a}	0.167	<0.001	2.76 ± 0.08
C18:2n6	8.21 ± 0.40 ^{a}	7.74 ± 0.28 ^{a,b}	7.79 ± 0.40 ^{a}	7.28 ± 0.40 ^{b}	0.087	<0.001	7.15 ± 0.24
C18:3n6	0.08 ± 0.09 ^{a,b}	0.06 ± 0.08 ^{b}	0.04 ± 0.07 ^{b}	0.17 ± 0.01 ^{a}	0.015	0.005	0.15 ± 0.01
C18:3n3	2.23 ± 0.16 ^{a}	1.99 ± 0.09 ^{b}	1.65 ± 0.08 ^{c}	1.30 ± 0.07 ^{d}	0.065	<0.001	1.77 ± 0.05
C20:0	1.02 ± 0.38 ^{a}	1.07 ± 0.04 ^{a}	0.97 ± 0.09 ^{a}	0.42 ± 0.37 ^{b}	0.065	0.001	0.98 ± 0.95
C20:1n9	2.21 ± 0.23 ^{a}	1.73 ± 0.23 ^{a,b}	1.58 ± 0.22 ^{b}	1.64 ± 0.36 ^{b}	0.063	0.003	4.25 ± 0.16
C20:3n6	0.11 ± 0.05 ^{a}	0.07 ± 0.06 ^{a,b}	0.06 ± 0.04 ^{b}	0.09 ± 0.02 ^{a,b}	0.008	0.035	0.06 ± 0.03
C20:3n3	0.38 ± 0.25 ^{a}	0.32 ± 0.20 ^{a}	0.18 ± 0.19 ^{b}	0.30 ± 0.04 ^{a}	0.034	0.050	0.41 ± 0.04
C20:4n6	0.19 ± 0.08 ^{a}	0.09 ± 0.10 ^{a,b}	0.05 ± 0.07 ^{b}	0.11 ± 0.03 ^{a,b}	0.015	0.009	0.14 ± 0.02
C22:0	nd	nd	nd	nd			nd

C22:1n9	nd	nd	nd	nd			nd
C20:5n3	0.55 ± 0.47	0.58 ± 0.26	0.74 ± 0.14	0.56 ± 0.36	0.057	0.629	1.06 ± 1.47
C22:2	4.23 ± 0.39 [a]	3.58 ± 0.33 [b]	3.14 ± 0.38 [b]	2.62 ± 0.34 [c]	0.122	<0.001	5.99 ± 0.18
C24:0	nd	nd	nd	nd			nd
C24:1n9	nd	nd	nd	nd			nd
C22:5n3	1.16 ± 0.15 [a]	0.98 ± 0.12 [b]	0.75 ± 0.07 [c]	0.58 ± 0.06 [d]	0.043	<0.001	1.09 ± 0.09
C22:6n3	12.91 ± 1.2 [a]	11.35 ± 1.12 [b]	10.04 ± 0.60 [b]	7.66 ± 0.98 [c]	0.385	<0.001	12.46 ± 0.59
SFA	30.43 ± 2.54 [d]	35.37 ± 2.38 [c]	39.47 ± 1.38 [b]	44.62 ± 2.55 [a]	1.012	<0.001	33.39 ± 1.39
MUFA	43.74 ± 0.98 [a]	41.44 ± 0.89 [b]	39.25 ± 0.61 [c]	37.34 ± 1.01 [d]	0.455	<0.001	42.31 ± 0.90
PUFA	25.82 ± 1.94 [a]	23.18 ± 1.77 [b]	21.28 ± 1.00 [b]	18.04 ± 1.70 [c]	0.579	<0.001	24.30 ± 1.67
n-3	17.23 ± 1.58 [a]	15.22 ± 1.46 [b]	13.34 ± 0.78 [c]	10.40 ± 1.35 [d]	0.504	<0.001	16.80 ± 1.57
n-6	8.59 ± 0.40 [a]	7.97 ± 0.45 [b]	7.94 ± 0.33 [b]	7.64 ± 0.37 [b]	0.090	<0.001	7.50 ± 0.26
n-3/n-6	2.00 ± 0.12 [a]	1.91 ± 0.14 [a]	1.68 ± 0.09 [b]	1.36 ± 0.12 [c]	0.049	<0.001	2.24 ± 0.20

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark); SD, standard deviation, SEM, standard error of the mean; SFA saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids. Different letters within a row indicate significant difference ($p \leq 0.05$). * Statistical analysis did not include BIO; nd = not detected.

3.5. Haematological Analyses

Hemoglobin (Hb) concentration, HCT, RBBC, MCV, MCH and MCHC showed no differences among the feeding groups (Table 8).

Table 8. Hematological parameters of Eurasian perch fed experimental and commercial control diet (mean ± SD, n = 9).

Items	Unit	CON	H20	H40	H60	SEM	*p*-Value	BIO *
Hb	(g/L)	51.5 ± 2.9	51.4 ± 2.6	50.5 ± 3.6	51.0 ± 2.8	0.426	0.987	51.8 ± 2.8
HCT	(l/L)	32.4 ± 6.9	31.9 ± 10.2	30.4 ± 6.1	28.8 ± 9.3	1.180	0.829	32.7 ± 7.5
RBBC	(T/L)	1.90 ± 0.3	1.75 ± 0.4	1.58 ± 0.2	1.81 ± 0.6	0.060	0.466	1.89 ± 0.4
MCV	(fl)	173.1 ± 43.4	185.5 ± 46.7	191.5 ± 25.8	165.9 ± 41.5	5.534	0.616	174.1 ± 25.4
MCH	(pg)	27.6 ± 4.5	32.5 ± 4.5	32.4 ± 5.0	33.8 ± 4.9	1.767	0.296	28.7 ± 6.8
MCHC	(g/L)	0.17 ± 0.04	0.18 ± 0.07	0.17 ± 0.04	0.20 ± 0.08	0.009	0.931	0.17 ± 0.05

Groups CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion, of H. *illucens* meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, Hb, hemoglobin concentration; HCT, hematocrit; RBCC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; SEM, standard error mean; *Statistical analysis did not include BIO; Different letters within a row indicate significant difference ($p \leq 0.05$).

3.6. Economic Analysis and Environmental Sustainability of Feeds

The FIFO index decreased proportionally with increased insect meal proportions, reaching 3.04 (CON), 2.17 (H20), 1.56 (H40) and 1.18 (H60). The H. *illucens* meal diets differed significantly with respect to ECR and EPI (Table 9), with cost increasing concurrent with H. *illucens* meal replacement. The inclusion of insect meal led to an overall increase of environmental sustainability parameters GWP, EU and LD and a reduction in freshwater demand.

Table 9. Economic and environmental sustainability parameters of European perch production using feeds differing in insect meal inclusion level (mean ± SD, n = 3).

Items	CON	H20	H40	H60	SEM	*p*-Value	BIO *
FIFO	3.04 ± 0.21 [a]	2.17 ± 0.12 [b]	1.56 ± 0.07 [c]	1.18 ± 0.06 [d]	0.214	< 0.01	-
GWP (kg CO_2-eq)	1.81	2.64	3.48	4.32	-	-	-
EU (GJ/MT)	15.35	24.80	34.26	43.71	-	-	-
LD (ha/MT)	0.06	0.08	0.11	0.13	-	-	-
WD (m^3/MT)	376	304	232	161	-	-	-
ECR	1.4 ± 0.10 [c]	1.71 ± 0.10 [c]	2.13 ± 0.10 [b]	3.13 ± 0.17 [a]	0.198	<0.01	2.62 ± 0.34
EPI	0.36 ± 0.01 [a]	0.36 ± 0.01 [a]	0.34 ± 0.01 [a]	0.26 ± 0.02 [b]	0.012	<0.04	0.35 ± 0.04

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of H. *illucens* meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, FIFO, fish-in fish-out ratio; ECR, economic conversion ratio; EPI, economic profit index, GWP, global warming potential; EU, energy use; LD, land demand; WD, water demand; Different letters within a row indicate significant difference ($p \leq 0.05$). * The statistical analysis did not include BIO.

4. Discussion

Intensive culture of Eurasian perch is still a young industry in Europe with the main producers being Ireland, France, Poland, Belgium and Denmark. Insects have been proposed as an efficient and high-quality alternative protein source for poultry [37,38], swine [11,39] and carnivorous fish [13,15–17,20,40] and interest in use of insect meals in perch diets is high. Insects are a viable source of protein and lipids [9,10] and a typical component of Eurasian perch natural diet. Nogales-Mérida [9], confirmed insects as an excellent source of several vitamins and minerals including iron, potassium, calcium and magnesium. Use of H. *illucens* insect meal is consistent with production of perch as an organic product, as insect meal can be produced locally on a variety of substrates [41,42].

The present study represents the first reported use of defatted black soldier fly H. *illucens* larva meal as an alternative feed ingredient for Eurasian perch reared in intensive culture. Bußler et al. [43] demonstrated that H. *illucens* is an appropriate insect species for insect meal production. It has a well-balanced essential amino acid profile, an average protein content of 55% DM and ~35% fat DM, which may be reduced to 5–9% by defatting, making it more digestible. However, complete FM replacement by insect meal has not been shown feasible. Henry et al. [44], reported that the maximum dietary replacement of FM by H. *illucens* meal ranges from 6 to 25%, depending on fish species, with higher inclusion levels reducing growth performance. Sealey et al. [45], reported up to 50% H. *illucens* inclusion without negative effects on growth of rainbow trout. Our study showed that there is no significant effect up to 400 g/kg of H. *illucens* in the perch diet on body weight or specific growth rate. Similar results were demonstrated by Renna et al. [15], where partially defatted H. *illucens* larva meal up to 40% of inclusion level was used in rainbow trout diet without negative effects on survival rate, growth performance, condition factor, somatic indices, physical quality or gut morphology. Magalhaes et al. [17], replaced 45% of the FM in diet of juvenile European seabass with up to 19.5% H. *illucens* meal corresponding to 22.5% protein without adverse effects on growth performance and feed utilization. Kroeckel et al. [20], reported that inclusion higher than 33% of defatted H. *illucens* larvae decreased protein digestibility, feed acceptance and growth performance of juvenile turbot. Lock et al. [12], showed that drying slightly defatted H. *illucens* meal (255 g/kg DM) at low temperature is the most suitable procedure and produced a good alternative feed for Atlantic salmon growth.

Proximate composition of fish is driven by endogenous (size, life cycle stage) as well as exogenous factors (water quality, feed) [46]. To minimize bias, we reared European perch under similar conditions. We found no significant differences in DM, CP and EE in whole-fish homogenate among tested H. *illucens* diets. This is in line with Gasco et al. [47], who found no significant difference in DM and CP content of European sea bass fed mealworm *Tenebrio molitor* at different diet proportions. Contrary results were obtained in rainbow trout fed T. *molitor*, in which increasing

the proportion of insect meal triggered significant decreases in DM, CP and EE [48], while increased enriched H. *illucens* prepupae content resulted in decline in DM and EE [45].

Reduction in DM and EE content may result from decreased nutrient availability [15], depending on insect species [23,42] or on its culture substrate [37,39]. Culture substrate also substantially affects insect ash content [49,50]. Although body ash content has been reported similar among fish consuming various insect meal diets [20,51], we found a significant difference among our diet groups, with the highest ash content in H60, while lower ash levels were observed in CON, H20 and H40 groups. This is in contrast to the proximate analysis of tested diets per se, in which the ash content decreased with increasing H. *illucens* inclusion. Kirchgessner and Schwarz [52] and Shearer [46] reported no effect of crude dietary ash on ash content of fish body, provided sufficient levels of essential elements are present. This suggests that the partially defatted H. *illucens* meal used in our study may lack some essential element or elements, although this complex mechanism is largely unexplored and needs further study. The GE content decreased significantly with increased *H. illucens* larva meal inclusion, reflecting the non-significant decrease in both CP and EE with higher *H. illucens* larva content.

We found total n-3 and n-6 fatty acid in Eurasian perch to decrease significantly with higher levels of *H. illucens* larva meal in the diet, reflecting lower fish oil content, with the n-3/n-6 ratio being inversely related to H. *illucens* inclusion. This is in agreement with findings of Borgogno et al. [51] and Renna et al. [15], who reported significant reduction of n-3/n-6 ratio in rainbow trout fed with *H. illucens* larva meal. The opposite effect was observed in Atlantic salmon fed H. *illucens* meal [13]. The differences among studies could be related to diet composition. In the present study, as well as those of Borgogno et al. [53] and Renna et al. [15], fish oil was used as a fat source, while Belghit et al. [54], used large quantities of rapeseed oil, which contain high level of n-6 polyunsaturated fatty acids contributing to maintain constant the n-3/n-6 ratio between insect meal based diets.

These comparisons underscore differences among insect species and culture media. We found increased H. *illucens* proportions to be associated with significantly higher SFA content in fish homogenate, reflecting that partially defatted H. *illucens* meal is rich in SFAs (lauric acid C12:0, myristic acid C14:0 and palmitic acid C16:0), while T. *molitor* larva meal is rich in MUFAs and n-6 PUFAs. A similar trend was observed in studies of Jian carp [55] and rainbow trout [13], fed H. *illucens* larva meal. The positive effect on HSI observed in the present study could be related to reduction of lipid storage in liver, as was demonstrated in Atlantic salmon [54].

Hematological parameters, essential tools in evaluation of fish welfare related to stress and immune status [56–58], are highly influenced by feeding regime [59]. Studies of FM substitutes such as cottonseed [60], soybean [61,62], housefly (*Musca domestica)* maggot [63] and cricket (*Gryllus bimaculatus)* [64], showed no significant effect of tested meals on hematological parameters of fish of various species. This reinforces our suggestion that dietary H. *illucens* larva meal does not impact welfare of Eurasian perch but further investigations of diet formulations and feeding strategies are needed to collect additional data for this new area of study and to obtain more comprehensive results on fish growth rate.

The fish-in fish-out ratio is a practical indicator of environmental sustainability [31]. This index uses a global average wet weight (whole fish) to fishmeal yield of 22.5% and wet weight to fish oil yield of 5%. A ratio >1 indicates net removal of fish globally. We found the FIFO ratio to be substantially reduced with increasing proportions of insect meal and that FIFO could be decreased by 49% in perch fed an insect-based diet without affecting growth. This downward trend is in agreement with forecast of Tacon and Marc [65].

Increasing H. *illucens* larva meal proportions in commercial fish feeds could lead to higher energy and land use and increased greenhouse gas production. A lower impact was found for freshwater use. Insect meal inclusion level, which does not affect growth parameters, led to a 144% increase in greenhouse gas production, 123% increase in energy demand and 77% increase in land use. Fresh water use was decreased by 38% compared to control. These findings suggest ongoing

monitoring of agricultural resources and related socio-economic and environmental impact during the shift in resource demands from the oceans onto the land.

Future studies should be focused on fine-tuning for optimal insect meal inclusion in the range of 40% to 60%, as well as evaluation of diets with a higher contribution of plant-based protein in combination with insect meal. Long-term studies of rearing fish to a higher market size (>200 g), in combination with sensory and texture analysis of the final product, should be carried out to explore full potential of insect-based diets for perch.

When the inclusion level was >60%, growth was significantly reduced compared with the control group, suggesting that incorporation of up to 40% H. *illucens* larva meal in the feed formulation for perch is feasible and can reduce reliance on marine resources. However, even if presents limitations, such as production cost and increased impact in some environment-related parameters, the partial replacement of fishmeal by insect protein will be more important in the future as getting enough amount of fishmeal will be difficult and culture of insects like a *H. illucens* using waste food means to convert non-resources to important protein resources is a promising solution to cope this problem.

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How Does Pikeperch *Sander lucioperca* Respond to Dietary Insect Meal *Hermetia illucens*? Investigation on Gut Microbiota, Histomorphology, and Antioxidant Biomarkers

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Effects of feeding dietary defatted black soldier fly (*Hermetia illucens*) larvae meal (HI) on intestine microbiota, and on histomorphology, oxidative enzyme activities in liver and intestine of pikeperch (*Sander lucioperca*) were investigated. Four isoproteic (45% crude protein) and isolipidic (18% ether extract) diets were formulated to include 0% (CO), 9% (HI9), 18% (HI18) and 36% (HI36) of HI as replacement for fishmeal at 0, 25, 50, and 100%, respectively, and were fed to triplicate groups of juvenile pikeperch (initial body weight, 68.7 ± 7.1 g) for 84 days. No adverse effects were detected on the intestine of pikeperch fed diet groups, in terms of histomorphology ($P > 0.05$), while fish fed free or low levels of HI (≤ 9% in diet) showed significant liver degeneration ($P < 0.05$). Dietary HI significantly affected the oxidative enzyme activities of catalase and glutathione peroxidase in the liver, and glutathione S-transferase in the intestine ($P < 0.05$), while activity of superoxide dismutase in both liver and intestine was HI-dose independent ($P > 0.05$). Feeding HI-containing diets positively modulated the richness and diversity of intestinal microbiota, especially for HI18 group ($P < 0.05$). Inclusion HI up to 18% (50% fishmeal replacement) in pikeperch diets increased abundance of *Clostridium*, *Oceanobacillus, Bacteroides,* and *Faecalibacterium* genera, whereas the predominant bacterium, *Cetobacterium* was found in control and HI36 groups. This study reveals the potential of HI as an immune and health booster for juvenile pikeperch.

Keywords: pikeperch, alternative ingredient, *Hermetia illucens*, microbiota, histomorphology, antioxidative

INTRODUCTION

Aquaculture is the largest global consumer of fishmeal production, accounting for 68–73% (Shepherd and Jackson, 2013; Tacon and Metian, 2015). Fishmeal is mainly derived from marine capture fisheries (70% in 2018) (FAO, 2020a), which has reached a plateau since the 2000s (Shepherd and Jackson, 2013) and has been projected that the ecological limits of stock will be

reached by 2037 (Froehlich et al., 2018a). Therefore, the current fastest growth of aquaculture in food-producing sectors (FAO, 2020a) and the continuous increasing trend, requires the development of novel aquafeed ingredients. Terrestrial crops have been used in aquafeeds more than other alternatives until recent (Tacon et al., 2011; Tacon and Metian, 2015) and, by 2050, the use of these feedstuffs in aquaculture will rise to twice the current level in a business-as-usual scenario, reaching 91 million tonnes (Froehlich et al., 2018b). However, crop-based feeds for aquatic animals introduce concerns regarding their nutritional properties and environmental consequences. An unbalanced essential amino acid profile, low palatability, and the presence of anti-nutritional substances could impair their inclusion in aquafeeds (Gatlin et al., 2007). Moreover, the expansion and intensification of the production of terrestrial crops will lead to tremendous environmental burdens pertaining to climate change, biodiversity loss, and increasing demand for arable land and water. Among such burdens, land use is considered the one that entails the greatest pressures on the planet (Foley et al., 2005, 2011; Boissy et al., 2011). Beyond terrestrial plant ingredients, fishery by-products and insect meals have shown the greatest potential to be protein-supplied to aquafeeds in the coming years (Hua et al., 2019; Gasco et al., 2020a). Although approximately 34% of the world's fishmeal production will be derived from fish by-products by 2030 (FAO, 2020a), this potential protein source will still not be able to meet the projected aquafeed demand by 2050 (Froehlich et al., 2018a). The efficiency of insect meal as a future aquafeed ingredient has already been identified, especially concerning the feasibility of costs, scalability, and processing technology (Hua et al., 2019). Globally, insect production is on the rise, and will reach approximately 1.2 million tonnes by 2025 and become price-competitive with fishmeal by 2023 (Hua et al., 2019; Gasco et al., 2020a). In addition, the development of production facilities and processing techniques would help to improve the environmental performance of insect meal as a sustainable aquafeed ingredient (van Huis and Oonincx, 2017). The use of seven insect species (two flies, two mealworms, and three cricket species) in fish diets has been authorised by the European Commission (Regulation No. 2017/893). Among these species, black soldier fly (*Hemertia illucens*), which belongs to the Diptera order, has received the most research interest (Hua, 2021). *Hemertia illucens* larvae meal possesses important nutritional profiles, especially amino acid profile which is close to that of fishmeal (Nogales-Mérida et al., 2019). As far as environmental impact is concerned, *H. illucens* production, if obtained using non-valorised substrates, entails significantly less arable land and water use than soybean meal (Smetana et al., 2019; Gasco et al., 2020b). Moreover, *H. illucens* meal-containing diets have shown lower environmental impacts associated with abiotic depletion, acidification potential, eutrophication potential, climate change, human toxicity potential, and marine aquatic ecotoxicity potential for Arctic char (*Salvelinus alpinus*) (Smárason et al., 2017) and lower water use for European perch (*Perca fluviatilis*) (Stejskal et al., 2020) than insect-free diets.

The substitution of fishmeal with *H. illucens* meal in aquafeeds for the largest fishmeal consumers has already been investigated, and substitution levels have been achieved that do not delay growth production of the tested species, including, white leg shrimp (*Litopenaeus vannamei*) (60% plausible substitution) (Cummins et al., 2017), Atlantic salmon (*Salmon salar*) (85–100%) (Lock et al., 2016; Belghit et al., 2018, 2019), European seabass (*Dicentrarchus labrax*) (45%) (Magalhães et al., 2017), barramundi (*Lates calcarifer*) (50%) (Katya et al., 2017), and rainbow trout (*Oncorhynchus mykiss*) (45%) (Sealey et al., 2011; Renna et al., 2017; Dumas et al., 2018). In addition, dietary *H. illucens* meal has been proved to modulate bacterial diversity and richness, which play essential roles in nutrition, immunology, and health status of fish, such as rainbow trout (*O. mykiss*) (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019; Rimoldi et al., 2021), and zebrafish (*Danio rerio*) (Zarantoniello et al., 2020b). The gut health benefit of insect-fed fish has been confirmed to be suitable for species that naturally feed on insect (Antonopoulou et al., 2019; Gasco et al., 2020c).

Pikeperch (*Sander lucioperca*) is one of the main percid species that has drawn a great deal of attention in aquaculture (Schulz et al., 2006). Aquaculture production of pikeperch reached 1557 tonnes in 2018, which was doubled that of 2009 (750 tonnes) (FAO, 2020b), and has mainly been established in intensive recirculation systems (Dalsgaard et al., 2013). However, pikeperch and other percid fish have so far received very little attention from feed manufacturers (Bochert, 2020). Although some commercial aquafeeds for percids have become available, salmonids-targeted feeds are more widely used in practice (Stejskal et al., 2016). Since European pikeperch aquaculture is moving toward an established freshwater aquaculture sector (Policar et al., 2019), it will be necessary to develop suitable and sustainable feeds for aforementioned sector. Dietary protein requirements of at least 43% have been reported for appropriate growth performance and feed utilization of pikeperch fingerling (Nyina-Wamwiza et al., 2005). In the nature, aquatic insects, i.e., larvae of lake flies (*Chironomidae*) (Diptera order), play an important role as food sources for the early ontogenetic stages of pikeperch (Vinni et al., 2009; Ginter et al., 2011; Kashinskaya et al., 2018; Huuskonen et al., 2019). Therefore, the use of *H. illuces* larvae meal has been hypothesised to be suitable for pikeperch aquaculture. The aim of present study is to investigate the effects of dietary defatted black soldier fly (*H. illucens*) (HI) on the diets of juvenile pikeperch (*S. lucioperca*) on intestinal microbiota, histomorphology, and oxidative enzyme activities. The outputs could provide information in the choice of an alternative aquafeed ingredient for the emerging percid farming industry in Europe.

MATERIALS AND METHODS

Ethics Statement

The experimental procedures were performed under European Communities Directive (No. 2010/63/EU) on the protection of animals used for scientific purposes and have been approved by the Czech Ministry of Health (MSMT-6744/2018-2).

Experimental Diets, Rearing Facilities, and Feeding Procedures

The feeding trial was conducted at the wet laboratory of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic. Defatted HI was obtained from a commercial source (Hermetia Geschäftsführungs GmbH, Baruth/Mark, Germany). Four isoproteic (approximately 45% crude protein) and isolipidic (approximately 18% ether extract) diets were formulated, comprising one fishmeal-based diet (CO) and three other diets, where HI was included at 9% (HI9), 18% (HI18), and 36% (HI36) to replace fishmeal at 25, 50, and 100%, respectively (**Table 1**). Experimental diets were prepared by a commercial feed producer (Exot Hobby s.r.o., Černá v Pošumaví, Czech Republic) using a dual-screw extruder (Saibainuo, China). Chemical composition of HI and experimental diets as well fatty acid (FA) composition of experimental diets are reported in **Tables 1**, **2**, respectively.

TABLE 1 | Ingredients and proximate composition of experimental diets.

Ingredients (g/kg, as it)	HI[a]	CO	HI9	HI18	HI36
Fishmeal[b]		300	225	150	0
HI		-	90	180	360
Soybean protein concentrate		75	75	75	75
Corn gluten meal		170	170	170	170
Soybean meal		150	150	150	150
Wheat meal		80	65	50	20
Merigel		60	60	60	60
Fish oil		60	60	60	60
Soybean oil		60	60	60	60
Vitamin mixture[c]		10	10	10	10
Mineral misture[d]		10	10	10	10
DL-Methionine		7	7	7	7
L-Lysine		8	8	8	8
Celite®		10	10	10	10
Proximate composition					
Dry matter (g/100g)	91.0	94.3	94.9	94.5	94.8
Crude protein (g/100g)	54.5	44.8	45.2	44.7	45.1
Ether extract (g/100g)	8.5	18.9	18.2	18.9	17.4
Ash (g/100g)	7.6	8.7	8.6	8.1	7.4
Chitin (g/100g)[e]	5.34	-	0.47	0.97	1.93
Nitrogen-free extract (g/100g)[f]	24.06	27.60	27.53	27.33	28.17
Gross energy (MJ/kg)	20.20	21.05	20.36	20.32	21.06

[a]Defatted Hermetia illucens larvae meal; [b]Purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (g/100g, as fed basis): 91.3 dry matter; 65.8 crude protein; 9.4 ether extract; and 15.5 ash; [c]Vitamin mixture (IU or mg kg^{-1} diet): DL-α tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B_{12}, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg (purchased from Granda Zootecnici S.r.l., Cuneo, Italy); [d]Mineral mixture (g or mg kg^{-1} diet): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt 40, g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (purchased from Granda Zootecnici S.r.l., Cuneo, Italy); [e]Estimated as described by Finke (2007); [f]Calculated as 100 - (CP + EE + Ash + Chitin).

The feeding experiment was conducted in a recirculation aquaculture system (total volume 11400 L), consisting of fifteen 250-L round conical plastic tanks (black walls, white bottom) connected to a mechanical drum filter (AEM 15, AEM-Products V.O.F., Lienden, Netherlands), sedimentation tanks (total volume 2600 l), a series of filtration sections (Bioakvacit PPI10), and a moving bed bio-filter (volume 4700 l, media BT10 Ratz Aqua & Polymer Technik, Remscheid, Germany), under controlled rearing conditions, with water temperature of 23.1 ± 1.0°C, photoperiod of 12h light – 12h dark, light intensity of 20–35 Lux, oxygen saturation of 98.4 ± 15.2%, and pH of 6.98 ± 0.28. Moreover, the concentration of nitrite-N, nitrate-N, and ammonia-N concentration were maintained at 0.42 ± 0.24, 48.8 ± 21.3, and 1.89 ± 0.58 mg/l, respectively.

The prepared diets were fed to triplicate groups of juvenile pikeperch (initial body weight 68.7 ± 7.1 g, with 50 individuals per tank) for 84 days. A combined feeding protocol of four meals per day, provided at 07.00, 09.00, 11.00, 13.00, by automatic feeders (EHEIM Twins, Deizisau, Germany), and one hand feeding, at 15.00 was adopted during the trial. Any unconsumed feeds were collected by siphoning and dried in an oven to calculate the exact feed intake.

Sampling Procedures

Fish Biometry

At the start and the end of the feeding trial, fish were individually weighed to calculate weight gain (WG) and feed conversion ratio (FCR):

WG (g) = final body weight-initial body weight

FCR = total feed supplied (g, Dry Matter)/WG

Antioxidative Enzyme and Histo-Morphological Analysis

After 84 days of the experiment, a total of 45 fish (3 individuals/tank) were randomly sampled, after 24 h of feed deprivation, and were euthanised by means of overdose anaesthesia (MS222, 125 mg/l).

Dissected livers and intestines from 15 fish/group were stored at −80°C for further antioxidative enzyme analysis. A similar number of samples, taken from another 15 fish/group, were fixed by immersion in a 10% buffered formalin solution for histo-morphological analysis.

Intestinal Microbiota

At the end of the experiment, three fish were randomly taken from each tank and euthanised by means of overdose anaesthesia (MS222, 125 mg/l). In order to ensure that all sampled fish had digesta throughout the intestinal tract, fish were deprived of feeds 12 h prior to sampling time. Fish exterior was wiped with 70% ethanol before abdomen was opened, whole intestine from each fish was removed from the abdominal cavity and digesta from proximal to distal intestine was squeezed gently into a 1.5 ml aseptic Eppendorf and immediately stored at −80°C for further analysis.

Analytical Methods

Diet Chemical Composition

Analysis of HI defatted meal and experimental diets for dry matter, crude protein, crude lipid, ash, and fatty acids (FAs) were performed as described elsewhere (Tran et al., 2021). Gross energy was determined by mean of a calorimetric bomb (IKA C7000, Stufen, Germany).

Oxidative Stress in Livers and Intestines

Oxidative stress biomarkers were evaluated in liver and intestine of each fish sample by means of spectrophotometer analysis (Varian Cary spectrophotometer, Santa Clara, CA, United States) as previously described by Elia et al. (2018). Briefly, superoxide dismutase (SOD) activity was measured in 50 mM Na_2CO_3, pH 10, 0.1 mM EDTA, 500 mM cytochrome C, and 1 mM hypoxanthine and xanthine oxidase. Reduction of cytochrome C by the xanthine/hypoxanthine system was measured versus a standard curve of SOD units at 550 nm. Catalase (CAT) activity was measured as the decrease in absorbance at 240 nm due to the consumption of H_2O_2. The assay was performed in an NaH_2PO_4 + Na_2HPO_4 buffer (100 mM, pH 7) and 12 mM H_2O_2. Glutathione peroxidase (SeGPx's) activities were measured by following the oxidation of NADPH at 340 nm and using 0.6 mM H_2O_2 or 0.8 mM cumene hydroperoxides (tot GPx) as substrates. Glutathione S-transferase (GST) was measured at 340 nm using as a substrate 1-chloro-2,4-dinitrobenzene (CDNB).

Histo-Morphological Analysis of Intestine and Liver

Samples of the anterior intestine were excised and flushed with a 0.9% saline solution to remove all the content. The collected samples were fixed in a 10% buffered formalin solution, routinely embedded in paraffin wax blocks, sectioned at a 5 μm thickness, mounted onto glass slides and stained with Haematoxylin & Eosin (HE). One slide per intestinal segment was examined by means of light microscopy and captured with a Nikon DS-Fi1 digital camera, coupled to a Zeiss Axiophot microscope, using a 2.5× objective lens. NIS-Elements F software was used to capture images.

Morphometric analysis was performed using Image®-Pro Plus software on ten well-oriented and intact villi. The evaluated morphometric indices were villi height (from the villus tip to submucosa) and villi width (across the base of the villus, but not including the brush border).

The observed histopathological findings were evaluated in all the organs, using a semi-quantitative scoring system as follows: absent (score = 0), mild (score = 1), moderate (score = 2), and severe (score = 3). Histopathological findings in intestine were assessed separately for each segment for mucosa (inflammatory infiltrates) and submucosa [inflammatory infiltrates and Gut-Associated Lymphoid Tissue (GALT) activation]. The total score of each gut segment was obtained by adding to the mucosa and submucosa scores. All the slides were blind assessed by two independent observers, and any discordant cases were re-examined, using a multi-head microscope, until unanimous consensus was reached.

Microbiome Analysis

DNA Extraction and 16S rRNA Amplicon Target Sequencing

Nucleic acid was extracted from the intestine content (500 mg as starting materials). Total DNA from the samples was extracted using a RNeasy Power Microbiome KIT (Qiagen, Milan, Italy), according to the manufacturer's instructions. One microlitre of RNase (Illumina Inc, San Diego, CA, United States) was added to

TABLE 2 | Fatty acid (FA) composition (as mg/g total FAs) of experimental diets.

*FAs	Experimental diets			
	CO	HI9	HI18	HI36
C12:0	0.4 ± 0^{a}	16.1 ± 0.3^{b}	25.7 ± 0.7^{c}	61.8 ± 3.4^{d}
C14:0	17.2 ± 0.1^{a}	20.1 ± 0.1^{b}	21.2 ± 0.1^{c}	27.5 ± 0.7^{d}
C16:0	102.7 ± 0.5^{a}	106.8 ± 0.3^{b}	105.2 ± 1.6^{b}	106.2 ± 0.9^{b}
C16:1	23.7 ± 0^{a}	23.9 ± 0^{ab}	24.0 ± 0.1^{b}	24.1 ± 0.1^{b}
C18:0	29.9 ± 0.2	30.2 ± 0.3	30.3 ± 1.7	28.1 ± 0.2
C18:1n9	201.3 ± 0.8^{c}	196 ± 0.2^{b}	195.6 ± 0.3^{b}	188.5 ± 0.9^{a}
C18:1n7	206.2 ± 3.5^{b}	196 ± 0.2^{a}	197.9 ± 3.8^{a}	194.5 ± 0.9^{a}
C18:2n6	257.6 ± 0.9^{d}	254.1 ± 0.4^{c}	251 ± 1.8^{b}	241.8 ± 1.0^{a}
C18:3n3	38.9 ± 0.2^{c}	37.3 ± 0^{b}	37 ± 0.2^{b}	34.3 ± 0.2^{a}
C20:1n9	33.0 ± 0.3^{c}	31.2 ± 0.1^{b}	31.0 ± 0.2^{b}	27.5 ± 0.1^{a}
C20:5n3 (EPA)	3.20 ± 0.01^{d}	3.10 ± 0.01^{c}	3.00 ± 0.01^{b}	2.60 ± 0.01^{a}
C22:6n3 (DHA)	48.2 ± 0.5^{d}	45.5 ± 0.2^{c}	39.1 ± 0.2^{b}	26.7 ± 0.5^{a}
∑n-3	91.4 ± 0.7^{d}	86.9 ± 0.3^{c}	80.1 ± 0.4^{b}	64.4 ± 0.6^{a}
∑n-6	268.1 ± 1.0^{d}	264 ± 0.5^{c}	259.8 ± 1.8^{b}	248.3 ± 1.0^{a}
∑SFA	164.6 ± 0.9^{a}	190.6 ± 0.7^{b}	200 ± 4.4^{c}	239.5 ± 4.4^{d}
∑MUFA	470.9 ± 2.5^{c}	453.6 ± 0.3^{b}	454.8 ± 3.6^{b}	440.2 ± 1.9^{a}
∑PUFA	360 ± 1.7^{d}	351.4 ± 0.7^{c}	340.4 ± 2.2^{b}	316.0 ± 4.9^{a}

**Only FAs > 10 mg/g total FAs (except for EPA) are presented; Different letters denote significant differences among the experimental groups (P < 0.05).*

digest the RNA in the DNA samples for an incubation period of 1 h at 37°C. DNA was quantified using Qubit ds and standardised at 5 ng/μ l.

DNA extracted directly from digesta samples was used to assess the microbiota, through amplification of the V3–V4 region of the 16S rRNA gene (Klindworth et al., 2012). The PCR products were purified according to the Illumina metagenomic standard procedure (Illumina Inc, San Diego, CA, United States). Sequencing was performed with an MiSeq Illumina instrument, with V3 chemistry, and 250 bp paired-end reads were generated according to the manufacturer's instructions.

Statistical Analysis

All data for antioxidative enzyme activities were tested for homogeneity of variance using Cochran, Hartley, Bartlett test. The effects of diet on oxidative stress in different organs were analysed separately, by means of one-way ANOVA, followed by Tukey test. Statistical analyses were performed using STATISTICA 12.0, with P-value < 0.05 as the significant difference.

Raw reads of microbiota were first joined, after sequencing, using FLASH software (Magoč and Salzberg, 2011), with default parameters, and were filtered, using QIIME 1.9.0 software and the pipeline as recently described (Biasato et al., 2018). Briefly, shorter reads (<300 bp) were discarded, using Prinseq. USEARCH software (version 8.1) was used for chimera filtering, and the Operational Taxonomic Units (OTUs) were picked, at a threshold of 97% similarity, using UCLUST algorithms. Taxonomy was assigned against 16S rRNA from Greengenes. The OTU table was rarefied at 10,144 sequences/sample. The OTU table displays the highest taxonomy resolution that was reached. When the taxonomy assignment was not able to reach the genus level, the family or phyla were displayed. R software was used to calculate the alpha diversity, while Weighted and Unweighted UniFrac distance matrix and OTUs table were used to find differences between samples, using permutational multivariate analysis of variance (Anosim) and analysis of similarity (Adonis) statistical test, considering the same function in R environment. Pairwise Wilcoxon test were used to determine any significant differences in alpha diversity or OTU abundance as a function of dietary insect meal. Principal component analysis (PCA) were plotted, using the *dudi.pca* function, through the *made4* package of R environment. Non-normally distributed variables were presented as median values (interquartile range, IR), and box plots represented the interquartile range between the first and the third quartile, with the error bars showing the lowest and the highest value. Pairwise Kruskal-Wallis tests were used to find any significant differences in microbial taxa abundance according to the dietary treatment. P-values were adjusted for multiple testing, and a false discovery rate (FDR) < 0.05 was considered as significant. The data generated from sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under the BioProject Accession Number PRJNA704237.

GraphPad Prism® software (version 8.0) was used to perform statistical analysis, for histo-morphometrical investigations. The Shapiro–Wilk test was used to test the normality of the data distribution before statistical analyses. Data were described by mean and standard deviation (SD), or median and IR depending on data distribution. Bivariate analysis was performed, by means of one way-ANOVA or Kruskall Wallis tests, to compare the intestine morphology and organs histopathology among different diet groups. P-values < 0.05 were considered statistically significant.

RESULTS

Diet Composition and Growth Production of Pikeperch

Formulated diets had a similar proximate composition, except for chitin which increased with the increase of HI inclusion (**Table 1**). The inclusion of dietary HI significantly altered the FA profile of experimental diets. As regards saturated FAs (SFA), lauric (C12:0), myristic (C14:0), and palmitic acid (C16:0) significantly increased with the increase of HI inclusion ($P < 0.05$). Monounsaturated FAs (MUFA), dominated by palmitoleic acid (C16:1), C18:1n9 and C18:1n7, were found to be significantly higher in CO than H36 ($P < 0.05$), while MUFAs

TABLE 3 | Growth performances and histopathological traits divided by diet groups.

	Experimental diets				
	CO	**HI9**	**HI18**	**HI36**	***P-value***
Growth performances					
Weight gain (g), *mean (SD)*	85.3^{a} (24.1)	84.8^{a} (23.7)	83.2^{a} (26.4)	62.8^{b} (18.3)	<0.001
FCR, *mean (SD)*	1.27^{b} (0.06)	1.28^{b} (0.07)	1.29^{b} (0.03)	1.81^{a} (0.15)	<0.001
Anterior gut					
Villi height (mm), *mean (SD)*	0.31 (0.07)	0.32 (0.07)	0.29 (0.05)	0.28 (0.07)	0.979
Villi width (mm), *mean (SD)*	0.03 (0.005)	0.03 (0.006)	0.03 (0.008)	0.11 (0.34)	0.065
Inflammation, *median (IR)*	0.00 (0.0–0.5)	0.00 (0.0–0.3)	0.00 (0.0–0.5)	0.00 (0.0–0.5)	0.967
Liver					
Degeneration, *median (IR)*	3.00^{a} (3.0–3.0)	3.00^{a} (2.0–3.0)	2.50^{b} (1.0–3.0)	2.50^{b} (1.0–3.0)	0.015
Inflammation	Absence of alterations				

SD, standard deviation; FCR, feed conversion ratio; IR, interquartile range. Values in the same row not sharing common superscript letter are significantly different.

in H9 and H18 remained comparable ($P > 0.05$). Increasing inclusion level of HI significantly reduced polyunsaturated FAs (PUFA) ($P < 0.05$). A similar trend was observed for EPA, DHA, linoleic acid, alpha-linolenic acid ($P < 0.05$) (**Table 2**).

At the end of the feeding trial, WG in fish fed HI36 (62.8, mean value) was significantly lower than the control group (85.3 g) ($P < 0.05$), whereas pikeperch fed HI9 (84.8 g) and HI18 (83.2 g) did not show significant difference with CO ($P > 0.05$). FCR of the CO group (1.27) was comparable with that of HI9 (1.28) and HI18 (1.29) ($P > 0.05$), but significantly lower than HI36 (1.81) ($P < 0.05$) (**Table 3**).

Oxidative Stress in Liver and Intestine

The results of oxidative biomarkers, SOD, CAT, SeGPx, and GST, in liver and intestine of pikeperch fed experimental diets are depicted in **Figure 1**. Dietary HI did not alter the SOD activities in either liver or intestine, CAT activities in liver, SeGPx activities in intestine, or GST activities in liver of pikeperch ($P > 0.05$). No significant difference was observed across experimental groups ($P > 0.05$) for liver, as regards CAT activities, whereas this biomarker was significantly lower in HI18 and HI36 than in HI9 ($P < 0.05$), but remained similar to CO ($P > 0.05$) in intestine. Even if did not differ from the CO group, among fish fed HI-containing diets, HI9 produced highest SeGPx activity in liver ($P < 0.05$), while the lowest activity was found in HI36 group ($P < 0.05$). A significant increase in the GST concentration was observed in intestine of pikeperch fed HI-containing diets, compared to CO ($P < 0.05$). Of the different insect-fed groups, HI9 showed a higher GST than HI18 ($P < 0.05$), while HI36 was remained intermediate position.

Histo-Morphology

Data regarding histopathological evaluation are reported in **Table 3**. Only few differences were observed for morphometry and histopathology of intestine among diet groups. Although there was no significant difference, a trend could be observed ($P = 0.065$) with HI36 group recording wider villi than the other groups. Thus, dietary HI inclusion did not induce any significant morphological changes in the pikeperch intestine, thereby suggesting no negative influence of such dietary HI on the physiological development of intestine.

Mild to severe multifocal to diffuse liver vacuolar degeneration was recorded in all treatments, and it was found to be greater in CO and HI9 group than in the HI18 and HI36 ones. Dietary HI did not show any evidence of inflammation of the liver of pikeperch (**Table 3** and **Figure 2**).

Microbiota

The total number of high-quality paired-end sequences obtained from 16S rRNA sequencing reached 1.916.822 raw reads. After the filtering, 1.295.693 reads passed the filters applied by QIIME, with a median value of 37.559 ± 15.565 reads/sample, and a mean sequence length of 443 bp. The rarefaction analysis and Good's coverage, expressed as a median percentage (97%), also indicated satisfactory coverage of all samples.

The result of the OTUs analysis showed that there was no significant difference in Shannon index ($P > 0.05$) among diet groups, while alpha-diversity of intestinal bacteria, associated with Chao1 and observed OTUs, in fish fed HI18 significantly increased relative to CO diet ($P < 0.05$) (**Figure 3**).

Adonis and Anosim statistical tests, based on weighted and on unweighted UniFrac distance matrix using the OTUs table, showed significant differences between diet groups as a administration of HI ($P < 0.002$). These differences were also observed when the PCA plot was produced at a genus level (**Figure 4**). It was also possible to observe a certain degree of separation, following diet groups. Microbiota of CO diet was near to the insect meal inclusion of 9%, while the microbiota of fish fed with 18 and 36% of HI was well separated (**Figure 4**).

The dominant OTUs, at the phyla level, were *Firmicutes* (mean values, 45–75%), regardless to dietary HI. Perch fed CO diet was enriched with *Proteobacteria* (26%), while *Bacteroidetes* (7–13%) was the prevalent phyla in fish fed HI-containing diets. As a result, *Clostridiaceae, Enterococcaceae,* and *Bacillaceae* were found to be the predominant families across fish fed diet groups. *Clostridium*, *Acetobacter*, *Cetobacterium*, *Plesiomonas*, *Acetobacter*, *Peptostreptococcaceae*, *Bacteroides,* and *Oceanobacillus* were, at the genus level, the most abundant genera found in intestine of perch considered in our study (**Figure 5**).

Dietary HI positively affected relative abundance of almost OTUs, compared with CO (FDR < 0.05), excepted for *Bacillus*, *Burkholderia,* and *Sporosarcina,* which were dominant in the CO group (**Figure 6**).

DISCUSSION

Oxidative Enzymes

Reactive oxygen species (ROS) is the production of aerobic metabolism processes, including superoxide, hydrogen peroxide, and lipid peroxides (Buetler et al., 2004). Excessive ROS compounds cause cellular and tissue damages (Rosa et al., 2008). The balance of ROS production ensures the normal physical function of any organism and is regulated by antioxidant systems (Rosa et al., 2008) involving two mechanisms, (i) enzymes that remove ROS, including SOD, CAT, and SeGPx; and (ii) antioxidative compounds, i.e., ascorbate, glutathione, scavenge free radicals (Passi et al., 2002). Antioxidative enzyme activities were documented to be tissue-specific in pikeperch, and liver was the most sensitive organ to the diet manipulation under recirculating aquaculture system (Policar et al., 2016). In the case of detoxification in the intestine, however, certain enzymes such as SOD were known to play a vital role (Tang et al., 2013). This study indicates that in liver of pikeperch dietary HI did not alter the SOD, CAT, or GST oxidative enzymes, while significantly reduced SeGPx activity, a result that is in agreement with those of previous study (Elia et al., 2018), who performed a trial on rainbow trout fed dietary HI. The significant reduction in the catalytic SeGPx efficiency in liver of pikeperch fed dietary HI could be explained by the presence of chitin (Elia et al., 2018). Indeed, increasing inclusion levels of HI increased chitin levels in diets (**Table 1**). In addition, declining in SeGPx activities, as a result of increasing dietary HI, could be attributed to different dietary PUFA levels

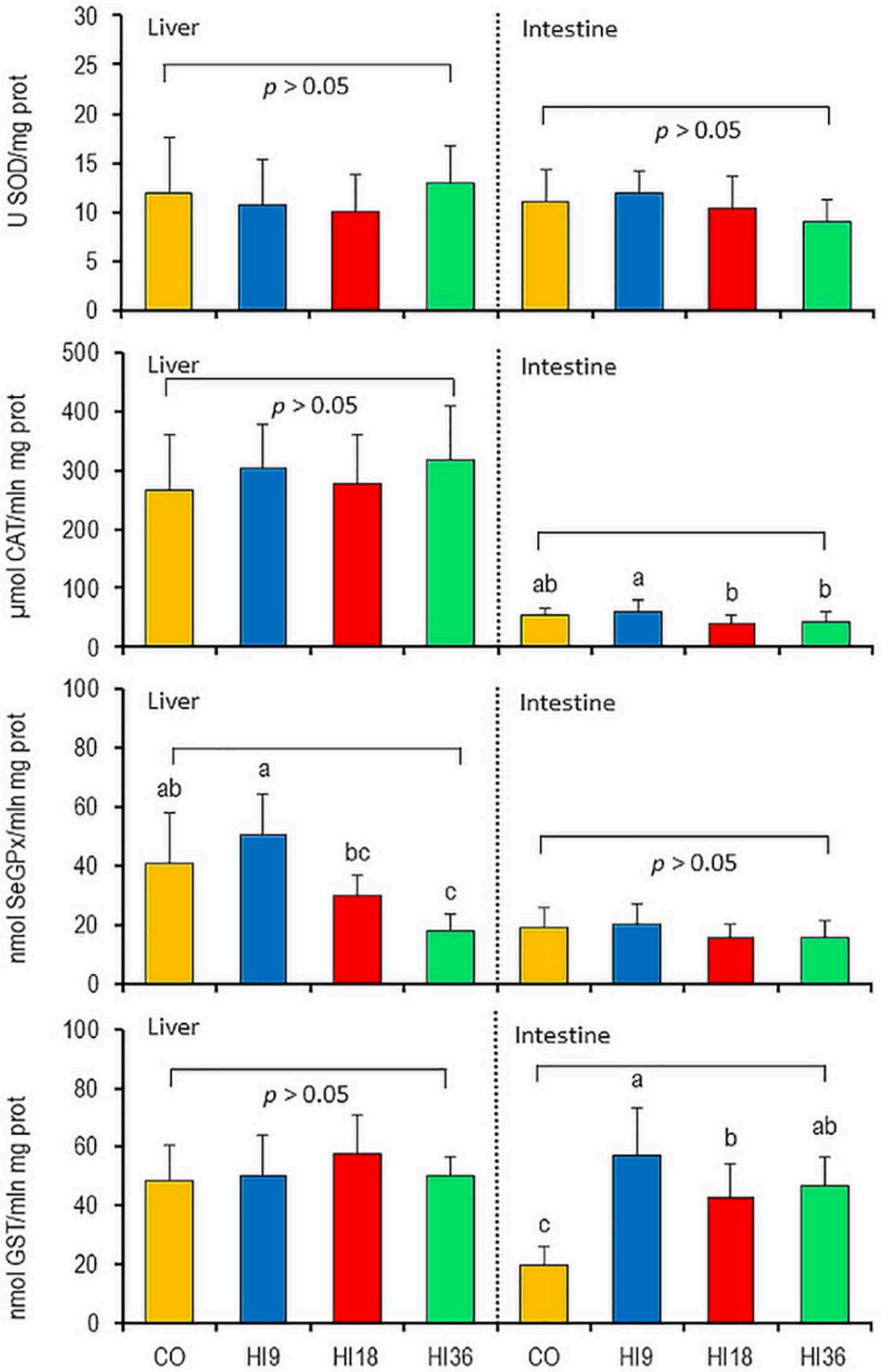

FIGURE 1 | Oxidative biomarkers in liver and intestine of pikeperch (*Sander lucioperca*) fed experimental diets. Values are presented as means ± standard deviation. Means with different letters are significantly different ($P < 0.05$) from each other.

(**Table 2**), which are highly susceptible to oxidation. In fact, Tocher et al. (2002) reported that a high dietary PUFA content increased lipid peroxidation in fish tissues, and consequently the SeGPx enzyme activity involved in reducing peroxides, including FA hydroperoxides and hydrogen peroxide, will be also high (Passi et al., 2002).

The present study indicates that the CAT activity in intestine of pikeperch was significantly higher for HI9 than

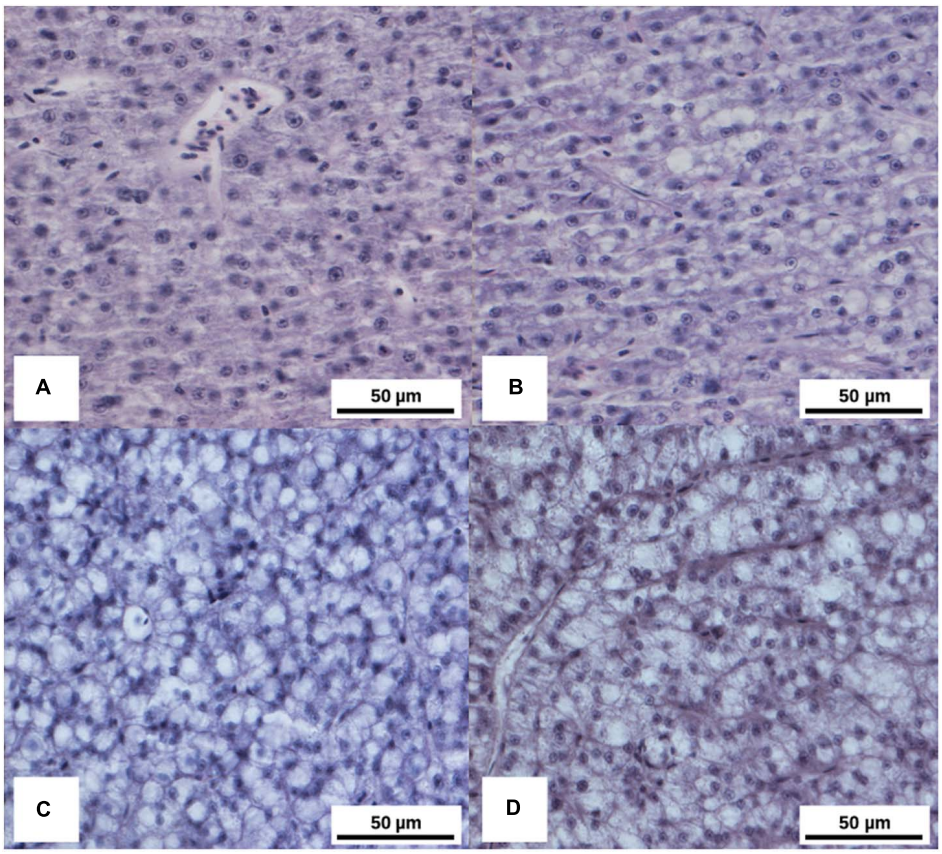

FIGURE 2 | Histopathological alteration of liver of pikeperch considered in the present study. **(A)** Normal liver, Haematoxylin & Eosin (H-e) stain, 40× magnification, for HI36 diet. **(B)** Mild and multifocal vacuolar degeneration (grade 1), H-e, 40× magnification, for HI18 diet. **(C)** Moderate and multifocal vacuolar degeneration (grade 2), H-e, 40× magnification, for the HI9 diet. **(D)** Severe and diffuse vacuolar degeneration (grade 3), H-e, 40× magnification, for CO diet.

for HI18 and HI36 groups. A similar phenomenon was reported for CAT activity in the intestine of rainbow trout fed insect meal (*T. molitor*), where a substitution level of 25% fishmeal displayed higher activity than the 50% level (Henry et al., 2018a). The CAT and SeGPx activities in the present study were similar for CO and HI9, and lower than for HI18, HI36 groups. This result indicates that substantial substitution of fishmeal with HI reduced antioxidant enzyme activities in pikeperch. This is in line with a previous finding pertaining to rainbow trout (*O. mykiss*) (Elia et al., 2018). The decline of these biomarkers in HI18 and HI36 groups could be related to an imbalance between ROS production and antioxidant capacity. A suitable concentration of antioxidants, such as chitin and other bioactive compounds (Ngo and Kim, 2014), may support antioxidant enzyme activities in HI9 compared to the other HI-contained diets (Henry et al., 2018a).

Glutathione S-transferase plays an essential role in scavenging free radicals and xenobiotics detoxification (Aksnes and Njaa, 1981; Li et al., 2010). Increased glutathione S-transferase activity in intestine, but not liver, was observed across diet groups in the present study (**Figure 1**), thus implicating that some of the compounds in HI may have stimulated the biotransformation pathway in intestine of pikeperch, which was also found in liver of tilapia (*Oreochromis niloticus*) fed cricket-based feeds (Ogunji et al., 2007). In fact, insect meals may contain harmful substances, i.e., heavy metals and pesticides (van der Spiegel et al., 2013). The absence of an alteration of the hepatic GST activities after administration of HI could be the result of factors other than xenobiotics (Collier and Varanasi, 1991) or tissue-specific response (Martínez-Álvarez et al., 2005).

We also observed numerically higher oxidative biomarkers in liver of pikeperch than in intestine (**Figure 1**), which was in agreement with recent findings (Policar et al., 2016), reporting that liver was one of the most susceptible tissue in response to artificial nutrition and controlled conditions.

Histo-Morphology

Dietary HI in our study did not induce any morphological or inflammatory changes in the intestine of pikeperch, a result that is in agreement with previous studies conducted on different fish species fed dietary insect meals (Elia et al., 2018; Zarantoniello et al., 2019; Zarantoniello et al., 2020a). The absence of intestinal and hepatic inflammation could be linked to anti-inflammatory properties regulated by dietary saturated fatty acids content, especially lauric acid (C12:0) and chitin

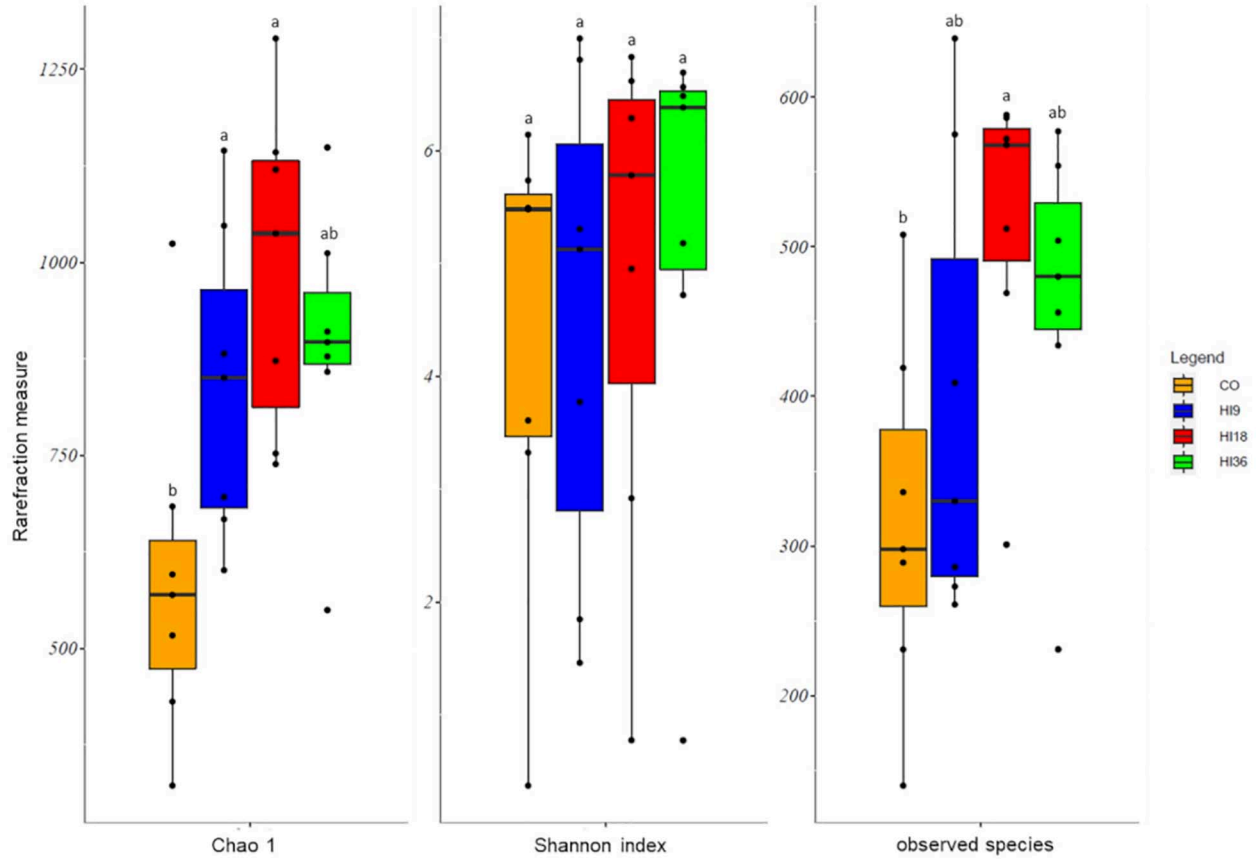

FIGURE 3 | Boxplots showing the alpha diversity rarefaction index across experimental diets. The boxes represent the interquartile range (IR) between the first and third quartiles, and the line inside the boxes represents the median. Whiskers denote the lowest and the highest values within 1.56 IR from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers. Different superscripts within boxplot indicate significant differences.

component (Henry et al., 2018b; Vargas-Abundez et al., 2019; Zarantoniello et al., 2019; Gasco et al., 2020b,c) which were found to be particularly high in HI and HI-containing diets in the present study. Although there were no significant differences (at P-value < 0.05), the villi were more expanded in the HI36 group than in the other groups (**Table 3**), and this was attributed to the presence of chitin. Chitin could stimulate the growth of villi thickness in tilapia (*O. niloticus*), probably due to its viscosity and water holding capacity (Kihara and Sakata, 1997). Chitin also induced the production of short-chain fatty acids, such as acetate, propionate and n-butyrate, and n-butyrate in particular was observed in intestine of tilapia (Kihara and Sakata, 1997), thereby increasing intestinal histo-morphology of fish, e.g., villi length and weight (Dawood, 2021). The large quantity of *Paenibacillus* genus in intestinal digesta of fish fed HI36 (**Figure 6**) could act as a probiotic for aquatic animal species (Midhun et al., 2017; Chen et al., 2019; Amoah et al., 2020), consequently enhancing intestinal health indices, including histomorphology (Dawood, 2021).

In contrast to recent findings, which reported that an increasing inclusion of insect meals induced a higher degree of hepatic vacuolization degeneration in fish (Li et al., 2017; Zarantoniello et al., 2019), the present study indicates that feeding pikeperch with $\leq$ 9% HI caused more severe hepatic degeneration than 18 or 36% did (**Table 3**), which could be related to a fatty liver status. Schulz et al. (2005) reported that a low level of palmitic acid (C16:0) yielded a higher hepatic lipid content. In the present study, the significantly lower palmitic acid in the control group than in the HI-containing groups could partly explain the hepatocellular vacuolization phenomenon. The mechanism to which palmitic acid affecting hepatic tissues remained to be elucidated. However, this FA promotes hepatocyte proliferation (Wang et al., 2011) and possess anti-inflammatory and antiviral effects (Librán-Pérez et al., 2019). On the other hand, the high content of dietary lauric acid (C12:0), high oxidation and low tissue deposition, was found to decrease liver lipid storage in freshwater Atlantic salmon (Belghit et al., 2019). This could explain the reduction in the adipose liver in pikeperch fed HI18 and HI36, compared to the control and HI9 diets (**Table 3**). Two FAs, linoleic and oleic acids, were confirmed to induce the occurrence of hepatic steatosis in sea bream (*Sparus aurata*) (Caballero et al., 2004). Moreover, owing to large molecular weight, oleic acid could produce a large lipid droplet while inrush hepatocyte (Bradbury, 2006).These FAs were found to

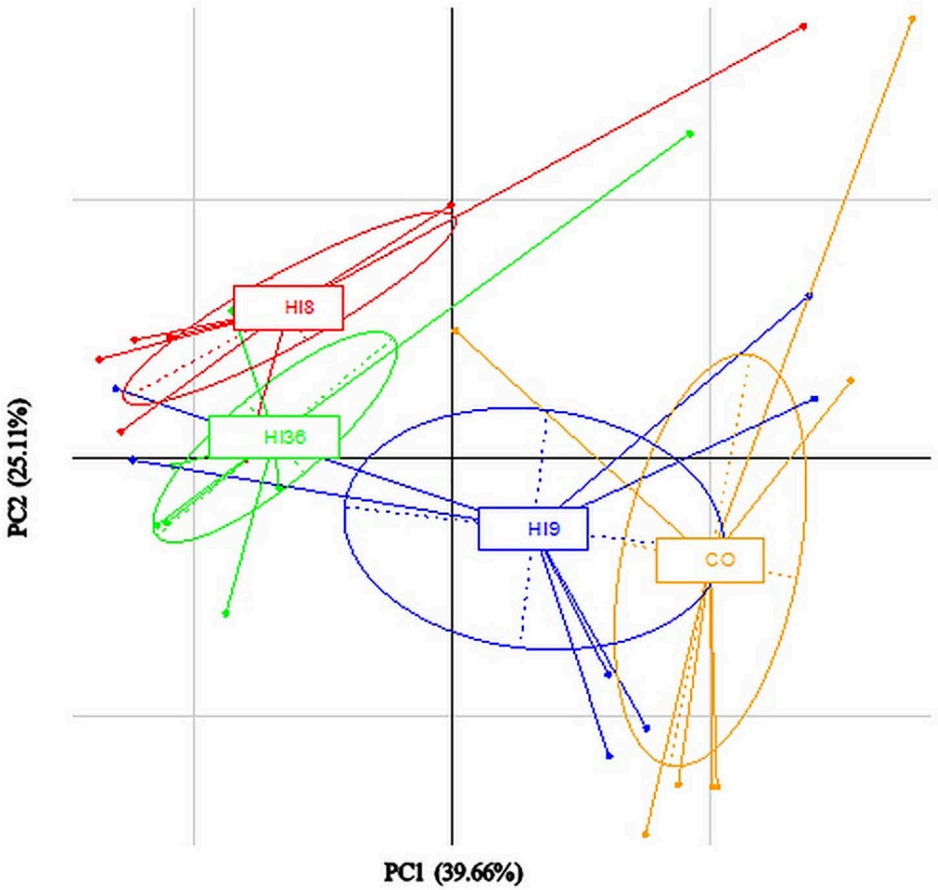

FIGURE 4 | Microbiota composition (PCA plots) in intestine of pikeperch (*Sander lucioperca*) fed experimental diets.

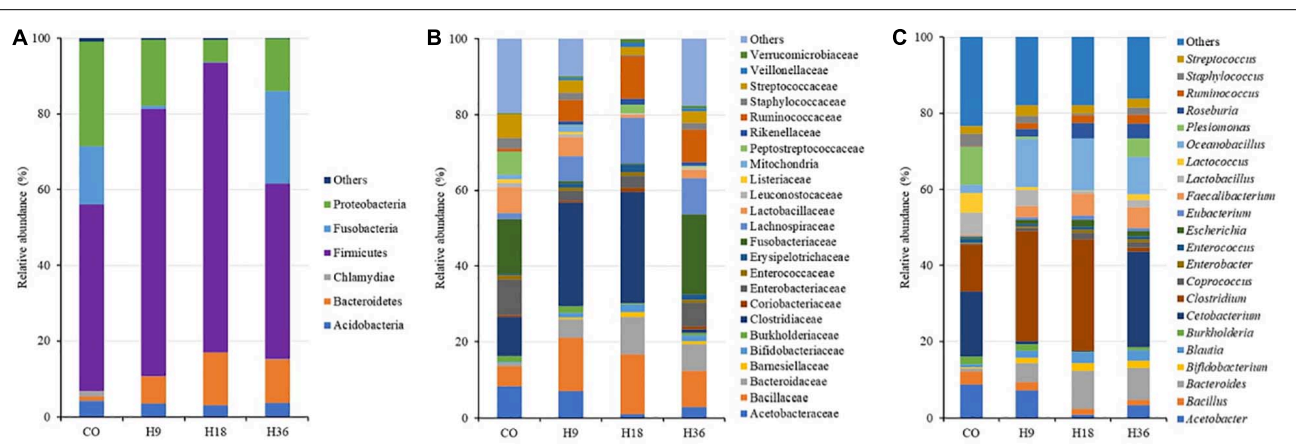

FIGURE 5 | Relative abundance (%) of the OTUs in the intestine of pikeperch fed experimental diets at phyla **(A)**, family **(B)**, and genus **(C)** level. Only bacteria with an overall abundance of ≥ 1% and ≥ 0.5% at phylum and family/genus level, respectively, were presented. The bacteria were pool as "Others," when lower than aforementioned abundance.

be significantly higher in CO than in HI18, HI36 (**Table 2**), which could indicate severe steatosis in livers of the former group (**Table 3**). High intakes of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to an inhibitor of lipid accumulation in livers of sea bream (*S. aurata*) (Caballero et al., 2004). Therefore, the change in the percentage of the different

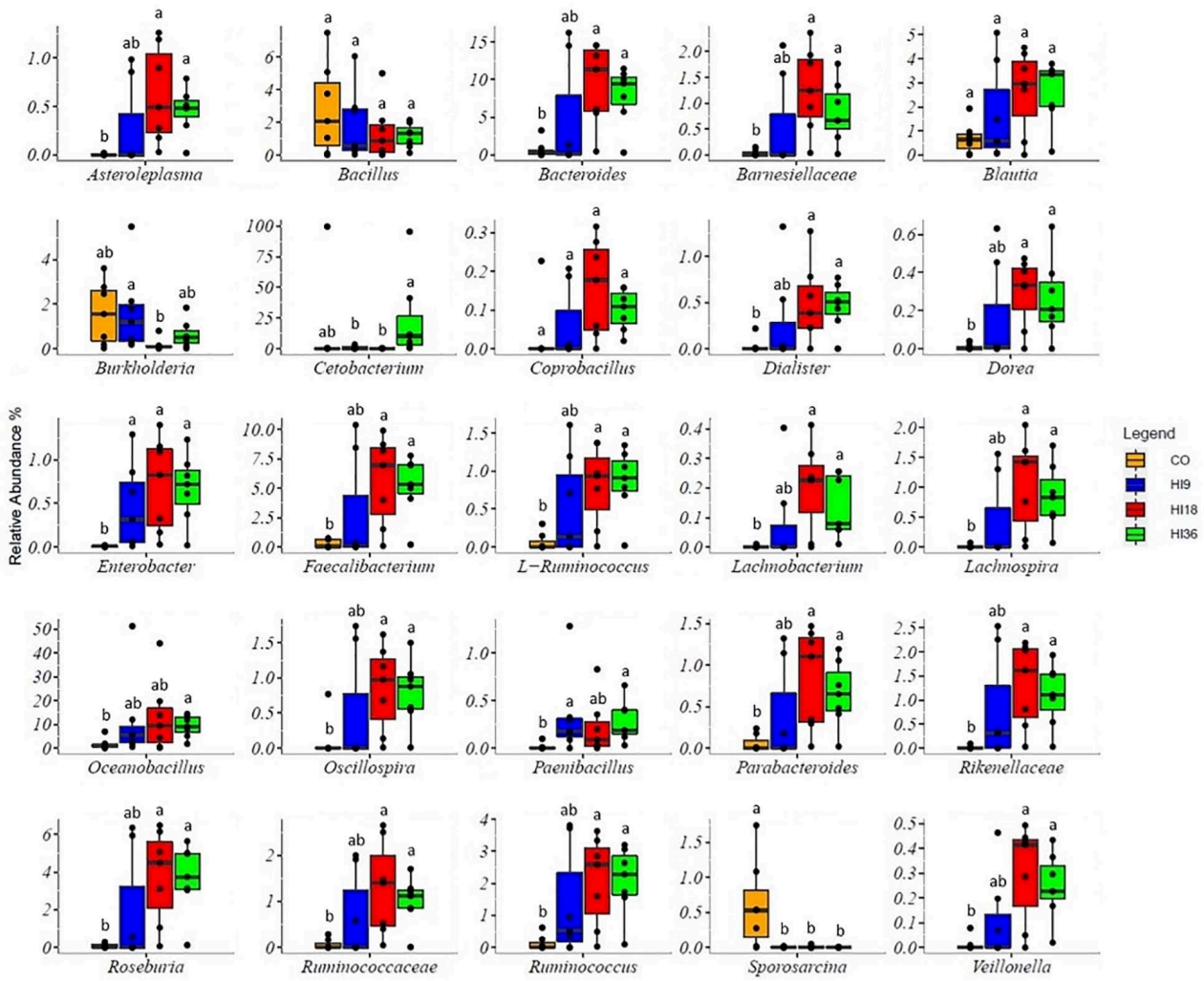

FIGURE 6 | Boxplots showing the relative abundance at the genus level of the OTUs in the intestine of perch fed experimental diets. Means with different letters are significantly different (FDR < 0.05) from each other.

FAs in the experimental diets, due to the inclusion of HI, could further explain the severity of hepatic vacuolization degeneration observed in perch fed CO and H9 diets.

Microbiota

The present study reveals that dietary HI enhanced microbial biodiversity indices in intestine of pikeperch, compared with insect-free diet, a result that is in line with recent findings on rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019), thereby contributing to gut health and health status of the host.

In agreement with previous studies on intestinal microbiota of percid fish and freshwater species, the present study reveals that *Firmicutes*, *Proteobacteria*, *Bacteroidetes* were the most dominant phyla in the intestine of pikeperch, regardless of the HI inclusion level (Li et al., 2014; Kashinskaya et al., 2018; Terova et al., 2019).

Our results show an abundance of *Clostridium* genus in fish fed HI9 and HI18, which was even greater than those fed CO and HI36. Members of the *Clostridium* genus are common effective microorganism used as probiotics in aquaculture (Nayak, 2010a,b). *Clostridium butyricum* has been shown to possess a pathogenic inhibition capacity in farmed fishes (Pan et al., 2008a,b; Gao et al., 2013), improve feed efficiency in shrimp (Duan et al., 2017; Li et al., 2019), and to be suitable for use as probiotics in farmed fish (Hai, 2015; Zorriehzahra et al., 2016). The greater prevalence of *Clostridium* and other probiotic-used bacteria in HI9, such as *Lactobaccillus* and *Bacillus* genera, than in HI36, could explain the difference in feed conversion ratio between these diets in present study. The *Bacteroides* and *Clostridium* genera are known to be the main taxa involved in production of fatty acids and vitamins (Balcázar et al., 2006). The abundant presence of these taxa could partially compensate for nutritional insufficiencies in HI-containing diets, and consequently resulted in a comparable growth rate among control, HI9 and HI18 diets, yet the offset may be not efficient for HI36 group.

It is worth noting that *Cetobacterium,* the most predominant bacterium in intestine of natural pikeperch (Kashinskaya et al., 2018) and other freshwater fish (Larsen et al., 2014), was detected in our captive pikeperch fed dietary HI. Similar findings were also observed in rainbow trout (Etyemez and Balcázar, 2015), common carp (van Kessel et al., 2011), and giant arapaima (Ramírez et al., 2018) fed commercial aquafeeds. It seems relevant that *Cetobacterium* is among the core bacteria in pikeperch.

Insect meal, in general, is a chitin-rich ingredient. The degradation and digestion of this substance require binary enzymes, including chitinase and β−N−acetylglucosaminidase, and involve various microbacteria derived from digestive tract of fish with a chitinase-produced capacity (Ray et al., 2012; Ringø et al., 2012). Among these chitin-degraded bacteria, the *Plesiomonas* and *Bacillus* genus were detected across treatment groups at a particularly low abundance (**Figure 4**). This finding implicates that pikeperch may not be able to degrade chitin. A limited presence of chitinase-producing bacteria was also observed in rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019) and this may help to explain the low or absent chitin digestibility in this species (St-Hilaire et al., 2007; Henry et al., 2015; Renna et al., 2017; Caimi et al., 2020).

In conclusion, HI, fed as a partial or total replacement of fishmeal did not induce any inflammation of liver or intestine, or any intestine degeneration, but did show signs of severe hepatic steatosis of pikeperch fed CO and HI9 groups. Dietary HI promotes antioxidative enzyme activities of CAT, GPx and GST, but not of SOD, in liver and, to a lesser extent, in intestine of pikeperch. The inclusion of HI up to 18% or 50% fishmeal replacement in pikeperch diets increased abundance of *Clostridium*, *Oceanobacillus, Bacteroides,* and *Faecalibacterium,* whereas the predominant bacterium, *Cetobacterium* was found in the control and HI36 groups. Because of the absence of inflammation in tissues, the evolution of antioxidative enzyme, and modification of the favourable microbiota observed in the present study, it is possible to assume that defatted HI could have an immunological effect on juvenile pikeperch. Further study on immune response and disease resistance of pikeperch fed insect meal could help to explore this efficiency.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Czech Ministry of Health (MSMT-6744/2018-2).

AUTHOR CONTRIBUTIONS

LG and VS: planning the experiment and editing the manuscript. HT: data analysis, writing, and editing of the manuscript. MP, MZ, and TG: wrote the manuscript. AE, EC, IF, CC, and FG: analysis and the first draft. All authors contributed to the article and approved the submitted version.

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European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): From a stable isotope perspective

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ABSTRACT

Stable isotope analysis was conducted to investigate stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$), diet-tissue discrimination factors of carbon ($\Delta^{13}C$) and nitrogen ($\Delta^{15}N$). Bayesian mixing models were performed to assess relative contribution of insect meal and other ingredients to the development of tissues of European perch (*Perca fluviatilis*). Accordingly, four experimental formulations, characterized by the increasing inclusion levels of yellow mealworm (*Tenebrio molitor*) larvae meal (TM) at 0, 6.8, 13.5 and 20.3% as replacement for fishmeal at 0 (TM0), 25 (TM25), 50 (TM50) and 75% (TM75), respectively, were fed to juvenile perch (initial bodyweight, 20.81 ± 3.36 g) in a recirculated aquaculture system for 105 days.

$\delta^{13}C$ and $\delta^{15}N$ of TM were -16.75 and 3.53‰ and significantly distinguished from other terrestrial and marine feed components ($P < 0.05$). Inclusion of dietary TM did not affect $\Delta^{13}C$ value in blood and liver ($P > 0.05$) but did reduce in muscle ($P < 0.05$), whereas $\Delta^{15}N$ was significantly increased with the increasing inclusion level of TM in all tissues ($P < 0.05$). The growth of perch had a significant negative relationship with diet-muscle $\Delta^{15}N$. The contribution of TM to muscle (7.7 ± 3.8%) was comparable to its dietary inclusion (6.8%) in TM25 but double in the blood (13 ± 6%). TM appeared to be an essential ingredient incorporated into liver, as its contribution was consistent or higher than dietary inclusion (TM25: 25.4 ± 12.1 vs. 6.8%; TM50: 31.1 ± 14.9 vs. 13.5%; and TM75: 29.4 ± 14.4 vs. 20.3%). The higher inclusion levels of TM (more than 6.8%) did not elevate its contribution to muscle, blood, and liver (probability, $P_{BIC} < 0.95$) but significantly decreased that of fishmeal in all tissues ($P_{BIC} > 0.95$). Soy-derived ingredients, soybean meal and soy protein, were an important ingredient in the development of all tissues regardless of dietary TM.

The present study provided insightful information on the role of various diet components in perch tissues, which could underlie further development of aquafeed formulations for emerging perch farming in Europe.

1. Introduction

European perch (*Perca fluviatilis*) has been identified as the promising candidate for intensive aquaculture with excellent nutritional value, especially beneficial fatty acids and increasing market demand (Stejskal et al., 2011; Toner, 2015; Stejskal et al., 2020b). Production of perch is mainly relied on re-circulating aquaculture systems and followed an upward trend, reaching approximately 700 t in, 2018 (FAO, 2020). Together with other percid fishes, perch farming moves towards an established aquaculture sector in Europe (Policar et al., 2019). In perch farming practice, salmonid aquafeeds and commercial perch feeds are commonly used (Bochert, 2020). These feeds become critical for the success of perch aquaculture with respect to profitability, meat quality traits and health status of farmed perch (Policar et al., 2019). Aquafeeds for carnivorous fish, including *P. fluviatilis*, require high protein sources, fishmeal traditionally derived from marine fish resources (Langeland et al., 2016). However, limited supply, continuous rise in price, and unsustainability of this marine-derived ingredient (Naylor et al., 2000; Foley et al., 2011; Tacon et al., 2011; Froehlich et al., 2018; Kok et al., 2020) have challenged the expansion of ever-growing aquaculture sector. Consequently, a number of alternatives aquafeed materials have been investigated. Among them, insect meals and by-products from

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fishery and aquaculture represent the most promising candidates to meet aquafeed demand over the next decades (Hua et al., 2019; Gasco et al., 2020). Along with the rise in production of by-products which will share approximately 35% global fishmeal production by 2030 (FAO, 2018), the output of insect meal production is on the global rise and forecasted to be price-competitive with fishmeal by 2023 (Hua et al., 2019).

Many insect meals have been investigated as replacement for fishmeal in aquatic animal diets, in which the black soldier fly (*Hemetia illucens*), common housefly (*Musca domestica*), and yellow mealworm (*Tenebrio molitor*) has drawn the most attention in the research (Hua et al., 2019; Fabrikov et al., 2020; Mastoraki et al., 2020). Gasco et al. (2019), in their review, reported that dietary insect meals significantly influenced growth performance, digestibility, and meat quality, especially the fatty acid profile of fed fish compared with insect-free diets. A meta-analysis indicated that a moderate inclusion level of insect meal was comparable with fishmeal diet in terms of growth performance, while effects of higher level on the growth rate of fed fish were insect-species-specific (Hua, 2021). Feeding European perch with 40% insect meal (*H. illucens*) inclusion levels did not affect growth performance, feed efficiency and hematological indices, but modified body fatty acid profile and hepatic somatic index (Stejskal et al., 2020). Those investigated indices mainly reflect integrated impacts of formulated diet, which consist of different ingredients, whereas the critical role of individual dietary components incorporated into fish and their tissues remained fragmentary (Yu et al., 2015; Cyrus et al., 2020). Understanding the importance of each ingredient in aquafeed formula to the development of particular tissues of fed organism could be informative for diet improvement, especially for new aquaculture species or raw materials. Stable isotope analysis could be a suitable way to address the importance of these individual ingredients.

Stable isotope techniques have become a valuable tool to investigate the diet proportion of various aquatic species in ecological studies (Post, 2002). Recently, the use of nitrogen and carbon stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$, respectively) and the Bayesian isotope mixing models (Parnell et al., 2013) has been employed in aquaculture nutrition research to explore further insights into the contribution of each ingredient in diet formula to the construction of particular tissues (Gamboa-Delgado and Le Vay, 2009; Enyidi et al., 2013; Gamboa-Delgado et al., 2013; Yu et al., 2015; Gamboa-Delgado et al., 2016; Cyrus et al., 2020; Nahon et al., 2020).

This study aimed to investigate the effects of *T. molitor* larvae meal as a replacement for fishmeal using stable isotopic values of different tissues by assessing diet-tissue isotopic discrimination factors, and modeling the contribution of a particular ingredient to the growth of three tissues, blood, liver and muscle, of juvenile perch.

2. Materials and methods

2.1. Ethics statement

The experimental procedures were conformed to the European Communities Directive (No. 2010/63/EU) and authorized by the Czech Ministry of Health (No. MSMT-6744/2018–2) regarding the protection of animals used for scientific purposes.

2.2. Experimental facilities and procedures

Experimental facilities and procedures of the present study were reported elsewhere (Tran et al., 2021). Briefly, four experimental diets, including a control diet (TM0), and three diets with TM replacement for fishmeal at 25, 50, and 75% (abbreviated diets as TM25, TM50, TM75). The main ingredients of experimental diets are presented in Table 1.

Each diet was fed to quadruplicate 180-L tank groups held juvenile European perch (bodyweight: 20.81 ± 3.36 g) (82 fish/tank), connected in a recirculating system. Parameters included photoperiod (12 h:12 h L:D), light intensity 58.6 lx, water temperature 22.44 ± 0.66 °C, pH 7.00 ± 0.29, oxygen saturation 80.41 ± 8.02%, ammonia-N 0.28 ± 0.16 mg/L, and nitrite nitrogen <0.45 mg/L were maintained throughout experimental period.

Table 1
Ingredients and proximate composition of fishmeal, *Tenebrio molitor* larvae meal, and experimental diets (Tran et al., 2021).

			Experimental diets			
	Fishmeal	TM	TM0	TM25	TM50	TM75
Ingredients (g/kg)						
Soybean concentrate			290	290	290	290
Fishmeal			271	203	135	68
Tenebrio molitor			–	68	135	203
Soybean meal			145	145	145	145
Corn flour			97	97	97	97
Fish oil			77	77	77	77
Rapeseed oil			58	58	58	58
Methionine[a]			8	8	8	8
Lysine[b]			5	5	5	5
Valine[c]			2	2	2	2
L-Threonine[d]			0.5	0.5	0.5	0.5
Vitamins & minerals[e]			8	8	8	8
Additives[f]			40	40	40	40
Proximate composition (dry basis)						
Dry matter (%)	96.5	95.0	94.8	95.7	95.6	95.6
Crude protein (%)	71.2	71.1	47.5	48.7	47.4	47.2
Crude lipid (%)	7.9	8.5	16.3	13.9	15.6	17.0
Ash (%)	14.0	7.1	8.9	9.0	8.3	7.6
Fibre (%)	1.24	2.8	2.0	2.0	2.2	2.3
Nitrogen-free extract (%)[g]	1.3	5.5	19.5	21.8	22.3	21.6
Gross energy (Mj/kg)[h]	20.1	21.1	21.0	20.8	21.2	21.5
Chitin[i]	–	4.8	–	0.33	0.65	0.97

[a] Adisseo, China.
[b] Inner Mongolia Eppen Biotech Co., Ltd.
[c] Ajinomoto Animal Nutrition Europe.
[d] Ningxia Eppen Biotech, China.
[e] Aminovitan Sak, Trouw Nutrition Biofaktory s.r.o, Czech Republic.
[f] Feed limestone (0.5%); Pentasodium triphosphate (Fosfa a.s, Czech Republic) (0.5%) and binder (NutriBind, Adisseo, China) (3.0%).
[g] Nitrogen-free extracts (NFE) = dry matter - (crude protein + crude lipid + ash + fibre).
[h] Gross energy (MJ/kg) as gross energy content of protein (23.6 MJ/kg), lipid (39.5 MJ/kg) and NFE (17.2 MJ/kg).
[i] Estimated from Basto et al. (2020) for defatted TM.

Fish were fed five times daily at 7.00, 9.00, 11.00, 13.00, and 15.00 with an excessive amount, using automatic feeders (EHEIM Twins, Deizisau, Germany). Fifteen minutes following each feeding, unconsumed feed was flushed from the funnel-like tank bottom and dried to determine feed intake. Fish mortality in each tank was recorded daily.

2.3. Sample collection and calculations

After 105 days, following 24 h starvation, fish from each tank were inspected for biometry, and thermal growth coefficient (TGC) was calculated with the formula: $TGC = ((W_f^{1/3} - W_i^{1/3}) / (T \times t)) \times 1000$, where W_f and W_i are final and initial weight (g), respectively, and T and t are water temperature (°C) and experimental duration (105 days), respectively.

Subsequently, three fish from each tank (12 fish/diet group) were randomly selected and sacrificed with an overdose (125 mg/L) Tricaine methanesulfonate (MS222, Sigma-Aldrich Chemicals, St Louis, MO, USA). Approximately 1 mL blood, and small piece of liver and dorsal muscle were sampled.

Experimental diets, ingredients, and perch's tissues were freeze-dried (Alpha 2–4 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground to a fine powder using an agate mortar and pestle. The samples were subsequently analyzed for

stable isotope ratios according to procedures described by Kiljunen et al. (2020). Briefly, approximately 0.5 mg sample was prepared in tin cups (D4057 Elemental Microanalysis, Okehampton, UK) and analyzed at the University of Jyväskylä (Jyväskylä, Finland) using a Thermo Finnigan DELTA^{plus}Advantage mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) connected to a FlashEA 1112 Elementar Analyzer. Northern pike (*Esox lucius*) tissue and birch leaves (*Betula pendula*) were used as internal standards. The results were presented as standard δ notation ($\delta^{13}C$, $\delta^{15}N$) as parts per thousand (‰) differences from the international standard. The percentage of carbon (%C), nitrogen content (%N), and C:N ratio (by weight) of samples were also derived from analysis.

Stable isotopes of carbon in animal tissue are ^{13}C depleted with the presence of lipid content in the samples (Post et al., 2007). Therefore, $\delta^{13}C$ values of fish tissues (except for muscle) were corrected according to Kiljunen et al. (2006) when the C:N ratio of specimens is greater than 3.5 (Skinner et al., 2016), and $\delta^{13}C$ values of ingredients and diets were corrected as described previously (Post et al., 2007), diet to tissue discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$) were calculated as follows: $\Delta X = \delta X_{tissue} - \delta X_{diet}$, where $X = \delta^{13}C$ or $\delta^{15}N$.

Bayesian mixing model framework (Parnell et al., 2013) in the *simmr* package of R environment (Parnell, 2016) was employed to estimate the contribution of feed ingredients to tissues of European perch. The mixing model employing *simmr* package has recently been used to estimate the proportional contribution of feed ingredients to rainbow trout fry tissue (Nahon et al., 2020). We performed Markov Chain Monte Carlo (MCMC) methods in the *simmr* package (Parnell et al., 2010) by running for 100,000 iterations, 10,000 burn-in rate, 100 thinning, and 4 chains. The model convergence was confirmed using Gelman–Rubin diagnostics (Gelman and Rubin, 1992). Due to the comparable isotopic ratios of soy protein and soybean meal (Table 2), we combined them as one source as previously recommended (Phillips et al., 2005). Other sources in the model consisted of fishmeal, TM and corn meal. The contribution of ingredients within diet groups and each ingredient across diet groups was compared, using the "compare_groups" and "compare_sources" function, respectively, in *simmr* package. The probability value (P_{BIC}) from comparison functions is considered a significant difference with $P_{BIC} > 0.95$ (Masson, 2011; Santana et al., 2020).

The mixing model assumes that the isotopic equilibrium of consumer tissue and its diets is reached (Gamboa-Delgado and Le Vay, 2009). Recent publications on aquatic species have reported that the muscle was in isotopic equilibrium with its feed ingredients within 10–90 days (Table 3). Blood and liver were reported to be faster than muscle in reflecting isotopic ratios of diet (Phillips and Eldridge, 2006). Therefore, our 105-days feeding trial of perch was long enough for feed ingredients to isotopically equilibrate with perch tissues.

The estimation of isotopically distinct feed components to the fish tissues using the isotopic mixing models requires corrected ingredient to tissue discrimination factors (Gamboa-Delgado and Le Vay, 2009; Parnell et al., 2010). The values for perch's tissue ($\Delta^{13}C$ (‰) and $\Delta^{15}N$ (‰)) was calculated according to Caut et al. (2009): $\Delta^{13}C = -0.248 \times \delta^{13}C_{ingredient} - 3.477$; $\Delta^{15}N = -0.281 \times \delta^{15}N_{ingredient} + 5.879$ (for muscle); $\Delta^{13}C = 0.77 \pm 0.30$ and $\Delta^{15}N = 1.61 \pm 0.34$ (for liver), and $\Delta^{13}C = 1.0 \pm 0.1$ and $\Delta^{15}N = 1.3 \pm 0.2$ for blood according to Matley et al. (2016), reporting for leopard coral grouper (*Plectropomus leopardus*), the same Perciformes order as perch in present study. The ingredient to tissue discrimination factors used in the mixing model are presented in Table 4.

Table 2
Stable isotope ratios, carbon (C) and nitrogen (N) concentration of main ingredients used in the experimental diets.

	Soy protein	Fishmeal	TM	Soy meal	Corn meal	SEM
$\delta^{13}C$ (‰)	-25.10^{d}	-20.07^{c}	-16.75^{b}	-25.51^{d}	-12.44^{a}	1.33
$\delta^{15}N$ (‰)	1.48^{d}	10.57^{a}	3.53^{b}	1.96^{d}	2.85^{c}	0.89
C (%)	45.28^{a}	47.17^{a}	47.54^{a}	41.60^{b}	45.21^{a}	0.06
N (%)	10.58^{c}	12.27^{a}	11.49^{b}	6.76^{d}	1.12^{e}	1.10
C:N ratio	4.28^{b}	3.85^{b}	4.14^{b}	6.16^{b}	40.96^{a}	3.94

Different superscripts indicate significant differences for specific rows. SEM, standard error of mean.

2.4. Statistical analyses

Data were checked for normal distribution (Shapiro-Wilks's test) and homogeneity of variances (Levene's test). The correlation between explanatory variables (diet-muscle discrimination of nitrogen and carbon) and TGC of fish were tested using *lm* function. ANOVA was used to test the differences, followed by Tukey's post-hoc test, when appropriate. All statistical analyses were performed using the R Statistic Package, R Development Core Team 2009–2021. Differences were regarded as significant at $P < 0.05$.

3. Results

The TGC of European perch exhibited a significant difference among diet treatments after a 105-day feeding trial. Fish fed TM0 and TM25 showed highest TGC (0.65 ± 0.02 and 0.65 ± 0.03, mean ± standard deviation, respectively), which is significantly higher than that of TM50 (0.56 ± 0.01) and TM75 (0.41 ± 0.01) ($P < 0.05$).

Isotope ratios of main feed ingredients used in experimental formulations were significantly different ($P < 0.05$), except soy protein and soy meal (Table 2). Carbon isotope $\delta^{13}C$ did not differ among experimental diets ($P > 0.05$), while increasing replacement levels of fishmeal by TM significantly reduce $\delta^{15}N$ value ($P < 0.05$) (Fig. 1).

Fishmeal replacement by TM significantly reduced $\delta^{15}N$ values ($P < 0.05$) in all tissues but increased $\delta^{13}C$ values in muscle and blood ($P < 0.05$). $\delta^{13}C$ in liver remained insect-dose independent ($P > 0.05$) (Fig. 1). Muscle was more enriched in ^{15}N than in blood and liver. The enrichment of ^{13}C was more pronounced in liver than blood and fillet (Fig. 1).

Diet-tissue discrimination factor, $\Delta^{13}C$ in blood (0.54–0.65‰) and liver (2.65–3.35‰) of European perch were not significantly affected by dietary treatments ($P > 0.05$). However, $\Delta^{13}C$ in muscle was significantly lower in TM-containing diets than TM0 ($P < 0.05$). European perch fed with each of the four experimental diets showed higher diet-tissue discriminations in liver than other tissues ($P < 0.05$). Dietary TM significantly increased discrimination of $\Delta^{15}N$ in all tissues of European perch ($P < 0.05$). Muscle of perch exhibited highest $\Delta^{15}N$ among other tissues ($P < 0.05$) (Fig. 2).

The correlation test indicated that there was a significant linear relationship between diet-muscle $\Delta^{15}N$ and TGC of perch (TGC = -0.03 $\Delta^{15}N + 0.57$, $P < 0.0001$, adjusted R-squared = 0.83, *F*-statistic: 74.05), while there was no significant correlation between TGC and $\Delta^{13}C$ in muscle ($P = 0.16$, adjusted R-squared = 0.07, *F*-statistic: 2.15).

After correcting for ingredient-tissue discrimination factor, the isotopic values of three tissues fell within mixing polygons mapped out by four main ingredients (Fig. 3), providing a sound basis for mixing models, and all important feedstuff were well incorporated in the model. Muscle values tended to fall closely to soy ingredients, while those of liver did to the center of the mixing polygons.

There was considerable variability in diet contribution estimates of the *simmr* model outputs among tissues and across diet treatments (Tables S1, Fig. 4). Regarding muscle development, soy ingredients were predicted to contribute most (63.8–80.3%, mean values) over other feed ingredients in all tissues ($P_{BIC} > 0.95$) (Fig. 4, Table S1). The contribution of soy was also notably higher than its proportion (43.5%) in experimental diets (Table 1). As expected, increasing replacement of fishmeal with TM significantly reduced the contribution of fishmeal

Table 3
Time (days) for feed ingredients reach isotopic equilibrium in muscle of aquatic animals in the literature.

Ingredients	Species	Life stage	Isotopic equilibrium (days)	Reference
Fish meal Pea meal	Shrimp (*Litopenaeus vannamei*)	Postlarvae	22	Martínez-Rocha et al., 2013
Fish meal Poultry by-products	Shrimp (*L. vannamei*)	Postlarvae	30	Gamboa-Delgado et al., 2014
Fish meal Soy concentrate	Shrimp (*L. vannamei*)	Postlarvae, juvenile	15	Gamboa-Delgado and Le Vay, 2009
Fish meal Poultry by-products	Catfish (*Ictalurus puncatus*)	Juvenile	30	García-Pérez et al., 2018
Fishmeal Corn meal	Catfish (*I. gariepinus*)	Larvae	30	Enyidi et al., 2013
Fishmeal Soybean meal	Catfish (*I. gariepinus*)	Larvae	28	Enyidi, 2012
Fish meal Soybean meal Beer yeast meal Corn gluten meal	Cobia (*Rachycentron canadum*)	Juvenile	24	Zhou et al., 2016
Fish meal	Rainbow trout (*Oncorhynchus mykiss*)	Fingerling	90	Beltrán et al., 2009
Fish meal Corn gluten meal	Rainbow trout (*O. mykiss*)	Fry	36	Nahon et al., 2020
Rotifers	Red drum (*Sciaenops ocellatus*)	Larvae	10	Herzka and Holt, 2000
Krill	Sockeye salmon (*O. nerka*)	Age 1^{+} (9.5–15.3 g)	40	Sakano et al., 2005
Fish meal Soybean meal	Tilapia (*Oreochromis niloticus*)	Fry	56	Zhou and Gu, 2020

Table 4
Discrimination factor (‰) of feed ingredients and tissues of perch used in the Bayesian mixing model.

Ingredient	Muscle		Liver		Blood	
	$\Delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$
Fishmeal	2.91 ± 0.02	1.50 ± 0.01	1.61 ± 0.34	0.77 ± 0.30	1.3 ± 0.2	1.0 ± 0.1
Soy	5.40 ± 0.02	2.80 ± 0.03				
Corn	5.08 ± 0.06	−0.39 ± 0.02				
T. molitor	4.89 ± 0.03	0.68 ± 0.02				

($P_{BIC} > 0.95$) but did not elevate that of the latter ($P_{BIC} < 0.95$) (Table S1). The estimated contribution of these animal-derived ingredients in muscle was considerably lower than their dietary inclusion levels. Except for TM0, corn meal appeared to be a less important ingredient in perch muscle as its contribution remained low (5.7–6.8%) regardless of dietary TM ($P_{BIC} < 0.95$) (Fig. 4, Table S1).

The development of liver of perch fed TM0 received the most significant contribution from corn meal (66.4 ± 3.5%) and significantly higher than fishmeal (30.5 ± 3.6%) and soy (3.1 ± 1.4%) ($P_{BIC} > 0.95$) (Fig. 4, Table S1). TM made up the second-largest proportion to perch liver (25.4–31.1%), following soy (32.5–38.5%), and there is no significant difference in the contribution of these ingredients across TM-containing diets ($P_{BIC} < 0.95$). In similarity to muscle tissue, increasing replacement fishmeal by TM accompanied statistical reduction in proportional contribution of the former to perch liver ($P_{BIC} > 0.95$), yet nonstatistical evidence for the latter ($P_{BIC} < 0.95$). The share of corn meal to the perch liver's growth (16.6–18.7%) was comparable with fishmeal across TM-containing diets.

Fishmeal, in the absence of dietary insect meal, was assimilated in the blood of perch (45.5 ± 4.2%) significantly more than that of soy (30.7 ± 1.8%) and corn meal (23.9 ± 5.2%) ($P_{BIC} > 0.95$). In the TM-containing diets treatments, soy had an immense contribution ($P_{BIC} > 0.95$), followed by fishmeal. TM, together with corn meal, remained a minor contributor to blood composition of perch fed with TM0, TM25 and TM50, but while fed with TM75, TM and fishmeal displayed an equal contribution ($P_{BIC} < 0.95$).

4. Discussion

This was the first study investigating isotopic signatures and proportional contribution of feed components to tissues of fish fed experimental formulations where fishmeal was partially substituted by insect meal. The study provided insightful findings on the importance of particular ingredients to the construction of perch tissues, a result underlies the effects of diet treatments on the production performance and nutrient assimilation in farmed perch. The outputs could offer an additional protein source choice for the future growing percid aquaculture sector (Policar et al., 2019; Stejskal et al., 2020; Tran et al., 2021). Our findings highlighted that there was a negative correlation between diet-muscle $\Delta^{15}N$ and fish growth performance. Insect meal (*T. molitor*) seemed not favorable for perch muscle and blood as its contribution to growth of these tissues was disproportional with increasing inclusion levels. In contrast, the share of TM in the liver remained significant in perch fed TM-containing diets. The present study also proposed helpful information for the ecological study of perch. Accordingly, a non-lethal sample of blood could be useful tissue for investigating food sources and, to a lesser extent, trophic position.

For isotope modeling purposes, dietary sources should obtain distinct isotope values. TM exhibited distinguishable isotope ratios compared to fishmeal and plant ingredients. In farming practice, *T. molitor* is the primary consumer of various plant substrates (Cortes Ortiz et al., 2016) and therefore more enriched in ^{15}N and ^{13}C from those diets (DeNiro and Epstein, 1978, 1981). This could explain a significantly higher nitrogenous isotopic signature of TM than soy and corn meal, which are classified as primary producers. On the other hand, fishmeal derived from marine catch enriches a substantial ^{15}N from marine food sources (Kusche et al., 2018).

Isotopic values of tested tissues strongly reflected those of respective diets, especially for $\delta^{15}N$, while $\delta^{13}C$ was slightly modified across tissues from respective diets. Diet-tissue $\Delta^{15}N$ for liver (ranged, 1.8–2.84‰) and muscle (2.97–4.63‰) in the present study was similar to an empirical study on *Totoaba macdonaldi* fed compound feeds under controlled conditions (2.8–4.9‰ and 1.2–4.4‰, respectively) (Zapata et al., 2016). Vollaire et al. (2007) reported that $\Delta^{15}N$ of 2.88‰ was observed in muscle of perch fed commercial feed, which is in our reported range, but that in liver (0.65‰) was slightly lower than the 1.80–2.84‰ observed in the present study. Regarding diet-tissue $\Delta^{13}C$ in muscle and liver, our results, ranged 0.47–0.74‰ and 2.65–3.35‰,

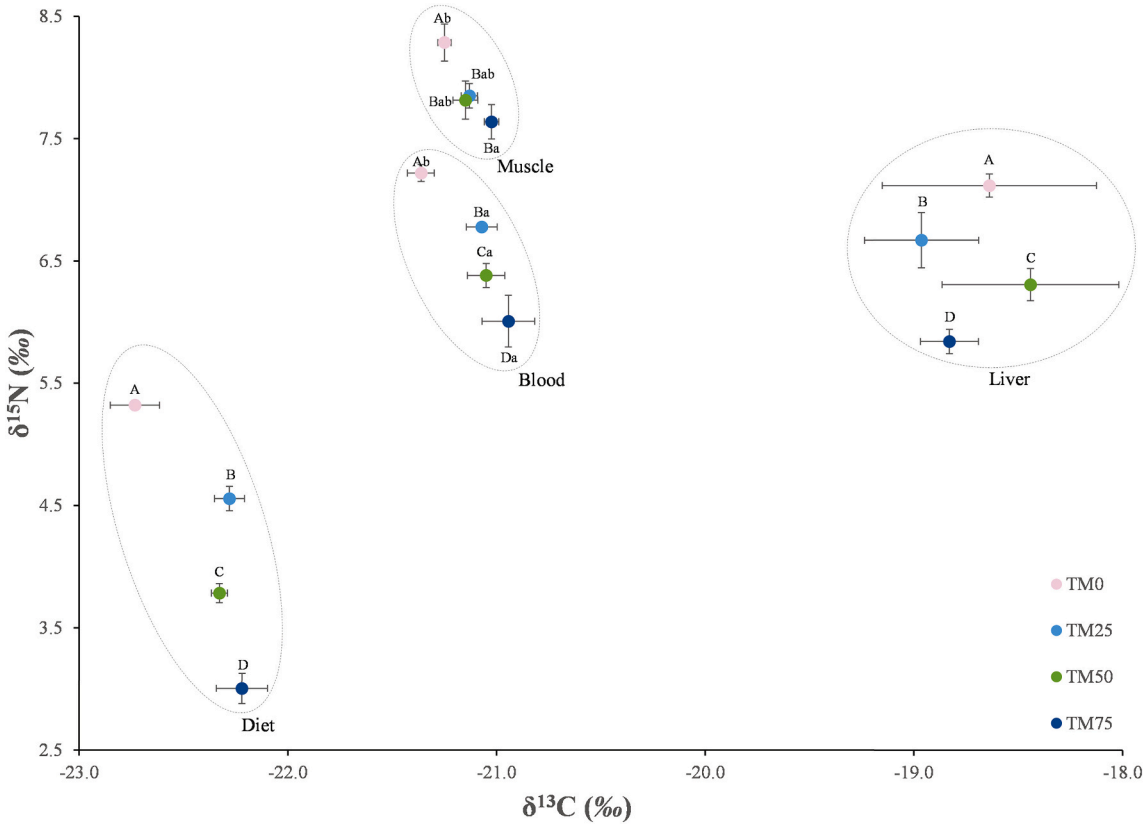

Fig. 1. Isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) of fillet, liver, and blood of European perch fed four experimental diets. Data were present as mean ± SD. Different lowercases and uppercases within the tissue group indicate significant differences in $\delta^{13}C$ and $\delta^{15}N$ values, respectively.

respectively, indicated relatively lower than published data (4.02‰ and 3.44‰, respectively) (Vollaire et al., 2007). The discrepancies could be due to several factors, including food quality (e.g., protein quality, dietary isotopic values, C/N), physiological status of tested animals, and diet kinetic effects (Vollaire et al., 2007; Kadye et al., 2020; Zhou and Gu, 2020).

The present study also investigated diet-blood discrimination values for both isotopic signatures and found in the range of 1.90–3.00‰ for $\Delta^{15}N$ and 0.54–0.65‰ for $\Delta^{13}C$, which is in consent with Cherel et al. (2005) for captive penguins fed fish, and with the published review (Caut et al., 2009) for mammals. Caut et al. (2009) indicated negative relationships between diet-tissue discrimination and respective dietary isotopic values. This was particularly found in our study where the highest $\delta^{15}N$ value in TM0 resulted in narrow discrimination of $\Delta^{15}N$ in somatic tissues, a similar phenomenon was observed for $\delta^{13}C$ isotopic values in tissues of perch fed TM25. While fish fed low dietary isotopes led to enlarge discrimination values. Moreover, the protein quality of diets strongly influenced nitrogen stable isotope incorporation, thereby $\Delta^{15}N$ discrimination (Mohan et al., 2016; Kadye et al., 2020). In the present study, the high nitrogen diet-tissue isotopic differences in all somatic tissues associated with increasing dietary TM could be due to the presence of chitin, which was reported to impair protein digestibility in insect-containing diets (Gasco et al., 2016). This evidence could also explain the negative relationship between growth performance as indicated by TGC of perch and nitrogenous diet-muscle discrimination, which is in agreement with earlier publications (Trueman et al., 2005; Beltrán et al., 2009; Lefebvre and Dubois, 2016).

In comparison with blood and liver in perch fed the same diet, muscle was found to be the most fractionated in diet-tissue nitrogen, which concurs with previous studies (Suzuki et al., 2005; Malpica-CruzLuis et al., 2012; Xia et al., 2013; Mohan et al., 2016; Zapata et al., 2016). The discrepancy in $\Delta^{15}N$ among tissues could be ascribed to a higher accumulation of heavier isotope in muscle than other tissues (Gamboa-Delgado et al., 2020), more essential amino acids contained in the latter than in the former tissues (Mohan et al., 2016), and typical traits of muscle relative to other tissues in fish (Pinnegar and Polunin, 1999).

The higher diet-tissue $\Delta^{13}C$ in liver than other tissues regardless of dietary TM observed in our study was in agreement with previously published work (Pinnegar and Polunin, 1999). DeNiro and Epstein (1978) suggested that the magnitude of carbon discrimination primarily relied on tissue's biochemical fractions (lipid, protein and carbohydrate). Liver of perch contained a greater proportion of lipid and glycogen than other tissues (Vollaire et al., 2007), which could explain the high departure of $\delta^{13}C$ in perch liver from respective diets in the present study. Our mixing model indicated that liver of perch received a high proportional contribution from TM and corn meal, which are distinguished from FM and soy ingredients, the main contributors to blood and muscle. In addition, we applied carbon isotopic correction (Kiljunen et al., 2006; Skinner et al., 2016) for liver tissue, as lipid synthesis in this tissue depleted the heavy carbon isotope at the expense of the lighter one (DeNiro and Epstein, 1978), this consequently leads to the convergence of $\delta^{13}C$ in liver, and thereby of $\Delta^{13}C$. Our study, therefore, implicated that blood and muscle, but not liver, are suitable tissues for evaluating the diet source since it was weekly ^{13}C enriched. Whereas muscle was particularly enriched in ^{15}N, rather than liver and blood, thereby this tissue will be useful for determining the trophic levels of perch (Caut et al., 2009). The present study also found that feeding insect-containing diets resulted in significantly lower diet-liver $\Delta^{13}C$ than insect-free diet did, which could be linked to diet quality, thereby fish catabolized fatty acid storage in the ^{13}C-depleted form (Nahon et al., 2020).

The present study provided insights into the incorporation of different ingredients into fish tissues with the presence or absence of

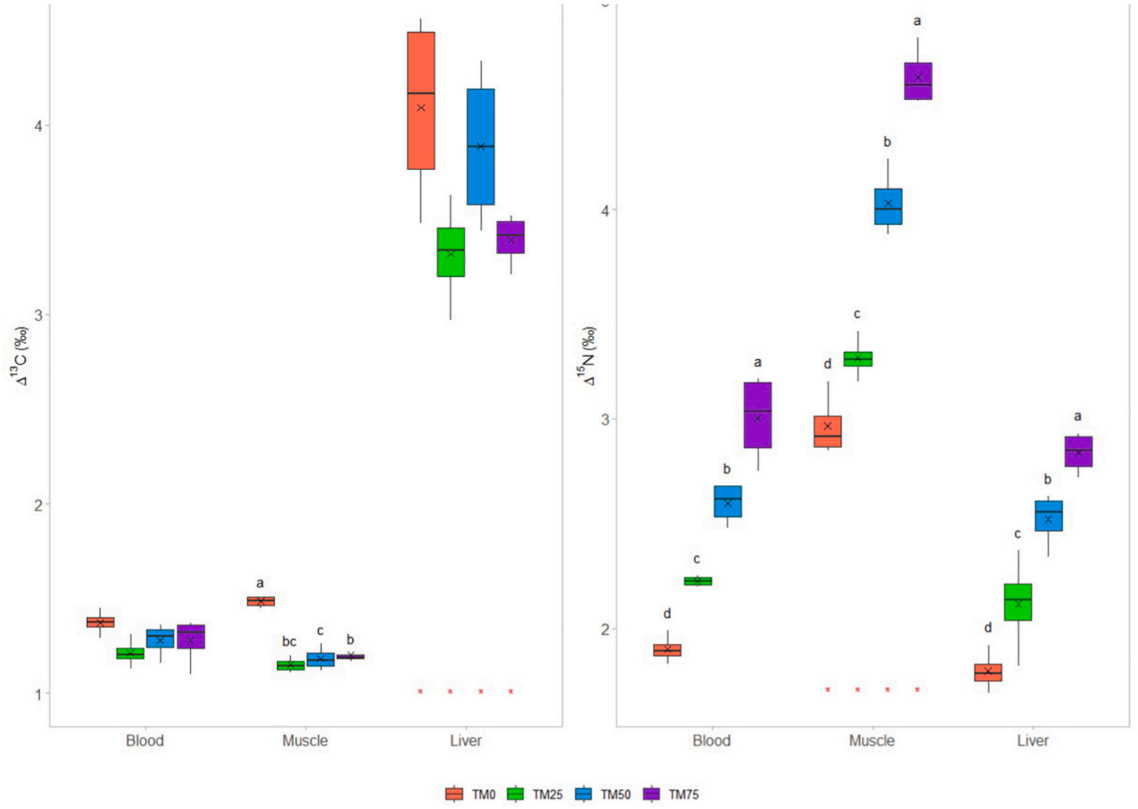

Fig. 2. Discrimination factors, $\Delta^{13}C$ (left) and $\Delta^{15}N$ (right) of European perch's tissues and experimental diets. The black "x" represents mean value. The horizontal line inside each boxplot represents the median separating the interquartile range. Different lower cases within tissue group indicate significant difference across diet treatments ($P < 0.05$). The red asterisks within diet group indicate significant difference ($P < 0.05$) compared to the other tissues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

insect meal (*T. molitor*). The *simmr* package, a stable isotope mixing model within a Bayesian framework, has been used in the ecological study and aquaculture nutrition to estimate the proportional contribution of ingredients to fish tissue (Nahon et al., 2020). Outputs of the mixing model are highly sensitive and require precise values of ingredient-tissue discrimination factor (Phillips and Gregg, 2001). In the present study, ingredient-muscle discrimination factors were corrected according to the decision diagram described by Caut et al. (2009). A similar approach was reported earlier while estimate ingredient contribution to tissue of cobia (*R. canadum*) (Zhou et al., 2016). A previous study (Nahon et al., 2020) recommended using different carbon and nitrogen discrimination factors in muscle for animal−/plant-derived ingredients. The distinction of our discrimination factors in muscle for animal and plant feedstuff, therefore, fits that assumption. All stable isotope values of perch tissues laid within the mixing polygons, indicating the sound reliability of *simmr* Bayesian mixing model. Overall, the model output showed that soy components, including soy protein and soybean meal, significantly contributed to all tissues, regardless of dietary TM. Indeed, these ingredients were included at a significant proportion in all experimental diets (43.5%). The previous study evidenced that ingredients with a high dietary proportion commonly accompanied a high contribution to sea cucumber due to opportunistic ingestion (Yu et al., 2015). On the other hand, high protein intake was documented to increase protein synthesis in vertebrates (Tsahar et al., 2008).

Perch fed 6.8% TM inclusion in diet received a matching contribution to muscle (7.7 ± 3.8%), but the utilization of TM in this tissue did not proportionally increase with its higher inclusion in TM50 and TM75 diets. A similar pattern regarding disproportion between dietary inclusion and predicted contribution of TM in blood and liver was observed. This phenomenon could be attributed to the presence of polysaccharides fraction, namely chitin (Table 1) and the limited ability of perch to degrade this non-protein nitrogen component (Langeland et al., 2016). The earlier study (Yu et al., 2015) has indicated that higher cellulose, one of polysaccharides fraction, and low cellulase activities in the digestive tract impaired seaweed (*Sargassum thunbergia*) utilization by sea cucumber. The unchanged contribution of TM to muscle, despite increased dietary inclusion, could also be linked to amino acid deficiency (Gamboa-Delgado and Le Vay, 2009). The amino acid profile of insect meal of *T. molitor* was found to be imbalanced, as indicated by the essential to non-essential amino acid ratio, being less than 1 (Nogales-Mérida et al., 2019).

Muscle has been confirmed to reflect the majority of isotopic compositions in whole-body fish (Zhou et al., 2016). Therefore, the growth of perch fed dietary groups could be closely linked to the proportional contribution of individual ingredients to muscle. Perch fed TM25, consisting of 45.3% soy ingredients and 6.8% TM, equivalent to proportional contribution to muscle of 79.9 ± 1.5% and 7.7 ± 3.8%, respectively, supported growth of perch relative to TM0. It appears to be apparent that the nutritional complement among ingredients in TM25 yielded comparable nutritional compositions, e.g., fatty acid profile (Tran et al., 2021), with TM0, thereby supporting the performance of perch. The higher administration of TM accompanying lower fishmeal contribution to muscle, resulted in growth delay of perch compared with TM-free diet. Soy protein has been known to be methionine and lysine deficient (Li et al., 2015), which can affect protein synthesis in fish muscle, thereby depressing fish growth (Abimorad et al., 2014). This suggests that soy, TM and corn meal could not cover the nutritional requirements of perch at the lower fishmeal availability.

Liver of perch tended to assimilate a large amount of corn meal (66.4 ± 3.5%) in the absence of TM, despite low dietary inclusion level (9.7%) and was significantly higher than that of fishmeal and soy. Lipid and

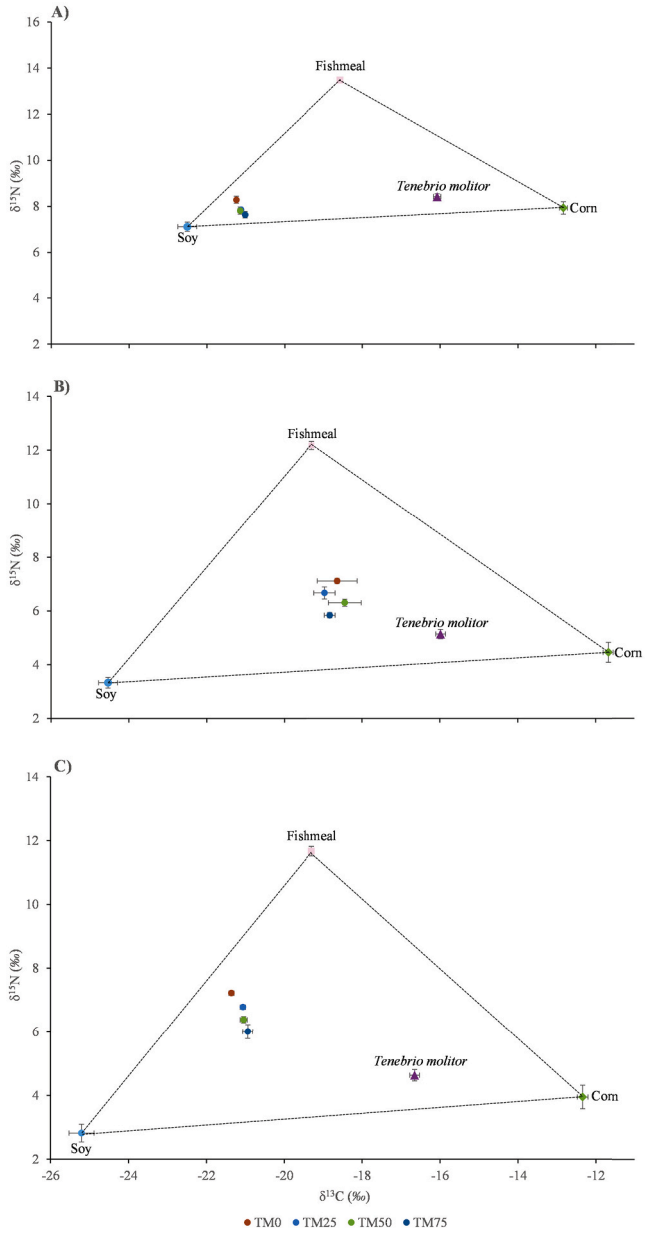

Fig. 3. Isospace plots of $\delta^{13}C$ and $\delta^{15}N$ signatures of four feed ingredients and tissues (muscle (A), liver (B) and blood (C)) of European perch fed for experimental diets.

glycogen were found the be more abundant in liver than other tissues in perch (Vollaire et al., 2007), which are resulted from glucose production (gluconeogenesis) and lipid synthesis (lipogenesis), with amino acids are the primary source of carbon (Ballantyne, 2001). Earlier studies have observed the important role of serine, glycine and alanine, leucine, and valine in these synthesis processes (French et al., 1981; Henderson and Sargent, 1981; Li et al., 2009). Although corn meal provided a comparable carbon quantity with fishmeal, the former contained a relatively higher content of the abovementioned essential amino acids than the latter ingredient (Al-Gaby, 1998; Allan et al., 2000; Herath et al., 2016; Moreno-Arias et al., 2018). Our result may elucidate the higher incorporation of corn meal than fishmeal in perch liver. TM represented a significant contribution to the composition of perch liver and higher compared to its dietary inclusion. This suggests the important role of TM in liver function, which may attribute to its carbon source and some amino acids necessary for the synthesis of biochemical fractions. In addition, fatty acids, especially oleic and linoleic, which are presented at approximately 60% total fatty acids in defatted TM observed in the present study (Tran et al., 2021), have been reported to be easily incorporated or act as an essential precursor for desaturation and elongation of fatty acid products (Xu et al., 2001; Xu and Kestemont, 2002). The disproportion between higher inclusion levels of TM and its contribution to liver could be explained by the deficiency of highly polyunsaturated fatty acids such as DHA and EPA, found in defatted TM (Nogales-Mérida et al., 2019; Tran et al., 2021). The high dietary insect meal was evidenced to induce lipid peroxidation and hepatic damage (Li et al., 2017).

Unlikely muscle and liver extracted from the lethal specimens. Blood has recently gained attention as a non-lethal sampling approach for bulk stable isotope analyses. The transport of amino acids derived from dietary protein plays an essential function in blood of fish (Barst et al., 2021) and protein was found at a great proportion in the blood of perch (Velíšek et al., 2009; Tran et al., 2021). Therefore, protein content, particularly amino acids, of individual ingredients underlie the relative contribution to blood composition. The present study indicated that fishmeal, at the absence of insect meal, made up 45.5 ± 4.2% share, followed by soy and cornmeal to the blood composition of perch. The superior amino acid profile of fishmeal over soy could demonstrate this phenomenon (Nogales-Mérida et al., 2019). However, soy ingredients surpassed fishmeal in terms of relative contribution to blood of perch, at the presence of insect meal. It is speculated that the combination of insect meal and soy ingredient at an appropriate ratio could stimulate the utilization of the latter in blood circulation. The encouragement of soy products in the blood of perch could be linked to the availability of specific amino acids. Leucine and phenylalanine amino acids are known to boost the assimilation and synthesis of protein in animal tissues (Gamboa-Delgado et al., 2020), which was found to be abundant in soy-derived ingredients (Deng et al., 2006) and insect meal (*T. molitor*) (Nogales-Mérida et al., 2019). The limited essential amino acids of TM, especially lysine and methionine, could be the reason that impairs the higher incorporation of TM into blood of perch.

5. Conclusion

The present study indicated that yellow mealworm (*Tenebrio molitor*) larvae meal had distinct isotopic signatures from fish meal, C_3 (soy-derived ingredients) and C_4 plant ingredients (corn meal), which can be employed in further studies using isotopic mixing models. For aquaculture and ecological studies, diet-tissue discrimination of nitrogen could be a valuable proxy to evaluate the protein quality of aquafeeds, trophic level investigation and the performance of fed organisms whereby muscle is a preferable tissue. In contrast, blood and muscle should be considered important tissues for exploring diet sources for European perch.

An inclusion level of 6.8% or 25% fishmeal replacement with insect meal (*T. molitor*) is recommended in diets for perch to ensure production performance and liver health. The further inclusion did not encourage its relative contribution to the development of three tissues (muscle, liver and blood), thereby impairing growth production. TM appeared to be less critical to the construction of muscle and blood as its dietary inclusion was disproportionally correlated with the relative contribution to these tissues. The role of TM was significant in liver by providing carbon source and important fatty acid, such as oleic and linoleic acids, but substantial inclusion of TM may induce liver damage for perch. The present study's findings revealed that the presence of non-nitrogen protein, chitin were critical factors affecting nutritional assimilation of insect meal in the growth of perch tissue and that the nutritionally complement among feed ingredients could be an important consideration for future feed formulation for perch farming. Our study also hinted at further studies to combine potential insect ingredients, or to supplement insect-containing diets with chitinase-produced bacteria to enhance the benefits of insect meals in aquafeeds.

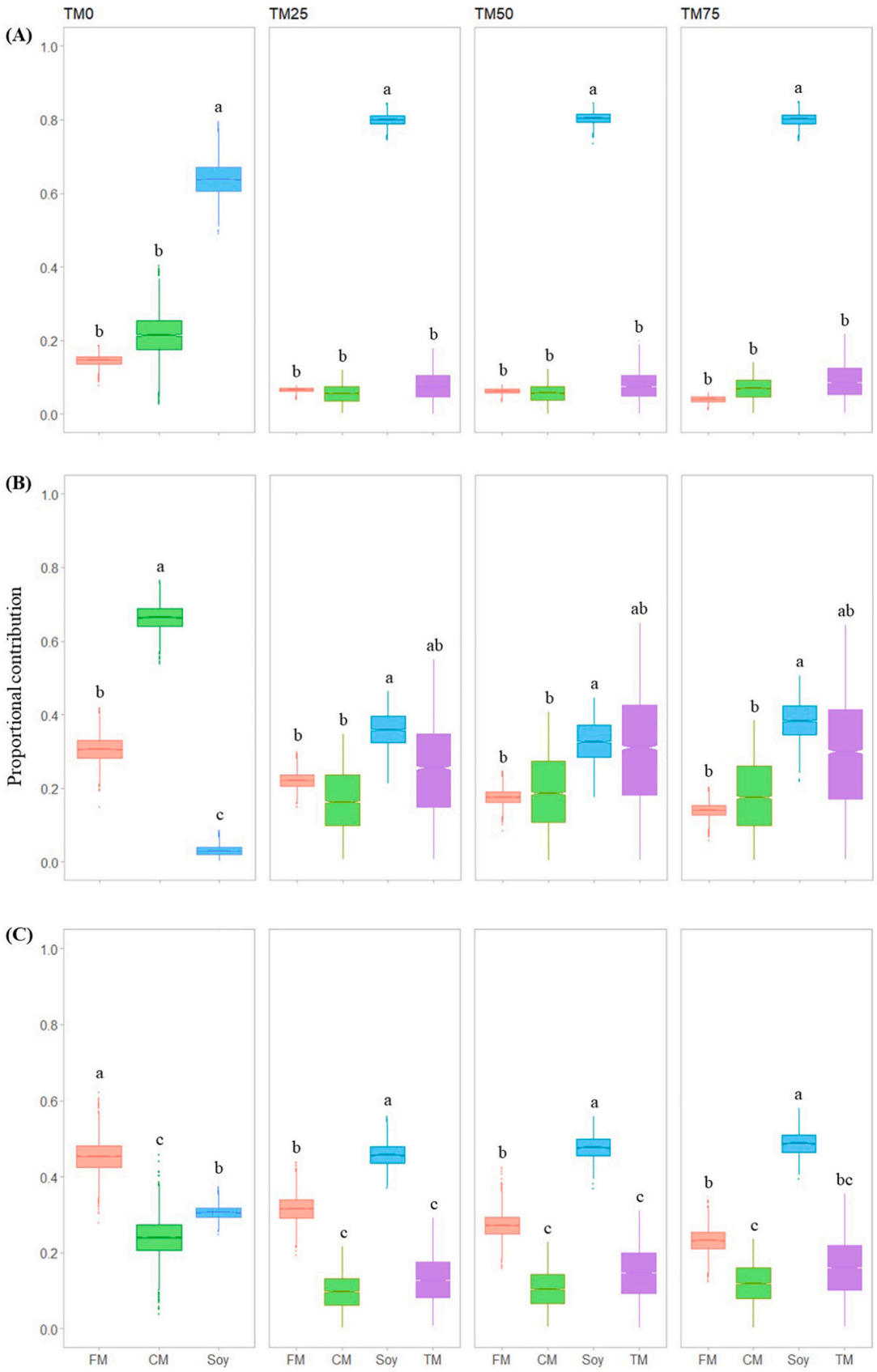

Fig. 4. Boxplots from Bayesian isotopic mixing models representing proportional contribution (mean, interquartile range) of individual feed ingredient to muscle (A), liver (B) and blood (C) tissues of European perch fed experimental diets.

Authorship statement

The manuscript, entitled "European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): from a stable isotope perspective". All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Journal of Aquaculture.

Authorship contributions

Vlastimil Stejskal: Conception and the revision of the manuscript.

Hung Quang Tran: Contribution to design, conduct experiment, analyse of the data, and manuscript preparation.

Hien Van Doan: Contribution to manuscript preparation.

Mikko Kiljunen: Contribution to analysis and manuscript preparation.

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.aquaculture.2021.737265.

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Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*?

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ABSTRACT

We assessed swimming capacity, energy expenditure, and physiological responses of European perch (*Perca fluviatilis*) fed four isonitrogenous and isoenergetic diets containing yellow mealworm (*Tenebrio molitor*) larvae meal at 0, 25, 50, and 75% substitution for fishmeal (abbreviated diets, TM0, TM25, TM50, and TM75). Each diet was fed to quadruplicate group of perch (initial biometrics, body weight 20.81 ± 3.36 g, total length 11.77 ± 0.72 cm) for 119 days. At the terminal of feeding trial following 24 h starvation, eighty fish (20 fish/diet group) were individually selected for swimming performance tests, which were conducted in a 10 L enclosed swimming tunnel with velocity increased from 5 cm/s in 2 cm/s increments every 60 s. Exercised fish, fish experienced swimming tests, and non-exercised fish, fish not involved in swimming tests were, at the same time, sampled for serum biochemistry, muscle traits. Whole-body of non-exercised fish were also analyzed for proximate composition and fatty acid profile.

Critical swimming speed (U_{crit}, cm/s and body length/s), oxygen consumption (MO_2, mg/kg/h), and energy cost of transport (COT, J/kg/m) of perch did not differ among diet treatments. Exercised perch significantly increased serum glucose and cortisol compared to non-exercised fish. Substitution of fishmeal by *T. molitor* larvae meal induced significant changes in aspartate aminotransferase across treatment groups, lactate dehydrogenase in TM0 and TM75, K^+ concentration in fish fed TM75, and muscle water content in TM50 of exercised compared to non-exercised perch. Oleic acid of whole-body fish had a significant linear correlation with the critical swimming speed of European perch. Since fish swimming behavior is an indicator of animal welfare, our findings suggest that dietary insect meals could ensure the welfare of farmed fish.

1. Introduction

The continuous growth of aquaculture industry as a consequence of increasing global seafood demand has pressured aquafeed ingredient inputs, which traditionally rely on finite marine fish for dietary protein sources (Froehlich et al., 2018; FAO, 2020). Insect meal possesses essential properties, namely good nutritional content, environmental suitability, consumer acceptance, scalability, and price competition, and thereby has been identified as the greatest potential as protein sources for aquafeeds over the next decades (Hua et al., 2019; Gasco et al., 2020b; Gasco et al., 2020a). Among seven insect species (two flies, two worms, and three crickets) approved for use in aquafeeds by the European Commission (Regulation 2017/893, 24 May 2017), black soldier fly (*Hemetia illucens*), common housefly (*Musca domestica*), and yellow mealworm (*Tenebrio molitor*) have received the most interests of research (Mastoraki et al., 2020; Hua, 2021).

The use of *T. molitor* larvae meal (TM) in fish feed has been investigated extensively. Substantial replacement of fishmeal by TM without detrimental impact on growth performance or feed conversion ratio has been reported, for example, red sea bream (*Pagrus major*) (100% substitution) (Ido et al., 2019), whiteleg shrimp (*Litopenaeus vannamei*) (100%) (Panini et al., 2017), African catfish (*Clarias gariepinus*) (80%) (Ng et al., 2001), yellow catfish (*Pelteobagrus fulvidraco*) (75%) (Su et al., 2017), and rainbow trout (*Oncorhynchus mykiss*) (67%) (Belforti et al., 2015). Dietary TM has been shown to alter fish immune responses (Su et al., 2017; Henry et al., 2018b; Henry et al., 2018a; Sankian et al., 2018; Song et al., 2018), gut microbiota diversity (Antonopoulou et al., 2019; Józefiak et al., 2019) and meat traits, including fatty acid profile,

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in mandarin fish (*Siniperca scherzeri*) (Sankian et al., 2018), rainbow trout (Belforti et al., 2015; Iaconisi et al., 2018), *P. bogaraveo* (Iaconisi et al., 2017), Nile tilapia (*Oreochromis niloticus*) (Sánchez-Muros et al., 2016), and European sea bass (*Dicentrarchus labrax*) (Gasco et al., 2016). Consequently, these alterations may influence the physiology of insect meal-fed aquatic animals. Swimming performance and metabolic activity assessment are considered important proxy for the physiological traits of fishes (Allen et al., 2021). The former variable is commonly assessed via critical swimming speed (U_{crit}) (Brett, 1964), while oxygen consumption (MO_2) is the measurement of the latter, and of other metallic endpoints, such as cost of transport (COT), the energy cost to transport unit of body mass over one unit of distance (McPhee and Janz, 2014). Those variables are sensitive indicators of physiological stress (Brett, 1972), dietary nutrition (Martos-Sitcha et al., 2018), and meat trait alteration of fish (Hammer, 1995; McKenzie et al., 1998; Wagner et al., 2004; Chatelier et al., 2006). In particular, muscle fatty acid profile was reported to affect swimming performance of Atlantic salmon (*Salmo salar*) (McKenzie et al., 1998; Wagner et al., 2004), European seabass (Chatelier et al., 2006), and Arctic charr (*Salvelinus alpinus*) (Pettersson et al., 2010). In addition, the fast-growing of aquaculture has placed the importance of introducing new farmed candidates and animal welfare, physiological indicators of swimming performance and metabolic rate are, therefore, beneficial for aquaculture guidelines, such as system design, towards better growth, health, and welfare (Martins et al., 2012; Allen et al., 2021).

European perch (*Perca fluviatilis*) is a novel candidate for aquaculture diversification in Europe and, along with other percids fish species, will promptly become an established aquaculture sector in Europe (Policar et al., 2019). Fillets of intensively cultured perch have excellent nutritional value, particularly beneficial fatty acids (Stejskal et al., 2011). Dietary insect meal (*Hermetia illucens*) was reported to significantly modify meat quality, especially the fatty acid profile of European perch relative to insect-free diet (Stejskal et al., 2020), which could alter the above-mentioned physiological indicators. These indicators become more critical for grow-out production of percids mainly held in recirculated aquaculture system (RAS), which is moving towards optimal operation system (Steenfeldt et al., 2015; Policar et al., 2019).

Thus far, the measure of swimming performance, metabolic activities of insect meal-fed European perch could provide useful information for the future farming practice of percid fish, especially RAS designed system and for other insect-fed fish species. The goal of the present study was to determine critical swimming speed, oxygen consumption, cost of transport, and physiological response of European perch fed dietary defatted mealworm *T. molitor* larvae meal. We also explored predictable factors which may influence the swimming performance of European perch.

2. Materials and methods

2.1. Ethics statement

The experimental procedures were performed under guidelines of the European Communities Directive (No. 2010/63/EU) on the protection of animals used for scientific purposes and have been approved by the Czech Ministry of Health (MSMT–6744/2018–2).

2.2. Experimental diets

Defatted TM was obtained from a commercial source (NovoProtein, FISHAG EDELHOF GmbH, Wien, Austria). Nutritional composition and fatty acid profile of TM are presented in Tables 1, 3.

Four isonitrogenous and isoenergetic diets were formulated, with the control diet (TM0) containing fishmeal as the main protein source and three diets with TM larvae substituted for fishmeal at 25, 50, and 75% (TM25, TM50, TM75) (Table 1). The experimental diets were produced at the EXOT HOBBY s.r.o., Czech Republic, using a commercial twin-screw extruder (Saibainuo, China). All finely grounded ingredients were mixed in a feed mixer HLJ–700/C (Saibainuo, China), followed by adding oil and water to form a mixture, subsequently extruded with 2-mm diameter pellets. Temperature during the extrusion process ranged 96–106 °C.

Table 1
Ingredients and proximate composition of *Tenebrio molitor* larvae meal and experimental diets.

	Fishmeal	TM	TM0	TM25	TM50	TM75
Ingredients (%)						
Soybean concentrate			29.0	29.0	29.0	29.0
Fishmeal			27.1	20.3	13.5	6.8
Tenebrio molitor			0.0	6.8	13.5	20.3
Soybean meal			14.5	14.5	14.5	14.5
Corn flour			9.7	9.7	9.7	9.7
Fish oil			7.7	7.7	7.7	7.7
Rapeseed oil			5.8	5.8	5.8	5.8
Methionine[a]			0.8	0.8	0.8	0.8
Lysine[b]			0.5	0.5	0.5	0.5
Valine[c]			0.2	0.2	0.2	0.2
L-Threonine[d]			0.05	0.05	0.05	0.05
Vitamins & minerals[e]			0.8	0.8	0.8	0.8
Additives[f]			4.0	4.0	4.0	4.0
Proximate composition (dry basis)						
Dry matter (%)	96.5	95.0	94.8	95.7	95.6	95.6
Crude protein (%)	71.2	71.1	47.5	48.7	47.4	47.2
Crude lipid (%)	7.9	8.5	16.3	13.9	15.6	17.0
Ash (%)	14.0	7.1	8.9	9.0	8.3	7.6
Fibre (%)	1.24	2.8	2.0	2.0	2.2	2.3
Nitrogen-free extract (%)[g]	1.3	5.5	19.5	21.8	22.3	21.6
Gross energy (Mj/kg)[h]	20.1	21.1	21.0	20.8	21.2	21.5

[a] Adisseo, China.
[b] Inner Mongolia Eppen Biotech Co., Ltd.
[c] Ajinomoto Animal Nutrition Europe.
[d] Ningxia Eppen Biotech, China.
[e] Aminovitan Sak, Trouw Nutrition Biofaktory s.r.o, Czech Republic.
[f] Feed limestone (0.5%); Pentasodium triphosphate (Fosfa a.s, Czech Republic) (0.5%) and binder (NutriBind, Adisseo, China) (3.0%).
[g] Nitrogen-free extracts (NFE) = dry matter - (crude protein + crude lipid + ash + fibre).
[h] Gross energy (MJ/kg) as gross energy content of protein (23.6 MJ/kg), lipid (39.5 MJ/kg) and NFE (17.2 MJ/kg).

2.3. Fish and rearing facilities

European perch juveniles were obtained by artificial propagation (Anapartner, Prague, Czech Republic) and transported in oxygenated 1 m^3 tanks to the Research Institute of Fish Culture and Hydrobiology. Fish underwent two weeks of adaptation to the experimental facility and were fed a commercial diet.

Eighty-two fish (body weight 20.81 ± 3.36 g, total length 11.77 ± 0.72 cm) were randomly assigned to each of sixteen black circular 180 L tanks (four per diet) connected in RAS. Water inflow of 6.5 L/min in combination with stone aeration created a constant clockwise flow of 4.6 cm/s. Other parameters included photoperiod of 12 h:12 h (light: dark), light intensity 58.6 lx, water temperature 22.44 ± 0.66 °C, pH 7.00 ± 0.29, oxygen saturation 80.41 ± 8.02%, ammonia-N 0.28 ± 0.16 mg/L, nitrite nitrogen <0.45 mg/L.

Fish were fed daily at 7.00, 9.00, 11.00, 13.00, and 15.00 using automatic feeders (EHEIM Twins, Deizisau, Germany) for 119 days. Unconsumed feed was removed after each feeding and dried to calculate daily feed intake.

2.4. Swimming experiment

At the conclusion of rearing, evaluation of swimming performance was conducted in a 10 L 40 × 10 × 10 cm swimming tunnel respirometer

(Loligo systems, Tjele, Denmark) submerged in a buffer tank that was connected to an aerated temperature-controlled 100 L reservoir tank allowing continuous water exchange. To ensure adequate dissolved oxygen concentration during swimming performance testing, the swimming chamber was connected to a buffer tank via a flush pump (20 L/min, Eheim GmbH, Deizisau, Germany). Throughout the swimming trial, dissolved oxygen remained above 70%, ensuring sufficient oxygen during swimming test (Hammer, 1995; Tudorache et al., 2008; Thomas and Janz, 2011). Dissolved oxygen and temperature in the swimming chamber were continuously recorded, using a fibreoptic oxygen probe and a temperature probe connected to a Witrox 1 (Loligo Systems, Tjele, Denmark). Water temperature was maintained at 23 ± 0.15 °C and light intensity at ~60 Lx at the surface of the swimming system. Because of the sensitivity of perch to external stressors (Jentoft et al., 2005; Langeland et al., 2016), the system was covered by black plastic sheeting to prevent disturbance from surroundings. The system was connected to AutoResp© software (Loligo systems, Tjele, Denmark) to control and record water velocity and dissolved oxygen in the swimming chamber.

Eighty fish (20 fish/diet group) were used in swimming tests following 24 h without feeding. Fish were selected rotationally among diet treatments as described previously (Wagner et al., 2004) to minimize time differences and any potential additional growth among groups.

Fish were lightly anesthetized with MS 222 (50 mg/L), and body weight was measured to the nearest 0.01 g and total length, width, and depth to the nearest 0.01 cm (Table 2). Individual fish were immediately transferred to the swimming tunnel and acclimated to test conditions for 30 mins with water flow velocity of 5 cm/s, which closely approximated that of the rearing tank.

At the start of the swimming test, the tunnel was completely closed (no water exchange with the surrounding bath). The initial velocity was set at 5 cm/s and increased in 2 cm/s increments every 60 s until fatigue. The small increments of velocity and time in our protocol were set to minimize stress of tested fish. A similar protocol has been conducted by Peterson (1974). The swimming test was terminated when the fish remained at the rear grid for more than 10 s.

Critical swimming speed (U_{crit}, cm/s) was calculated according to Brett (1964):

$U_{crit} = U_{max} + (T_{max}/T_{interval} * U_{interval})$, where U_{max} is highest velocity recorded at fatigue (cm/s); $U_{interval}$ is velocity interval (2 cm/s); T_{max} is spent time at fatigued velocity; and $T_{interval}$ is the time interval (60 s).

Oxygen consumption (MO_2, mg/kg/h) was calculated as.

$MO_2 = ([O_2]_{t0} - [O_2]_{t1}) * (V/t) * (1/BW)$, where $[O_2]_{t0}$ is oxygen concentration at the start of the swimming test (mg O_2/L), $[O_2]_{t1}$ is oxygen concentration at the terminal of the swimming test (fish fatigue) (mg O_2/L); V is volume of swimming chamber minus volume of fish (L). In fish, 1 kg is equivalent to 1 L (Boldsen et al., 2013); t = t1 – t0 (hours); BW, body weight.

Cost of transport (COT, J/kg/m) was calculated as described by McPhee and Janz (2014).

$COT = (MO_2 * 14.2)/U_{swim}$, where oxycaloric value of 14.1 J/mg O_2; U_{swim} is corresponding swimming speed (m/s).

Table 2
Morphometrics (Mean ± SD, $n = 20$/group) of European perch fed experimental diets for 119 days used in the swimming performance test.

Diets	Body weight g	Total length cm	Condition factor*
TM0	84.22 ± 9.85[a]	18.40 ± 0.62[a]	1.35 ± 0.08
TM25	84.82 ± 10.33[a]	18.19 ± 0.72[a]	1.41 ± 0.11
TM50	82.86 ± 10.25[a]	18.03 ± 0.60[a]	1.41 ± 0.10
TM75	70.70 ± 8.01[b]	17.16 ± 0.56[b]	1.40 ± 0.11

Different superscripts within a column indicate significant difference at $P < 0.05$.
*Condition factor = (Body weight/total length^3) × 100.

2.5. Sampling procedures

After a 119-day feeding trial fish not used for swimming tests, hereafter non-exercised fish, and fish experienced swimming tests till fatigue, hereafter exercised fish, were sampled as following procedures:

Twelve non-exercised fish (12 fish/diet group) and exercised fish (20 fish/diet group) were euthanized using an overdose of 125 mg/L (Dezfuli et al., 2013) MS222. Approximate 1 mL of blood was drawn from the caudal vein and centrifuged at 3000 RPM for 10 min with obtained serum was held at −80 °C for further analysis. Subsequently, those fish were dissected for assay of post-mortem pH and water content. Fish muscle was assessed for pH using Testo 206-pH 2 (Testo Inc., Lenzkirch, Germany) inserting into muscle, then scale-off muscle was ground in a mincer (IKA Mill A11 Basic, Staufen, Germany), subsequently oven-dried (105 °C) to constant weight for water content.

Forty-eight non-exercised fish (12 fish/group) were taken and stored at −20 °C for subsequent analysis of whole-body proximate composition.

2.6. Chemical and biochemical analyses

Feed samples were finely ground (IKA Mill A11 Basic, Staufen, Germany) and analyzed for dry matter (AOAC, n. 934.01), crude protein (AOAC, n. 984.13), crude fibre by the Henneberg -Stohmann method (AOAC, n. 920.86), and ash content (AOAC, n. 942.05) according to (AOAC, 2000). Lipid and fatty acid profiles in both diets and whole-body fish were determined according to the methodology described by Mráz and Pickova (2009).

Serum biochemical parameters and blood ions (Na^+, K^+, Cl^-) were analyzed using an auto-analyzer Architect c4000 (Abbott Laboratories, Illinois, USA) with commercial reagent kits (Abbott Diagnostics, Illinois, USA). Cortisol concentration was analyzed using a reagent kit and automated immune-analyzer Immulite 2000 XPi (Siemens Healthineers, Siemens Healthcare GmbH, Erlangen, Germany).

2.7. Statistical analyses

All statistical analyses were performed using the R Statistic Package; R Development Core Team 2009–2019, available at www.r-project.org/. All data were assessed for normality using Shapiro-Wilk test and homogeneity of variance using Levene's test. Effect of fish weight, length, width, depth on critical swimming speed, oxygen consumption, and cost of transport was tested with Covariance Analysis (ANCOVA), and no significant influence [U_{crit}, (weight: *F-statistic* = 0.85, *P-value* = 0.36; length: 0.75, 0.39; width: 0.36, 0.55; depth: 1.49, 0.23); MO_2 (weight: 2.97, 0.09; length: 0.34, 0.94, width: 2.0, 0.16; depth: 1.56, 0.22), COT (weight: 0.77, 0.08; length: 0.75, 0.39; width: 0.54, 0.37; depth: 0.36, 0.87)] was found. Significant differences in swimming speed, oxygen consumption, cost of transport, blood biochemistry, meat traits, diet composition among diet treatments were verified using one-way ANOVA followed Tukey's honestly significant difference (HSD) as a post hoc test, when appropriate. Student's *t*-tests were used to test significant differences in blood biochemistry, muscle traits between exercised and non-exercised groups. Correlation matrix analysis with respect to critical swimming speed was performed on 'ggcorrplot' package (Kassambara, 2019). Correlation between nutritional composition (of diets, fish muscle, and whole-body fish) vs. U_{crit} as well as between MO2, COT vs. U_{crit} were assessed with linear models using 'lm' function. Significantly differences were considered at $P < 0.05$.

3. Results

3.1. Fish growth and proximate composition

After a 119-day feeding trial, fish fed experimental diets had a similar survival rate, ranged 98.48–99.09%. Morphometrics of fish used

in swimming tests is shown in Table 2. European perch fed TM75 showed significantly lower growth performance than other treatment groups ($P < 0.05$). Although fish growth was impaired when *T. molitor* larvae meal was substituted for 75% of fishmeal, condition factor did not differ among dietary treatments ($P > 0.05$). Feed conversion ratio significantly higher in fish fed TM75 diet (1.77) than does TM0 (1.15), TM25 (1.19), and TM50 (1.33) (standard error of the mean, 0.07).

TM contained a high amount of monounsaturated constitute of oleic acid, accounting for 35.10% total fatty acids, while possessed low levels of essential fatty acids, docosahexaenoic acid (DHA) (0.01%) and eicosapentaenoic acid (EPA) (0.04%) (Table 3). Replacement fishmeal by TM significantly increased oleic acid (OA), linoleic acid (LA), and decreased EPA, DHA ($P < 0.05$) (Table 3).

There was no significant difference ($P > 0.05$) on proximate composition of whole-body perch fed experimental diets, except for moisture, palmitic acid, and total saturated fatty acids (Table 4). Feeding perch with 50% fishmeal replaced by TM resulted in significantly higher moisture content compared to the fishmeal group ($P < 0.05$) and significantly reduced C16:0 and total SFA ($P < 0.05$). (See Table 5.)

Water content and pH were not statistically different in both specimen groups across diet treatments ($P > 0.05$) (Table 5).

3.2. Swimming performance

Critical swimming speed, both in cm/s and BL/s, did not differ across treatment groups ($P > 0.05$) (Fig. 1). The values of U_{crit} in the present study were 106.4 cm/s (interquartile range, 97.42–117.23) and 5.94 BL/s (5.43–6.49).

3.3. Oxygen consumption and cost of transport

No significant difference on MO_2 and COT across diet treatments was found ($P > 0.05$). There was a positive quadratic relationship ($P < 0.001$, $F = 36.01$, adjusted R-square $= 0.47$) for oxygen consumption and respected critical swimming speed. In contrast, negative quadratic model ($P < 0.001$, $F = 169.5$, adjusted R-square $= 0.81$) was observed for cost of transport (Fig. 2).

3.4. Physiological responses

Serum biochemistry indices of exercised and non-exercised fish fed experimental diets were found insect meal-dose independent ($P > 0.05$), except serum Cl^- where exercised fish fed TM75 showed significantly higher concentration than does control group ($P < 0.05$) (Fig. 3).

The fatigued swimmers significantly increased serum AST, glucose, and cortisol regardless of dietary treatment compared to non-swimmers ($P < 0.05$). This was also observed for serum K^+ concentration in TM50 ($P < 0.05$). Exercised fish fed TM0 and TM75 also significantly elevated LDH serum activities relative to non-exercised specimens ($P < 0.05$), while this pattern was not significant in TM25 and TM50 groups ($P > 0.05$).

Exercised fish significantly increased muscle water content compared with non-exercised fish fed TM50 ($P < 0.05$).

Table 4
Whole-body composition (% as wet weight) and fatty acid profile (as a percentage of total fatty acid) of European perch *Perca fluviatilis* fed experimental diets for 119 days.

	TM0	TM25	TM50	TM75	SEM
Moisture	66.66^{b}	67.27^{ab}	68.81^{a}	67.92^{ab}	0.27
Crude protein	19.66	19.71	19.82	19.07	0.24
Crude lipid	11.85	11.57	10.85	11.80	0.24
Ash	3.58	3.27	3.42	3.25	0.10
Fatty acid profile					
C16:0	14.43^{ab}	15.86^{a}	14.92^{ab}	13.25^{b}	0.33
C16:1	6.30	7.51	7.26	6.23	0.27
C18:0	1.22	1.20	1.24	0.99	0.04
C18:1n9	44.07	42.10	41.52	39.08	0.92
C18:2n6	14.74	14.56	15.07	15.14	0.11
C18:3n3	3.03	3.26	3.10	3.16	0.22
C20:5n3 (EPA)	1.79	1.74	1.96	1.69	0.07
C22:6n3 (DHA)	6.82	6.27	6.98	5.88	0.28
SFA	19.08^{ab}	20.44^{a}	19.61^{ab}	17.14^{b}	0.46
MUFA	52.24	51.50	50.78	47.18	1.19
PUFA	27.87	27.31	28.61	35.23	1.62
n-3	11.87	11.48	12.23	10.90	0.40
n-6	16.00	15.82	16.39	24.33	1.94
PUFA/SFA	1.46	1.34	1.46	2.22	0.17
n-3/n-6	0.74	0.73	0.75	0.59	0.04

Fatty acids with less than 1% total fatty acids in experimental diets (C10:0, C12:0, C13:0, C14:1, C15:0, C15:1, C17:0, C17:1, C16:3, C18:1n9 trans, C18:1n7, C18:2n6 trans, C18:3n6, C20:0, C21:0, C20:3n6, C20:3n3, C20:4n6, C22:0, C24:0, C24:1n9, C22:5n6) were not presented in the table but included in fatty acids group calculation. Data are means and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly ($P < 0.05$).

Table 3
Fatty acid composition (as a percentage of total fatty acid) of fishmeal, defatted *Tenebrio molitor* larvae meal and experimental diets.

	Fishmeal[a]	*TM*	TM0	TM25	TM50	TM75	SEM
C14:0	7.9	1.30	1.57	1.57	1.49	1.47	0.03
C16:0	23.0	20.68	9.30	9.18	8.95	9.14	0.07
C16:1		2.05	2.04	2.00	1.94	1.98	0.02
C18:0	5.3	9.08	2.68	2.57	2.59	2.69	0.03
C18:1n9 (OA)	8.4	35.10	46.65^{b}	46.07^{ab}	47.27^{ab}	49.77^{a}	0.52
C18:2n6 (LA)	1.1	25.02	17.14^{b}	17.43^{b}	17.80^{b}	18.89^{a}	0.22
C18:3n3 (LNA)	0.2	0.96	6.19	6.07	6.23	4.31	0.51
C20:1n9	0.3	0.26	3.02^{a}	2.92^{a}	2.03^{ab}	0.41^{b}	0.36
C20:5n3 (EPA)	14.1	0.04	2.54^{a}	2.38^{b}	2.15^{c}	2.06^{c}	0.06
C22:6n3 (DHA)	16.1	0.01	4.26^{a}	3.96^{b}	3.43^{c}	3.23^{c}	0.13
SFA	36.1	33.32	15.25	14.96	14.62	14.95	0.12
MUFA	20.6	38.95	52.70	53.06	53.63	54.42	0.35
PUFA	37.3	26.55	31.89	31.51	31.01	29.90	0.45
n-3	34.7	1.05	13.26	12.67	12.08	9.88	0.57
n-6	2.7	25.50	18.63^{b}	18.84^{b}	18.93^{b}	20.01^{a}	0.17
PUFA/SFA	1.03	0.77	1.69	1.70	1.69	1.71	0.04
n-3/n-6	12.9	0.04	0.71	0.67	0.64	0.49	0.03

[a] Barroso et al. (2014) (Proximate composition (% dry matter), crude protein: 73%; ether extract: 8.2%; ash: 18%; nitrogen-free extract: 0.8%). Fatty acids with less than 1% total fatty acids in experimental diets (C10:0, C12:0, C13:0, C14:1, C15:0, C15:1, C17:0, C17:1, C16:3, C18:1n9 trans, C18:1n7, C18:2n6 trans, C18:3n6, C20:0, C21:0, C20:3n6, C20:3n3, C20:4n6, C22:0, C24:0, C24:1n9, C22:5n6) were not presented in the table but included in fatty acids group calculation. LNA: linolenic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Data are means and pooled standard error of the mean (SEM). Means in the same row with different superscripts letters differ significantly ($P < 0.05$).

Table 5
Muscle properties of exercised (n = 48) and non-exercised (n = 48) European perch fed *T. molitor* larvae meal diets. Data are mean ± SD.

Variable	Experimental diets			
	TM0	TM25	TM50	TM75
pH				
Non-exercised	6.74 ± 0.04	6.74 ± 0.06	6.77 ± 0.04	6.80 ± 0.01
Exercised	6.77 ± 0.03	6.71 ± 0.10	6.78 ± 0.01	6.73 ± 0.10
Water content (%)				
Non-exercised	74.44 ± 0.16	74.27 ± 0.36	74.72 ± 0.54[b]	73.96 ± 1.98
Exercised	74.76 ± 0.64	75.53 ± 1.06	75.61 ± 0.59[a]	75.28 ± 0.81

Superscripts indicate significant differences between exercised and non-exercised perch within a diet group ($P < 0.05$).

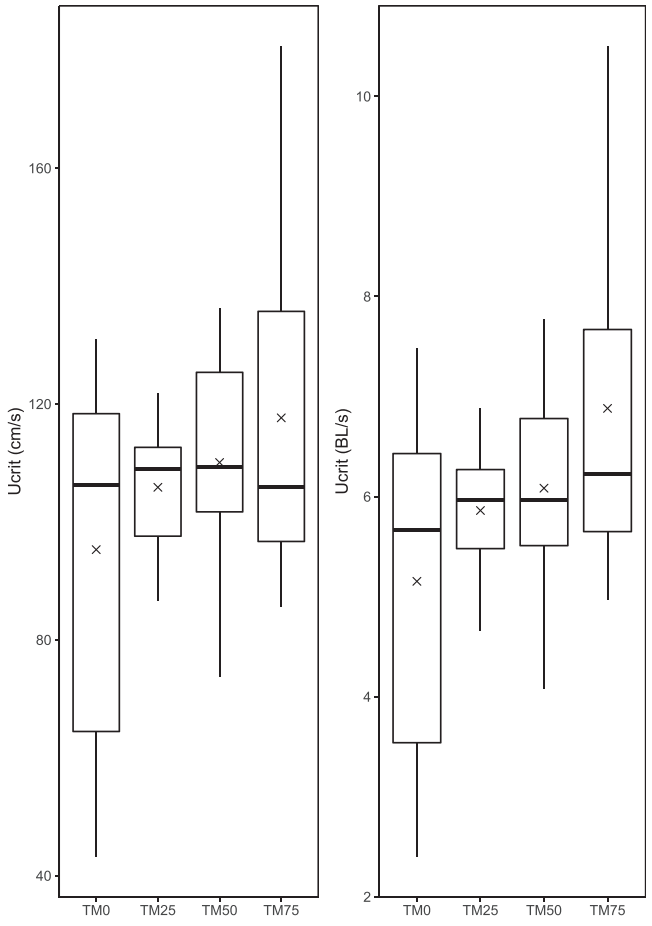

Fig. 1. Critical swimming speed (U_{crit}, cm/s and BL/s) of European perch (*Perca fluviatilis*) fed experimental diets. The black 'x' in boxes represents the mean value, the horizontal line within boxes represents median separating interquartile range (upper quartile and lower quartile).

3.5. Correlation matrix between nutritional factors and critical swimming speed

A significant correlation in both diet and whole-body composition relative to swimming performance was observed (Fig. 4). Three factors that influenced the perch's swimming performance in the present study included DHA, EPA, and MUFA matrixes. There was a negative linear correlation between DHA and U_{crit} (estimated correlation = −0.96, P = 0.04), similar model was found EPA (estimated correlation = −0.96, P = 0.04). While U_{crit} increased linearly with increasing dietary MUFA (estimated correlation = 0.96, P = 0.04). No significant relationship among other dietary composition (protein, lipid, gross energy) on critical swimming speed ($P > 0.05$). We found a significant and strong linear correlation between oleic acid in whole-body fish and swimming capacity (estimated correlation = −0.98, P = 0.02). Other whole-body proximate composition did not significantly influence fish swimming ($P > 0.05$) (Fig. 4).

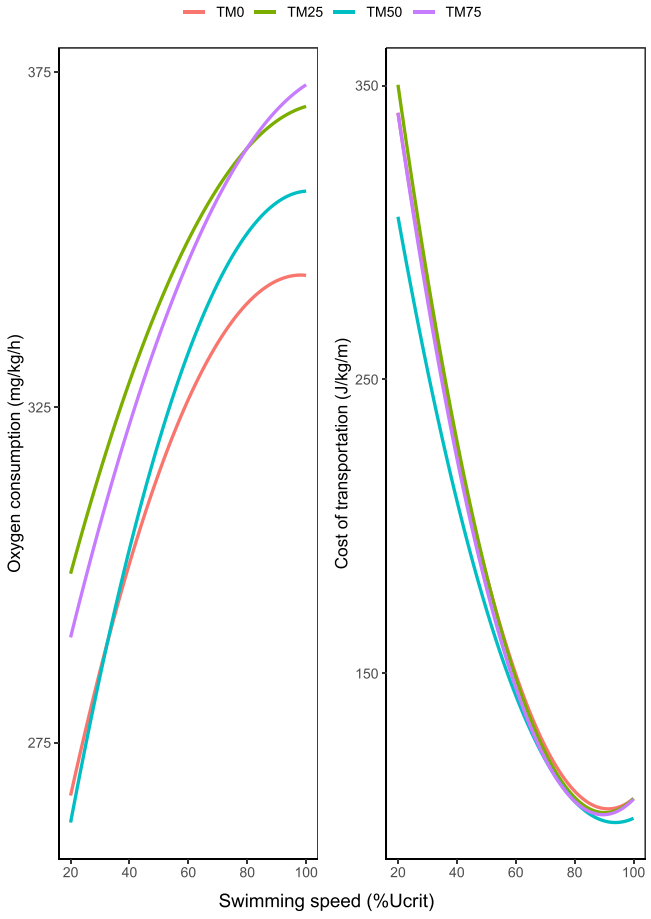

Fig. 2. Oxygen consumption (MO_2, mg/kg/h) and cost of transport (COT, J/kg/h) with respect to critical swimming speed of European perch (*Perca fluvialtilis*) fed experimental diets.

4. Discussion

As a part of many published works investigating the effects of insect meals as the alternative protein source in diets for aquatic animals, our work contributed findings on swimming performance, metabolism rate, cost of transport of European perch, which was insect meal levels independent. This could be an important implication for future percid aquaculture gearing towards established aquaculture sector in Europe (Policar et al., 2019), since swimming performance of fish represents a useful indicator of farmed fish welfare (Martins et al., 2012), the use of insect meals in aquafeeds could benefit future aquaculture industry in term of animal welfare. We also explored insights on dietary and fish composition factors which could influence swimming capacity of fish and found that fatty acids rather than other macronutrients in diets and fish body significantly was the significant influencers.

In accordance with our results, previous studies have confirmed that dietary protein sources did not affect swimming capacity (Wilson et al., 2007; Chai et al., 2013), cost of transport (Wilson et al., 2007), and oxygen consumption (Gerile and Pirhonen, 2017) of fish. Known as a long-distance migrant species, *P. fluviatilis* exhibited high swimming

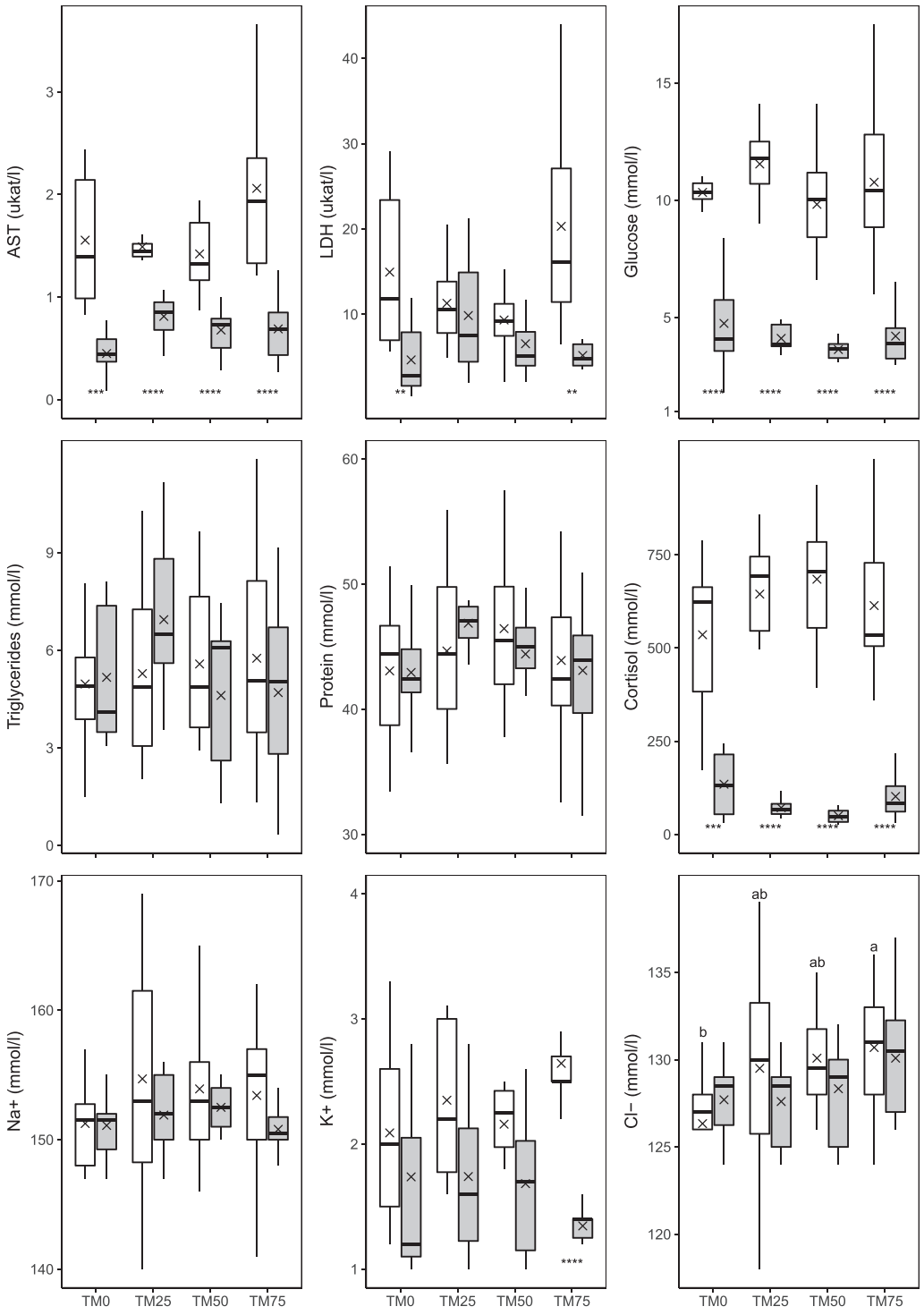

Fig. 3. Serum biochemistry of exercised (white boxplots) and non-exercised (grey boxplots) European perch (*Perca fluviatilis*) fed experimental diets. The black '*' shows the significant difference between exercised and nonexercised fish (Studenr's *t*-test, $^{**}P < 0.001$, $^{***}P < 0.0001$; $^{****}P < 0.00001$). Different lowercase letters denote significant differences in serum biochemistry indices of exercised fish fed experimental diets ($P < 0.01$). Significant differences in serum biochemistry in non-exercised perch were absent. AST = aspartate aminotransferase; LDH = lactate dehydrogenase.

speeds. Tudorache et al. (2008) evaluated the critical swimming speed of 17.8 cm perch and reported a U_{crit} of 113.04 cm/s or 6.35 BL/s. Our findings were also consistent with recent findings (Cano-Barbacil et al., 2020), reporting U_{crit} of 97.7 cm/s or 5.97 BL/s for 16.37 cm length perch, similar to our data (interquartile range, 97.42–117.23 cm/s or 5.43–6.49 BL/s). Stejskal et al. (2009) reported oxygen consumption of European perch (48.3–333.6 g, body weight) at 23 °C was 261.9–279.7 mg O_2/kg/h. Similarly, perch (18.5–56.5 g) kept at the same temperature consumed 150.1–278.5 mg O_2/kg/h (Zakęś et al., 2003). Those were aligned with our oxygen consumption results at 20% U_{crit} (interquartile range, 265.2–302.0 mg/kg/h).

The contrasting trends, positive for MO_2 and negative for COT

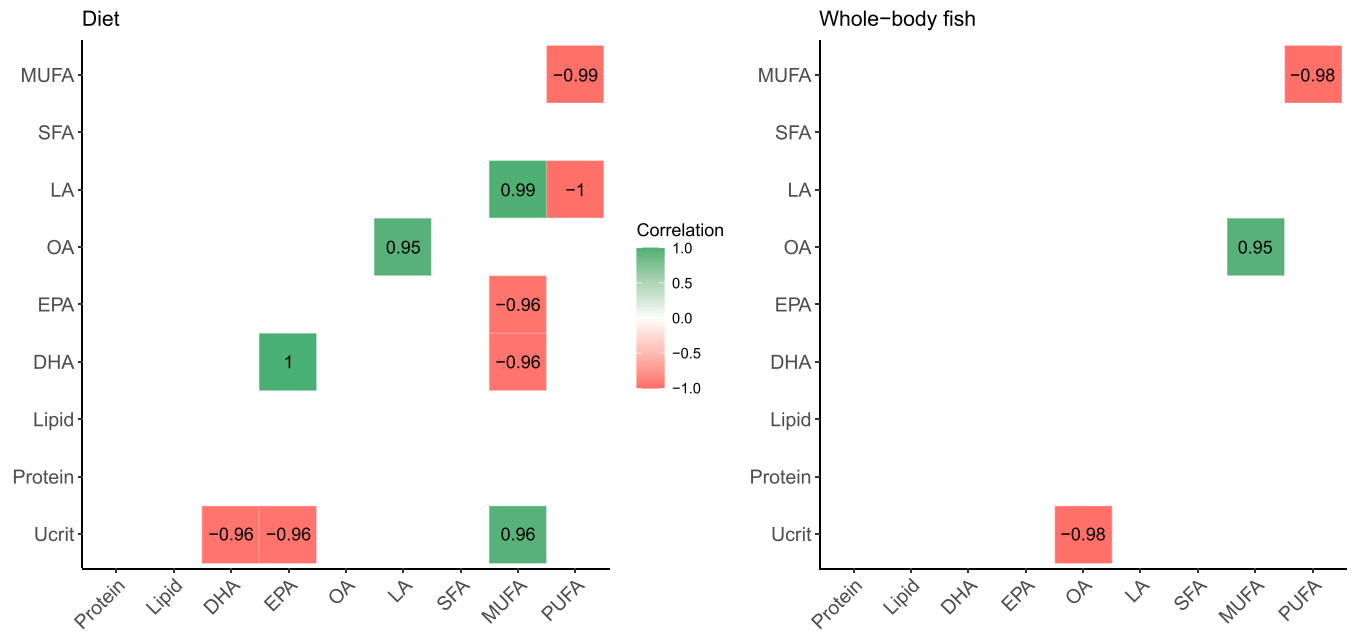

Fig. 4. Correlation matrix between critical swimming speed and nutritional composition of diet, whole-body fish. The red and blue boxes indicate significantly negative and positive correlations, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relative to critical swimming speed, in our study has also been reported earlier in fish species (Brett, 1964; Thomas and Janz, 2011; Yan et al., 2013; McPhee and Janz, 2014; Martos-Sitcha et al., 2018; Rubio-Gracia et al., 2020). In any case, oxygen consumption increased with the velocity acceleration of the swimmer in response to current flow drag (Martos-Sitcha et al., 2018). This demonstrates that perch tended to take up more oxygen when swimming against the higher water flow while using less energy to transport one unit of body mass over one unit of distance. The relationship between U_{crit} and MO_2 in our study was well described by a positive quadratic model, to which oxygen consumption of perch sharply rocketed until reaching approximately 70–80% U_{crit} and then slightly increased till fatigue. This is a common trend for many fishes to experience swimming exercise as there was a shift from aerobic to anaerobic energy use at this critical swimming speed point (Webb, 1971; Moves and West, 1995; Burgetz et al., 1998).

During aerobic swimming, fish generate energy from triglyceride catabolism up to about 80% critical swimming speed, while anaerobic activities initiated, fuelling glycogen from glycogenolysis through glucose, subsequently transited through circulation and distributed to targeted tissues (Hammer, 1995; Moves and West, 1995). Therefore, the measure of triglycerides and glucose in serum could provide a useful explanation of how perch mediates energy during their swimming exercise. We found the concentration of triglycerides in serum was consistent in non-exercised fish as well as in exercised groups, a similar pattern was also observed for glucose. This could explain similar swimming capacity of perch fed dietary insect meal. In addition, the high swimming capacity of fish across diet groups could also be attributed to continuous exposure to mild water velocity of 4.6 cm/s for 119 days of experimental fish, as this form of training had a positive effect on U_{crit} (Hammer, 1995).

At the dietary level, our matrix analysis indicated that critical swimming speed is dietary protein, lipid independent (Fig. 4). The studies on the effect of protein and lipid sources on swimming capacity strongly supported our findings (Wilson et al., 2007; Regan et al., 2010; Gerile and Pirhonen, 2017). We also highlighted that dietary fatty acids, particularly DHA, EPA, and monounsaturated fatty acids, rather than other macro-nutrients, significantly affected European perch swimming performance. This is consistent with a previous study on Atlantic salmon (McKenzie et al., 1998). The negative relationship of dietary n-3 PUFA relative to swimming speed in our study is unexpected and contradicts with the result from Wagner et al. (2004). In fact, diet-mediated alterations in the fatty acid composition of fish were documented to significantly affect physiological performance, including critical swimming capacity in seabass (Chatelier et al., 2006). Fatty acid composition of fish generally mirrors that of diet (Turchini et al., 2009), but the extent to which this occurs depends on the number of factors. The present study, however, found slightly modified DHA, EPA, and some MUFA constitutes of whole-body fish from the respective diet (Tables 3, 4). This was because European perch exhibited a high capacity of biosynthesis of DHA from EPA and C18 precursors (Xu et al., 2001; Xu and Kestemont, 2002). Consequently, a considerable increase in DHA, and a remarked decrease in EPA and linolenic acid in whole-body perch compared to respective diets (Tables 3, 4) were observed. McKenzie et al. (1998) suggested that 18 carbon unsaturated fatty acids were responsible for altering the swimming capacity of salmon. Our correlation analysis indicated that oleic acid strongly influenced exercise activities of European perch, following a negative correlation, which is in agreement with the finding of Wagner et al. (2004). Accordingly, oleic acid may, in combination with other fatty acids, impair carnitine palmitoyl transferase activities - the enzyme enhancing fatty acid metabolism in the red muscle (Wagner et al., 2004).

Fish after exposure to exhaustive exercise or stressors, regulated assortment of physiological changes involving primary, secondary, and tertiary response (Barton, 2002). The primary response was the elevation of cortisol level from the head kidney into blood, while the second response resulted in alteration in blood chemistry, tissue composition, ion concentrations, and increased glucose, and the whole-body organism performance was referred to as the third response (Eissa and Wang, 2016). As expected, exercised perch in our study showed nearly double glucose and ten-fold the cortisol concentration of non-exercised perch (Fig. 3). The previous studies on perch *P. fluviatilis* following acute stress also reported significant elevation of these products (Acerete et al., 2004; Jentoft et al., 2005). Cortisol levels of non-exercised perch in our study (interquartile, 46.9–116.0) were comparable with the 45 ng/mL

(124 mmol/L) reported by Acerete et al. (2004) and relatively higher than the basal level in fish (Barton, 2002). This suggests the high susceptibility of European perch with stressors and low capacity of acclimatization. Increase glucose concentration in blood excreted from the liver is a typical stress response of fish to source fuel energy during exercise (Moves and West, 1995).

Electrolyte Na^+ was consistent across diet treatments and within fish treatment group, while K^+ in fish fed TM75 diet was significantly increased in exercised compared to non-exercised fish. Increased potassium concentration has been recorded in exercised rainbow trout, possibly resulting from loss of K^+ from swimming-involved muscles during depolarization, which replaces K^+ with NH_4^+, and subsequent K^+ take-up by erythrocytes (Nielsen and Lykkeboe, 1992; Wicks et al., 2002). This elevation could also be related to decreased blood pH and oxygen pressure (Cnaani et al., 2014) and osmoregulatory dysfunction (Imanpoor et al., 2017). We also observed significant elevation on Cl^- concentration in exercised groups fed TM75 compared to TM0. This may be due to the absorption of Cl^- during lactate and/or anion in the white muscle (Wood, 1991).

In the present study, the independence of hematological indices on dietary mealworm meal supports previous studies of common carp (*Cyprinus carpio* var. Jian) (total protein, AST, glucose, and triglyceride) (Li et al., 2017), *D. labrax* (glucose, protein, and triglyceride) (Magalhães et al., 2017), *S. scherzeri* (total protein, AST, and triglyceride) (Sankian et al., 2018), rockfish (*Sebastes schlegeli*) (triglyceride, protein, and AST) (Khosravi et al., 2018), and *O. niloticus* (total protein, glucose) (Tubin et al., 2020). Aspartate aminotransferase is a non-plasma-specific enzyme used as a proxy for liver damage (Gharaei et al., 2011), involved in protein synthesis (Masola et al., 2008) and glucose production via gluconeogenesis (Tejpal et al., 2009). We evaluated aminotransferase activity in serum of exercised and non-exercised perch and found fatigued perch fed experimental diets significantly elevated AST activities relative to non-exercised specimens (Fig. 3). The present work also showed no significant effects on hematological glucose or protein concentration of either exercised or non-exercised perch fed diet treatments. Therefore, the source of AST's elevation could be hepatic cell damage, with subsequent release into the blood circulation. Acute exercise induced hepatic injury, thereby increased AST enzyme activity in mammals (Zhao et al., 2010; Ruhee et al., 2020). This, in conjunction with significantly higher lactate dehydrogenase, a biomarker of cell injury (Gharaei et al., 2011), in TM0 and TM75 (Fig. 4), could further confirm the susceptibility of cells of perch fed fishmeal and insect meal diets.

Dietary *T. molitor* meal had no significant effect on muscle water content of non-exercised perch (Table 5), confirming results observed in blackspot sea bream (Iaconisi et al., 2017), mandarin fish (Sankian et al., 2018), rainbow trout (Iaconisi et al., 2018), and rockfish (Khosravi et al., 2018). Similarly, muscle water content of fatigued perch did not differ among experimental diets (Table 5). This is in agreement with Regan et al. (2010), who reported no significant effect of diet on muscle water content of exercised chinook salmon held in freshwater. The significant difference in muscle water content of two groups of perch fed TM50 could be linked to lipid depletion, as they show an inverse relationship (Kause et al., 2002). The muscle pH of non-exercised perch in the present study was similar to that reported by (Komolka et al., 2020) for farmed European perch. Our diets did not affect muscle pH, which is similar to observations of rainbow trout (Iaconisi et al., 2018), but Iaconisi et al. (2017) reported significantly lower pH in the muscle of sea bream fed a diet containing 50% *T. molitor* replacement of fishmeal compared to a fishmeal diet. Basal pH value is species-specific as well as associated with dietary stressors, anaerobic glycolysis, and buffer substances or free amino acid retention in exercised fish (Bugeon et al., 2003; Iaconisi et al., 2017; Komolka et al., 2020).

Our study suggests the potential use of insect meal as an aquafeed ingredient for farmed fish welfare. Future aquaculture of percid fish could consider the high swimming behavior and susceptibility of European perch to stressors for an adequately designed and operated RAS system. Attention should also be paid to diet formulation, especially for the fatty acid profile, which was the critical factor that affects critical swimming speed of European perch.

Authorship statement

Manuscript title: Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*?. All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Aquaculture Journal.

Authorship contributions

Tran Quang Hung: Contribution to design, conduct experiment, analyze of the data, and manuscript preparation.

Vlastimil Stejskal: Conception and the revision of the manuscript.

Hien Van Doan: Contribution to manuscript preparation.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736610.

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