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Zastosowanie inżynierii genomowej i hormonalnego odwracania płci w rozrodzie lipienia europejskiego (*Thymallus thymallus*)

Application of Genome Engineering and Hormonal Sex Reversal in the Reproduction of European Grayling (*Thymallus thymallus*)

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Lista publikacji

Publikacja 1:

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Publikacja 4:

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Streszczenie

Zanieczyszczenia środowiska, silna presja antropogeniczna oraz utrata integralności genetycznej związana z mieszaniem się pul genowych (zanieczyszczenie genetyczne) różnych populacji doprowadziły do znacznego spadku liczebności lipienia europejskiego (*Thymallus thymallus*) w wielu regionach jego naturalnego występowania. Właściwe gospodarowanie tym gatunkiem powinno opierać się na zarybianiu wód materiałem pochodzącym wyłącznie z lokalnych populacji tarlaków, natomiast w sytuacjach, gdy jest to niemożliwe, alternatywą może być wykorzystanie sterylnego, triploidalnego materiału zarybieniowego, który nie będzie krzyżował się z populacjami autochtonicznymi, a jednocześnie pozwoli na utrzymanie wysokiej atrakcyjności łowisk wędkarskich. Niestety, technologia produkcji triploidów u lipienia pozostaje na wczesnym etapie rozwoju. Dlatego też, głównym celem niniejszej rozprawy doktorskiej było określenie wpływu trzech zestawów chromosomów (3n) na rozwój gonad, przeżywalność, tempo wzrostu i atrakcyjność wędkarską lipienia europejskiego oraz próba opracowania warunków produkcji wyłącznie samiczych triploidalnych osobników tego gatunku.

Uzyskane wyniki wykazały, że osobniki triploidalne charakteryzowały się porównywalną do diploidów przeżywalnością. Tempo wzrostu było u triploidów istotnie wyższe jedynie w fazie osiągania dojrzałości płciowej przez diploidalne osobniki. W warunkach wspólnego podchowu osobniki triploidalne wykazywały nieco wyższe tempo wzrostu i lepszą kondycję, jednak różnice te nie były istotne statystycznie. Wyniki połowów wędkarskich wskazały, że osobniki triploidalne utrzymują wysoką atrakcyjność wędkarską i nie odbiegają, a nawet lekko przewyższają pod tym względem diploidalne lipienie. Analiza gonad wykazała, że jajniki triploidalnych samic były silnie zredukowane (tworzyły je pasma tkanki łącznej) i pozbawione gamet, natomiast jądra w przypadku wielu triploidalnych samców miały typowy dla tych gonad wygląd jednak nie zaobserwowano spermacji. Biorąc pod uwagę powyższe, istotnym elementem badań była ambitna próba uzyskania wyłącznie samiczych triploidalnych lipieni. Zastosowanie gynogenezy z wykorzystaniem nasienia pstrąga tęczowego (*Oncorhynchus mykiss*) pozwoliło na uzyskanie potomstwa złożonego wyłącznie z samic (all-female). Niestety, ale pierwsze próby hormonalnej maskulinizacji z zastosowaniem androgenów (OHA i MT) nie doprowadziły do uzyskania w pełni funkcjonalnych neosamców, u części osobników obserwowano jedynie cechy interseksualne.

Podsumowując, produkcja triploidalnego lipienia europejskiego stanowi obiecujące narzędzie biotechnologiczne wspierające ochronę zasobów genetycznych tego gatunku przy jednoczesnym zachowaniu jego wysokiej wartości użytkowej w wędkarstwie rekreacyjnym. Praca ta stanowi podstawę dalszych badań nad rozwojem technologii produkcji triploidalnego, samiczego materiału zarybieniowego lipienia europejskiego.

Słowa kluczowe:
lipień europejski; ochrona integralności genetycznej; triploidyzacja; gynogeneza; maskulinizacja hormonalna.

Abstract

Environmental pollution, intense anthropogenic pressure, and the loss of genetic integrity resulting from the mixing of gene pools (genetic contamination) among different populations have led to a significant decline in the abundance of European grayling (*Thymallus thymallus*) across many regions of its natural distribution. Effective management of this species should be based on stocking programs using fish originating exclusively from local broodstock populations. However, when this is not feasible, the use of sterile triploid stocking material may represent a viable alternative, as such fish are unable to interbreed with native populations while still maintaining the high attractiveness of recreational fisheries. Unfortunately, the technology for producing triploid grayling remains at an early stage of development. Therefore, the main objective of this doctoral dissertation was to determine the effects of possessing three chromosome sets (3n) on gonadal development, survival, growth performance, and angling attractiveness of European grayling, as well as to explore the possibility of developing a method for producing all-female triploid individuals of this species.

The obtained results demonstrated that triploid fish exhibited survival rates comparable to those of diploids. Growth performance was significantly higher in triploids only during the period when diploid individuals reached sexual maturity. Under communal rearing conditions, triploid fish showed slightly higher growth rates and better condition than diploids; however, these differences were not statistically significant. Recreational fishing results indicated that triploid individuals maintained high angling attractiveness and were at least comparable to, and in some cases slightly more attractive than, diploid grayling. Gonadal analyses revealed that the ovaries of triploid females were severely reduced, consisting mainly of connective tissue strands and lacking gametes. In contrast, the testes of many triploid males displayed a typical morphological appearance; however, spermiation was not observed. Given these findings, an important component of the study was the ambitious attempt to produce all-female triploid grayling. The application of gynogenesis using rainbow trout (*Oncorhynchus mykiss*) sperm enabled the production of exclusively all-female offspring. Unfortunately, the initial attempts at hormonal masculinization using androgens (OHA and MT) did not result in the production of fully functional neomales, and intersex characteristics were observed in some individuals.

In conclusion, the production of triploid European grayling represents a promising biotechnological tool for conserving the genetic resources of this species while maintaining its high utility value in recreational fisheries. This dissertation provides a foundation for further research aimed at developing technologies for the production of triploid, all-female stocking material of European grayling.

Keywords: European grayling; genetic integrity conservation; triploidization; gynogenesis; hormonal masculinization.

1. Wstęp

1.1. Zanieczyszczenia środowiska

Zanieczyszczenie środowiska stanowi jedno z kluczowych wyzwań współczesnej cywilizacji, będąc konsekwencją rosnącej antropopresji na zasoby naturalne (Rockström i in., 2009; Vörösmarty i in., 2010; Díaz i in., 2019; UNEP, 2021). Degradacja ekosystemów, wynikająca z intensywnej działalności antropogenicznej, polega głównie na wprowadzaniu do środowiska zanieczyszczeń chemicznych, fizycznych i biologicznych. Zanieczyszczenia te mogą mieć zarówno charakter punktowy, jak i rozproszony, a ich wpływ na środowisko ma często charakter długotrwały. Ponadto interakcje między różnymi typami zanieczyszczeń mogą prowadzić do efektów synergicznych, które nasilają ich negatywny wpływ na środowisko (Vörösmarty i in., 2010; Fuller i in., 2022). Szczególne znaczenie w degradacji ekosystemów wodnych odgrywają zanieczyszczenia biologiczne, ze względu na trudności w ich wykrywaniu i kontroli oraz nieprzewidywalność ich skutków środowiskowych. Zanieczyszczenia biologiczne obejmują żywe organizmy oraz ich formy przetrwalnikowe, takie jak bakterie, wirusy, grzyby, pierwotniaki, pasożyty oraz inne organizmy makroskopowe, wprowadzane do środowiska wodnego głównie w wyniku działalności antropogenicznej, które mogą powodować negatywne skutki sanitarne i ekologiczne (Ashbolt, 2004; Peeler i in., 2011).

1.2. Zanieczyszczenia genetyczne i ich przykłady

W grupie zanieczyszczeń biologicznych, na szczególną uwagę zasługują zanieczyszczenia genetyczne, definiowane jako obecność i rozprzestrzenianie się obcego materiału genetycznego w środowisku naturalnym. Obecnie ten typ zanieczyszczeń uznawany za jedno z największych zagrożeń dla środowiska wodnego wynikającego z działalności człowieka (Karlsson i in., 2016; Naylor i in., 2021). W przeciwieństwie do klasycznych form zanieczyszczeń biologicznych zanieczyszczenia genetyczne zazwyczaj pozostają trudne do wykrycia, a ich skutki ujawniają się dopiero w dłuższej perspektywie czasu oraz mają często charakter nieodwracalny, ponieważ prowadzą do trwałych zmian w pulach genowych populacji (Laikre i in., 2010; Frankham i in., 2017). W ekosystemach wodnych zanieczyszczenia genetyczne stanowią szczególnie istotny problem wynikający z wymuszonego przez zmiany klimatyczne niekontrolowanego rozprzestrzeniania się materiału genetycznego oraz mniej lub bardziej celowego wprowadzania do środowiska

naturalnego obcego materiału genetycznego na przykład w wyniku ucieczek ryb i bezkręgowców wodnych z obiektów produkcji akwakultury (Rhymer i Simberloff, 1996; Jonsson i Jonsson, 2006) lub zarybień wód otwartych (Rhymer i Simberloff, 1996; Laikre i in., 2010).

Głównym mechanizmem zanieczyszczenia genetycznego jest introgresja czyli proces trwałego włączania materiału genetycznego jednego gatunku lub populacji do puli genowej innego gatunku lub populacji, zachodzącym w wyniku hybrydyzacji oraz kolejnych krzyżowań wstecznych mieszańców z osobnikami form rodzicielskich (Martin i Jiggins, 2017; Hibbins i Hahn, 2019). Wykazano, że napływ genów pochodzących od ryb hodowlanych może prowadzić do utraty lokalnych adaptacji środowiskowych dzikich populacji (McGinnity i in., 2003; Bolstad i in., 2017). Przypadki zanieczyszczenia genetycznego wynikającego z introgresji obserwowano u wielu gatunków ryb łososiowatych na świecie w ostatnich kilkudziesięciu latach, m.in. pstrąga potokowego (*Salmo trutta m. fario*), pstrąga tęczowego (*Oncorhynchus mykiss*), łososia clarka (*Oncorhynchus clarkii*), a także u palii (*Salvelinus alpinus*) oraz innych gatunków z rodzaju *Salvelinus* spp. Zjawiska te obejmują zarówno wymieszanie genów w obrębie jednego gatunku (mieszańce wewnątrzgatunkowe), jak i hybrydyzację międzygatunkową, często prowadzącą do powstawania krzyżówek o obniżonej żywotności, ograniczonej płodności lub braku zdolności do przetrwania w środowisku (Allendorf i in., 2001; Muhlfeld i in., 2009; Todesco i in., 2016). Metodą, która sprzyja mieszaniu się pul genowych różnych populacji jest zarybianie wód materiałem uzyskanym od tarlaków z innych czasem odległych geograficznie i genetycznie populacji czy też ryb z udomowionych stad (Hindar i in., 1991; Sušnik i in., 2004; Hansen i Mensberg, 2009; Araki i Schmid, 2010). Ryby udomowione i dziko żyjące tego samego gatunku często różnią się istotnie pod względem genetycznym, fenotypowym i fizjologicznym, co jest bezpośrednim efektem procesu udomowienia polegającego na wielopokoleniowej selekcji w warunkach hodowlanych (Araki i Schmid, 2010; Lorenzen i in., 2012; Teletchea, 2015). Krzyżowanie osobników hodowlanych z rybami z dzikich populacji prowadzi do trwałej modyfikacji ich puli genowej, obniżając zdolność adaptacji do lokalnych warunków środowiskowych, co jest obecnie uznawane za jedno z największych zagrożeń dla przetrwania autochtonicznych populacji ryb (Allendorf i in., 2001; McGinnity i in., 2003; Frankham, 2015). Problem ten dotyczy w szczególności

gatunków ekologicznie wrażliwych, takich jak ryby łososiowate, których ochrona często wymaga prowadzenia działań zarybieniowych.

Dynamiczny rozwój akwakultury na świecie wiąże się ze wzrostem ryzyka niekontrolowanego przedostawania się organizmów hodowlanych do środowiska naturalnego, co może prowadzić do zaburzeń ekologicznych oraz genetycznego zanieczyszczenia dzikich populacji. Do najbardziej znanych przykładów zanieczyszczeń genetycznych w ekosystemach wodnych należą przypadki krzyżowania się dzikich populacji łososia atlantyckiego z osobnikami pochodzącymi z hodowli morskich, będące następstwem masowych ucieczek ryb z farm akwakultury w Norwegii, Szkocji i Kanadzie (Naylor i in., 2005; Glover i in., 2017). Szacuje się, że w samej Norwegii w latach 2000–2015 liczba hodowlanych łososi atlantyckich uciekających corocznie z farm akwakultury wynosiła od kilkuset tysięcy do nawet kilku milionów osobników (Glover i in., 2012; Glover i in., 2017). Udokumentowano, że proces ten prowadzi do włączania alleli hodowlanych łososi do pul genowych dziko-żyjących osobników czego konsekwencją bywa obniżenie przystosowania ryb z dzikich populacji do lokalnych warunków środowiskowych oraz zmniejszenia ich różnorodności genetycznej (Glover i in., 2013; Karlsson i in., 2016; Glover i in., 2017; Bolstad i in., 2017). W Norwegii potwierdzono udział genów pochodzących od ryb hodowlanych w 51 spośród 109 badanych populacjach dzikiego łososia atlantyckiego, przy czym w części rzek udział genów pochodzenia hodowlanego przekraczał 40% (Glover i in., 2013; Karlsson i in., 2016). Incydenty ucieczek ryb z hodowli odnotowuje się także w Polsce. W wielu rzekach Pomorza stwierdzono obecność pstrągów tęczowych i źródlanych pochodzących z hodowli (Witkowski i Kotusz, 2008). W 2017 roku na Pomorzu, w rejonie Darłowa, doszło do masowej ucieczki z obiektów hodowlanych do rzeki Wieprzy i dalej do Morza Bałtyckiego około 50 tysięcy obcych dla lokalnej fauny jesiotrów syberyjskich (*Acipenser baerii*) oraz rosyjskich (*Acipenser gueldenstaedtii*), co wzbudziło obawy dotyczące potencjalnej hybrydyzacji z restytuowaną populacją rodzimego jesiotra ostronosego (*Acipenser oxyrinchus*) (Arciszewski i Skóra, 2017).

W Polsce najbardziej znanym przykładem zanieczyszczenia genetycznego jest introdukcja pelugi (*Coregonus peled*) w latach 80. XX wieku, w wyniku której doszło do trwałego zanieczyszczenia puli genowej rodzimych populacji siei (*Coregonus* spp.) w licznych jeziorach północnej Polski (Mamcarz, 1992; Popović i in., 2016). Istotnym problemem pozostają również wieloletnie zarybienia materiałem hodowlanym ryb

łososiowatych, w tym troci wędrownej (*Salmo trutta m. trutta*) oraz pstrąga potokowego, prowadzące do osłabienia lokalnego przystosowania populacji dzikich oraz zaniku naturalnej struktury genetycznej stad (Wenne i in., 2016; Bernaś i Wąs-Barcz., 2020). Podobne zjawiska obserwowano również w Hiszpanii, gdzie intensywne zarybienia hodowlanym materiałem pstrąga potokowego doprowadziły do szeroko rozpowszechnionej introgresji oraz znaczącego ograniczenia unikalnej zmienności genetycznej rodzimych populacji iberyjskich, co uznawane jest za jeden z najbardziej wyraźnych przykładów negatywnych skutków genetycznych niekontrolowanych zarybień w Europie (Aparicio i in., 2005; Almodóvar i in., 2006).

1.3. Lipień europejski jako gatunek zagrożony

Lipień europejski (*Thymallus thymallus* Linnaeus, 1758; Actinopterygii: Salmoniformes: Salmonidae: Thymallinae) jest ważnym pod względem ekologicznym oraz rekreacyjnym przedstawicielem ryb łososiowatych zamieszkującym wody Europy (Northcote, 1995; Persat, 1996; Koskinen i in., 2001; Sušnik i in., 2004; Weiss i in., 2013). Gatunek ten jest bioindykatorem jakości wód płynących, wykazując wysoką wrażliwość na podwyższoną temperaturę wody, niedobór tlenu oraz obecność zanieczyszczeń (Sušnik i in., 2004; Turek i in., 2010; Marić i in., 2012; Weiss i in., 2013; Hayes i in., 2021; Vom Berge i in., 2025). Jednocześnie lipień należy do najbardziej pożądanych gatunków ryb przez wędkarzy muchowych, przekładając się na istotne korzyści ekonomiczne dla wielu regionów Europy (Cove i in., 2018; Lyach i Remr, 2019, 2020). Niestety, w ostatnich dekadach lokalne populacje lipienia doświadczyły znacznego spadku liczebności i ograniczenia zasięgu występowania wskutek degradacji siedlisk, regulacji hydrotechnicznych, silnej presji drapieżników oraz nadmiernej eksploatacji wędkarskiej (Northcote, 1995; Persat, 1996; Koskinen i in., 2001; Sušnik i in., 2004; Marić i in., 2012; Weiss i in., 2013). W wielu regionach Europy gatunek ten uznaje się obecnie za zagrożony wyginięciem (HELCOM, 2013; IUCN, 2024).

1.4. Zanieczyszczenie genetyczne populacji lipienia europejskiego - konsekwencje i przeciwdziałanie

Obecnie, jednym z największych wyzwań w ochronie lipienia jest utrata integralności genetycznej jego dzikich populacji, wynikająca z masowo prowadzonych zarybień przy użyciu materiału pochodzącego z losowo wybranych źródeł, często ze stad

hodowlanych utrzymywanych w warunkach kontrolowanych przez wiele pokoleń (Araki i Schmid, 2010; Dawnay i in., 2011; Meraner i in., 2013). W efekcie wiele dzikich populacji lipienia doświadczyło wymieszania pul genowych z rybami pochodzącymi z allochtonicznych stad lub populacji, co spowodowało pogorszenie ich zdolności adaptacyjnych do lokalnych warunków środowiskowych (Koskinen i in., 2002; Sušnik i in., 2004; Duftner i in., 2005). W skrajnych przypadkach zjawisko to doprowadziło do całkowitego zaniku lokalnych populacji tego gatunku w wielu regionach Europy (Avise i Hamrick, 1996; Turek i in., 2010; Huff i in., 2011). Jeżeli lipień europejski ma uniknąć losu wielu populacji pstrąga potokowego i troci wędrownej, konieczne jest prowadzenie kompleksowych badań nad strukturą genetyczną lokalnych populacji, tworzenie banków genów oraz opracowanie zasad produkcji bezpiecznego materiału zarybieniowego. Działania te mogą umożliwić odbudowę liczebności populacji lipienia w wodach Polski przy jednoczesnym ograniczeniu ryzyka dalszego zacierania lokalnych różnic genetycznych i utraty naturalnej zmienności populacyjnej. Z tego powodu, obserwuje się w ostatnich latach rosnące zainteresowanie wykorzystaniem narzędzi biotechnologicznych w celu produkcji materiału zarybieniowego, którego wprowadzenie do środowiska nie wiązałoby się z ryzykiem zanieczyszczenia genetycznego dzikich populacji (Piferrer i in., 2009). Materiał taki powinien wykazywać zdolność do przeżycia, wzrostu i funkcjonowania w warunkach naturalnych, przy jednoczesnym braku zdolności do efektywnego rozmnażania się. Takimi cechami charakteryzują się uzyskane w warunkach kontrolowanych osobniki triploidalne, które uznaje się za funkcjonalnie sterylne (Piferrer i in., 2009).

1.5. Proces spontanicznej triploidyzacji i występowanie triploidalnych ryb w naturze

U niektórych gatunków ryb triploidyzacja stanowi naturalny element ich biologii. Do tej pory naturalne triploidy opisano przede wszystkim u niektórych przedstawicieli ryb rodzaju *Carassius* oraz u piskorza amurskiego (*Misgurnus anguillicaudatus)*, u których w obrębie populacji stwierdzono współwystępowanie różnych cytotypów czyli osobników różniących się liczbą zestawów chromosomów w komórkach, obejmujących formy diploidalne, triploidalne i tetraploidalne (Zhang i Arai, 1999; Nam i in., 2004; Gui i Zhou, 2010). U piskorza amurskiego opisano również populacje, których osobniki, produkują zarówno haploidalne jak i diploidalne gamety, co często prowadzi do powstawania triploidalnego potomstwa (Zhang i Arai, 1999; Gui i Zhou, 2010). U ryb triploidy spontanicznie mogą powstawać zarówno w obrębie jednego gatunku, jak

i w wyniku hybrydyzacji międzygatunkowej (Zhang i Arai, 1999; Piferrer i in., 2009). Triploidalne osobniki hybrydowe obserwowano między innymi u mieszańców *Cobitis elongatoides* i *Cobitis taenia* oraz pomiędzy *Cobitis elongatoides* i *Cobitis tanaitica* (Janko i in., 2007). Pojedyncze spontanicznie powstałe osobniki triploidalne opisano u wielu gatunków ryb, takich jak na przykład: *Hesperoleucus symmetricus* (Gold i Avise, 1976), rekin piaskowy (*Ginglymostoma cirratum*) (Kendall i in., 1994). Także wśród ryb łososiowatych triploidalne osobniki obserwowane są zarówno w populacjach dzikich, jak i w stadach hodowlanych (Thorgaard i Gall, 1979; Aegerter i Jalabert, 2004; Ocalewicz i Dobosz, 2009). Wśród ponad 4 000 łososi atlantyckich pochodzących z 55 norweskich farm około 2% osobników stanowiły spontaniczne triploidy (Glover i in., 2015). Badania nad spontaniczną triploidyzacją wykazały, że wzrost częstości występowania osobników triploidalnych jest powiązany z obniżoną jakością ziaren ikry wykorzystanej do zapłodnienia (Aegerter i Jalabert, 2004; Flajšhans i in., 2007).

1.6. Indukcja triploidalnego rozwoju w warunkach kontrolowanych

Ryby triploidalne można także uzyskać w warunkach kontrolowanych (Thorgaard, 1986; Pandian i Koteeswaran, 1998; Piferrer i in., 2009). Najpowszechniejszą metodą jest ekspozycja zapłodnionej ikry tuż po aktywacji na działanie udaru środowiskowego (szoku fizycznego lub chemicznego) (Thorgaard, 1986; Pandian i Koteeswaran, 1998). Kolchicyna, subletalne temperatury lub wysokie ciśnienie hydrostatyczne destabilizują mikrotubule wrzeciona kariokinetycznego, co uniemożliwia zakończenie drugiego podziału mejotycznego oocytu i wyrzucenie z oocytu drugiego ciałka kierunkowego zawierającego haploidalny zestaw chromosomów. W wyniku takiego działania, w jajku znajdują się dwa zestawy matczynych chromosomów (2n) i jeden zestaw chromosomów ojcowskich (1n) (Piferrer i in., 2009). Triploidalne osobniki można uzyskać także poprzez krzyżowanie tetraploidalnych samic z diploidalnymi samcami lub rzadziej poprzez polispermię, czyli zapłodnienia pojedynczej komórki jajowej przez więcej niż jeden plemnik, przy czym metody te różnią się skutecznością i mają głównie zastosowanie eksperymentalne (Chourrout, 1984; Ueda i in., 1986; Blanc i in., 1987; Meyer i Hershberger, 1991; Arai i Fujimoto, 2013; Zhou i Gui, 2017).

Obecność dodatkowego zestawu chromosomów u ryb triploidalnych prowadzi do istotnych zaburzeń rozwoju i funkcjonowania układu rozrodczego, które u samic objawiają się znaczącym zahamowaniem rozwoju jajników mających u dorosłych ryb

zazwyczaj postać pasm tkanki łącznej (Benfey, 1999; Tiwary i in., 2004; Benfey, 2016). W tak zbudowanych jajnikach w wielu przypadkach nie stwierdzono występowania komórek rozrodczych, natomiast w pozostałych obserwowano jedynie nieliczne oogonia i niedojrzałe oocyty (Benfey, 1999; Piferrer i in., 2009). Natomiast, u triploidalnych samców obserwuje się rozwój asymetrycznych jąder o rozmiarach zbliżonych do gonad osobników diploidalnych, produkujących ograniczoną liczbę aneuploidalnych plemników na różnych etapach dojrzewania, cechujących się ograniczoną zdolnością zapładniającą (Benfey, 1999; Maxime, 2008; Lahnsteiner i Dünser, 2024).

1.7. Ryby triploidalne w akwakulturze komercyjnej

Ze względu na istotnie ograniczoną płodność, triploidy stały się obiektem komercyjnej produkcji w akwakulturze. Hodowla takich ryb pozwala na wyeliminowanie negatywnych skutków dojrzewania płciowego, które w przypadku ryb diploidalnych wiąże się z redystrybucją energii metabolicznej w kierunku rozwoju gonad i procesów gametogenezy kosztem wzrostu somatycznego. W konsekwencji ryby diploidalne w okresie około tarłowym cechują się spowolnionym tempem wzrostu, niższą przeżywalnością oraz obserwuje się u nich pogorszenie jakości surowca mięsnego (Ihssen i in., 1990; Pandian i Koteeswaran, 1998; Tiwary i in., 2004; Piferrer i in., 2009). Najbardziej znanymi przykładami triploidów komercyjnie produkowanych w akwakulturze są łosoś atlantycki (*Salmo salar*) oraz pstrąg tęczowy (Piferrer i in., 2009; Fraser i in., 2012; Benfey, 2016).

1.8. Ryby triploidalne w akwakulturze zachowawczej i ich znaczenie wędkarskie

Ryby triploidalne są powszechnie uznawane za bezpieczny materiał zarybieniowy, umożliwiający intensyfikację wędkarstwa rekreacyjnego bez ryzyka trwałej ingerencji w pulę genową dzikich populacji (Piferrer i in., 2009). Strategia ta jest spotykaną praktyką w zarządzaniu wodami otwartymi na świecie, m.in. w USA, Kanadzie, Wielkiej Brytanii czy Francji (Benfey, 1999; Dillon i in., 2000; Piferrer i in., 2009). W Stanach Zjednoczonych triploidalne ryby karpiowate, takie jak amur biały (*Ctenopharyngodon idella*), są wykorzystywane do biologicznego zwalczania roślinności wodnej; ich sterylność gwarantuje, że nie staną się gatunkiem inwazyjnym i nie rozmnożą się niekontrolowanie w przypadku ucieczki (Allen i Wattendorf, 1987; Pauley i in., 1988; Piferrer i in., 2009). Ze względu na potencjalne zagrożenie ekologiczne związane z introdukcją płodnych osobników, w wielu stanach USA do zarybień dopuszczane są

wyłącznie certyfikowane ryby triploidalne. Weryfikacja ich ploidalności prowadzona jest w ramach federalnego programu National Triploid Grass Carp Inspection and Certification Program, nadzorowanego przez U.S. Fish and Wildlife Service, którego celem jest eliminacja ryzyka wprowadzania do środowiska płodnych osobników diploidalnych (Zajicek i in., 2011; USFWS, 2026). W przypadku ryb łososiowatych agencje stanowe w Idaho od 2001 r. nakazują stosowanie do zarybień wyłącznie sterylnych pstrągów tęczowych w celu ochrony lokalnych populacji przed introgresją genetyczną (Teuscher i in., 2003; Koenig i in., 2011). Kluczowym przykładem wykorzystania triploidów do zarybień, jest program Brytyjskiej Agencji Środowiska (Environmental Agency), która wdrożyła zastępowanie diploidalnego materiału pstrąga potokowego triploidalnym (Pawson, 2003; Piferrer i in., 2009). Triploidalny Pstrąg potokowy w rzekach Wielkiej Brytanii wykazuje cechy szczególnie pożądane: ryby te nie podejmują wędrówek tarłowych, pozostając na swoich stanowiskach żerowania, co zapobiega niszczeniu tarlisk przez ryby hodowlane (Piferrer i in., 2009). Brak wydatku energetycznego na rozwój gonad powoduje, że osobniki te zachowują lepszą kondycję, atrakcyjniejsze ubarwienie oraz wyższą jakość mięsa przez cały rok, co czyni je atrakcyjnymi dla wędkarzy także zimą i wiosną (Piferrer i in., 2009). Badania wykazały, że ich łowność jest porównywalna z łownością ryb diploidalnych, a pod koniec sezonu komercyjnego może być nawet wyższa (Pawson, 1991; Piferrer i in., 2009). Podobne korzyści z wpuszczania triploidalnego materiału zarybieniowego odnotowano w jeziorach wysokogórskich Idaho, gdzie wyłącznie samicze populacje triploidalnego pstrąga tęczowego osiągają wyższą przeżywalność i wyższą łowność, stanowiąc efektywne narzędzie zarządzania łowiskami rekreacyjnymi (Koenig i in., 2011; Teuscher i in., 2003). Opisane powyżej cechy behawioralne i użytkowe triploidów dowodzą, że zarybienia triploidami skutecznie łączą ochronę integralności genetycznej lokalnych ekotypów z wysoką wartością sportową łowisk (Dillon i in., 2000; Piferrer i in., 2009).

Łowność oraz waleczność ryb podczas holu są istotnymi cechami wartości użytkowej materiału zarybieniowego, ponieważ determinują efektywność zarybień w wodach otwartych, wpływając na atrakcyjność rekreacyjną łowisk (Miko i in., 1995; Yule i in., 2000; Haddix i Budy, 2005; Losee i Phillips, 2017; Harmon i in., 2018). W kontekście wykorzystania osobników triploidalnych, które charakteryzują się sterylnością i ograniczonym ryzykiem oddziaływania genetycznego na populacje rodzime, ich przydatność wędkarska budzi rosnące zainteresowanie, jednak dane

dotyczące porównań łowności z formami diploidalnymi są niedostępne dla wielu gatunków (Brock i in., 1994; Simon i in., 2011; Pease i in., 2023). W odniesieniu do sukcesu wędkarskiego, badania nad pstrągiem tęczowym w wodach otwartych najczęściej wskazują na niższą ogólną łowność (catchability) mieszanych płciowo populacji triploidalnych w porównaniu do diploidalnych (Brock i in., 1994; Rutz i Baer, 1996; Dillon i in., 2000; Koenig i in., 2011; Pease i in., 2023). Co istotne, wyższą skumulowaną łowność oraz lepsze tempo wzrostu obserwuje się u triploidalnych populacji składających się wyłącznie z samic (all-female), co wiąże się z ich mniejszą podatnością na drapieżnictwo i brakiem migracji związanych z tarłem (Josephson i in., 2001; Solomon, 2003; Pease i in., 2023). Oprócz łowności również tempo wzrostu oraz osiągane rozmiary ciała należą do najważniejszych cech użytkowych ryb wykorzystywanych w zarybieniach rekreacyjnych, ponieważ w istotnym stopniu wpływają na atrakcyjność wędkarską łowisk, satysfakcję wędkarzy oraz efektywność gospodarki rybacko-wędkarskiej. Choć teoretycznie ryby triploidalne powinny wykazywać szybsze tempo wzrostu i osiągać większe rozmiary końcowe ze względu na brak inwestycji energii w rozwój gonad (Benfey, 1999; Maxime, 2008), dostępne obserwacje wskazują, że w praktyce ich wskaźniki wzrostu są często zbliżone lub nawet niższe niż u diploidów (Quillet i Gaignon, 1990; Ojolick i in., 1995; Simon i in., 2011). Wyższe tempo wzrostu triploidów u gatunków takich jak pstrąg tęczowy, pstrąg źródlany czy łosoś atlantycki ujawnia się zazwyczaj dopiero w czasie osiągania dojrzałości płciowej przez ich diploidalnych rówieśników (Boulanger, 1991; Galbreath i in., 1994; Sheehan i in., 1999; Oppedal i in., 2003; Teuscher i in., 2003). Niemniej jednak wyżej opisane korzyści z wpuszczania triploidalnego materiału często zanikają, gdy ryby różniące się liczbą zestawów chromosomów są hodowane razem lub wypuszczane razem do środowiska naturalnego, co może wynikać z mniej agresywnego zachowania triploidów podczas żerowania (Carter i in., 1994; McCarthy i in., 1996; Garner i in., 2008; Koenig i in., 2011).

1.9. Produkcja triploidalnego samiczego materiału zarybieniowego

Biorąc pod uwagę nieliczne doniesienia dotyczące produkcji nasienia przez triploidalne samce ryb łososiowatych, jedynym sposobem uzyskania bezpiecznego materiału zarybieniowego jest produkcja wyłącznie triploidalnych samic. Proces ten jest wieloetapowy i obejmuje: 1) opracowanie skutecznych warunków triploidyzacji; 2) uzyskanie diploidalnej linii all-female (gynogeneza); 3) produkcję neosamców;

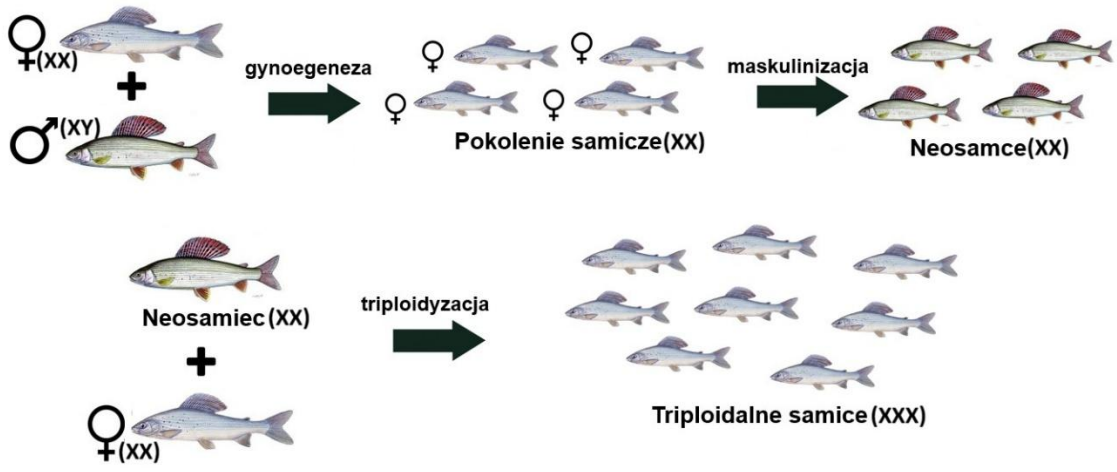
4) wykorzystanie neosamców do krzyżowań prowadzących do uzyskania triploidalnego materiału all-female (Pandian i Koteeswaran, 1998; Sheehan i in., 1999; Piferrer, 2001).

Gynogeneza jest formą rozmnażania, w której potomstwo dziedziczy wyłącznie genom matki (Jaylet i Ferrier, 1978; Chourrout, 1986; Polonis i in., 2018). Zjawisko to występuje naturalnie m.in. u amazonki (*Poecilia formosa)* oraz karasia srebrzystego (*Carassius auratus gibelio)* (Schlupp, 2005; Gui i Zhou, 2010). Może być również indukowane sztucznie poprzez aktywację jaj ryb nasieniem, którego materiał genetyczny jest inaktywowany promieniowaniem UV. Do aktywacji ikry można wykorzystać zarówno nasienie homologiczne, jak i heterologiczne. Powstała po aktywacji jaj naświetlonym nasieniem zygota jest haploidalna i umiera najczęściej jeszcze przez wykluciem. Ekspozycja na udar środowiskowy, czyli tak jak w przypadku indukowanej triploidyzacji powoduje zatrzymanie w jaju drugiego ciałka kierunkowego i w konsekwencji diploidyzację gynogenetycznej zygoty (Pandian i Koteeswaran, 1998). W przypadku gatunków o systemie determinacji płci typu XX/XY, do których należą ryby łososiowate, potomstwo uzyskane w wyniku gynogenezy posiada wyłącznie chromosomy płci pochodzące od samicy (XX), co umożliwia uzyskanie populacji złożonej wyłącznie z samic (all-female) (Devlin i Nagahama, 2002; Yano i in., 2013). Właściwość ta stanowi podstawę wykorzystania gynogenezy w programach hodowlanych ukierunkowanych na produkcję żeńskich linii hodowlanych oraz triploidalnego materiału all-female.

Kolejnym etapem produkcji triploidalnych samic jest hormonalna maskulinizacja (odwrócenie płci) gynogenetycznych samic (XX) prowadząca do uzyskania funkcjonalnych neosamców zdolnych do produkcji nasienia. W przypadku ryb łososiowatych, produkcja neosamców polega na zastosowaniu głównie 11β-hydroksyandrostendion (OHA) oraz 17α-metylotestosteron (MT) podawanych drogą immersji lub pokarmową we wczesnych etapach różnicowania gonad (Feist i in., 1995; Piferrer, 2001; Devlin i Nagahama, 2002). MT jest syntetycznym androgenem naśladującym działanie testosteronu i stymulującym różnicowanie jąder podczas gonadogenezy (Baker i in., 1988; Pandian i Sheela, 1995; Devlin i Nagahama, 2002). Natomiast OHA, jako naturalny metabolit testosteronu i prekursor androgenów, może ulegać konwersji do biologicznie aktywnych form przy udziale enzymów steroidogenezy, indukując różnicowanie się gonad w kierunku samczym (Baroiller i in., 1999; Sharma i in., 2022; Schiffer i in., 2024).

Integracja biotechnologicznych metod triploidyzacji, gynogenezy i maskulinizacji umożliwiła produkcję jednopłciowych, samiczych i funkcjonalnie sterylnych stad o wysokiej wartości hodowlanej oraz zarybieniowej w przypadku m. in. pstrąga tęczowego, łososia atlantyckiego i pstrąga potokowego (Galbreath i in., 1994; Solomon, 2003; Piferrer i in., 2009; Preston i in., 2013). Biorąc pod uwagę istotną rolę ekologiczną oraz wysoką wartość wędkarską lipienia europejskiego, a także narastające problemy związane z zanieczyszczeniem pul genetycznych, mogące prowadzić do zaniku autochtonicznych populacji za istotne uznałem zastosowanie nowoczesnych biotechnik rozrodu ryb w celu opracowania metody produkcji bezpiecznego z genetycznego punktu widzenia materiału zarybieniowego również w odniesieniu do tego gatunku (rys. 1).

Badania dotyczące triploidyzacji lipienia rozpocząłem w 2017 roku, dołączając do zespołu profesora Konrada Ocalewicza zajmującego się zastosowaniem technik inżynierii genomowej, w tym triploidyzacji, gynogenezy i androgenezy u ryb łososiowatych (Ocalewicz i in., 2010; Jagiełło i in., 2017). Prowadzone w latach 2017–2020 doświadczenia pozwoliły na opracowanie, a następnie opublikowanie warunków skutecznej triploidyzacji zygot lipienia europejskiego (Hliwa i in., 2022). Informacje opublikowane w tym artykule dały początek dalszym badaniom, wyniki których zamieszczone zostały w niniejszej rozprawie doktorskiej.



Rysunek 1. Produkcja triploidalnych samic lipienia (XXX).

2. Cel i zadania badawcze

Głównym celem mojej rozprawy doktorskiej było określenie wpływu trzech zestawów chromosomów (3n) na rozwój gonad, przeżywalność, tempo wzrostu i atrakcyjność wędkarską lipienia europejskiego oraz próba opracowania warunków produkcji wyłącznie samiczych triploidalnych osobników tego gatunku.

Zadania badawcze, które zostały zaplanowane w trakcie realizacji celu głównego pracy obejmowały:

1. Analizę makroskopową i porównawczą struktury oraz stopnia rozwoju gonad u diploidalnych i triploidalnych lipieni europejskich, obejmującą ocenę ich funkcjonalności rozrodczej (Publikacja 2).
2. Ocenę przeżywalności, tempa wzrostu oraz konkurencji pokarmowej diploidalnych i triploidalnych lipieni (Publikacja 3).
3. Porównanie łowności oraz waleczności diploidalnych i triploidalnych lipieni w standaryzowanych warunkach imitujących środowisko rzeczne (Publikacja 3).
4. Opracowanie i optymalizację procedury indukcji gynogenetycznego rozwoju u lipienia europejskiego w celu uzyskania wyłącznie samiczego potomstwa (Publikacja 1).
5. Opracowanie i ocena skuteczności hormonalnej maskulinizacji lipienia europejskiego z zastosowaniem 11β-hydroksyandrostendionu (OHA) oraz 17α-metylotestosteronu (MT) w celu uzyskania funkcjonalnych neosamców (Publikacja 4).

3. Materiały i metody

3.1. Ryby, pochodzenie oraz warunki chowu i hodowli

Lipienie europejskie i pstrągi tęczowe wykorzystane w badaniach pochodziły ze stad tarłowych utrzymywanych w Zakładzie Hodowli Ryb Łososiowatych w Rutkach, należącym do Instytutu Rybactwa Śródlądowego w Olsztynie. Tarlaki w wieku 3 oraz 4 lat utrzymywano w stawach hodowlanych o objętości 3000 dm^3, przy przepływie wody wynoszącym 5 dm^3/s. Obiekty zasilane były wodą pochodzącą z rzeki Raduni. W trakcie prowadzenia doświadczeń temperatura wody wynosiła od 0°C do 20°C, natomiast poziom natlenienia utrzymywano powyżej 80% nasycenia.

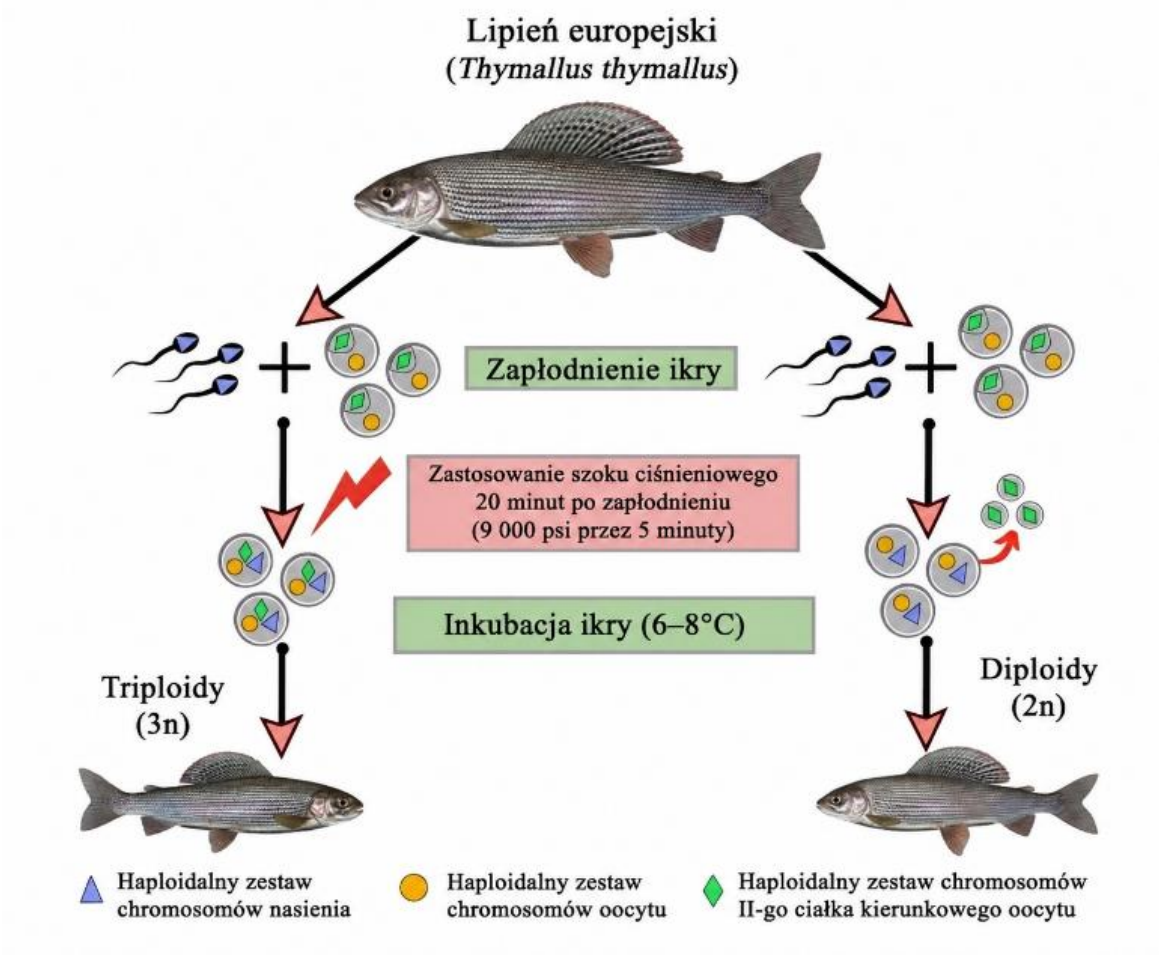
3.2. Produkcja triploidalnych lipieni

Indukcję triploidalnego rozwoju lipienia europejskiego (rys. 2) przeprowadzono w Zakładzie Hodowli Ryb Łososiowatych w Rutkach podczas dwóch sezonów tarłowych, 23 kwietnia 2020 r. oraz 22 kwietnia 2021 r. Proces triploidyzacji wspomnianego gatunku polegał na 5 minutowej ekspozycji ikry na działanie hydrostatycznego szoku ciśnieniowego o wartości 9000 psi, dokładnie 20 minut po jej zapłodnieniu w temperaturze 10°C, przy zastosowaniu urządzenia TRC-APV (TRC Hydraulics Inc., Kanada). Szczegóły procedury znajdują się w publikacji 3.

3.3. Weryfikacja skuteczności indukowanej triploidyzacji

Weryfikację skuteczności indukowanej triploidyzacji przeprowadzono na podstawie wyników analizy wielkości erytrocytów i ich jąder oraz z wykorzystaniem cytometrii przepływowej. Metody te są powszechnie stosowane w badaniach poliploidalnych ryb (Ewing i Scalet, 1991; Garcia-Abiado i in., 1999; Hliwa i in., 2022). Badania erytrocytów wykonywano na rozmazach pełnej krwi obwodowej, które po uprzednim utrwaleniu w metanolu barwiono 10% roztworem Giemsy. Następnie przeprowadzano analizę mikroskopową z wykorzystaniem mikroskopu NIKON Eclipse E600 wyposażonego w kamerę oraz oprogramowanie NIS-Elements BR 3.2. Analizę cytometryczną wykonano przy użyciu analizatora ploidalności CyFlow® Ploidy Analyzer współpracującego z oprogramowaniem CyView™ v1.8.0.82. Materiał biologiczny stanowiły fragmenty płetwy tłuszczowej pobrane od ryb przeznaczonych do dalszych doświadczeń. Próbki bezpośrednio po pobraniu poddawano procedurze przygotowania z wykorzystaniem zestawu CyStain™ UV Precise T kit, umożliwiającego ocenę zawartości DNA metodą cytometrii przepływowej. Szczegółowy opis metodologii

oraz procedur wykorzystanych do weryfikacji ploidalności przedstawiono w publikacjach 2 i 3.



Rysunek 2. Graficzne podsumowanie procesu triploidyzacji lipienia europejskiego.

3.4. Makroskopowa analiza stopnia rozwoju gonad u diploidalnych i triploidalnych lipieni

Makroskopową analizę stopnia rozwoju gonad oraz ich funkcjonalności przeprowadzono u diploidalnych (n=40) i triploidalnych (n=40) osobników w wieku 25 miesięcy. Gonady po odsłonięciu sfotografowano, a następnie oszacowano ich barwę, rozmiar, kształt, spermację (u samców) lub obecność oocytów (u samic). Szczegółowy opis gonad znajduje się w publikacji 2.

3.5. Analiza przeżywalności, tempa wzrostu oraz konkurencji pokarmowej u diploidalnych i triploidalnych lipieni

Analizę tempa wzrostu diploidalnych i triploidalnych lipieni przeprowadzono w dwóch eksperymentach. Pierwszy eksperyment, którego celem było oszacowanie przeżywalności i tempa wzrostu od stadium narybku (0,15 g) do dorosłości trwał od czerwca 2021 r. do września 2023 r (28 miesięcy). Podczas tego eksperymentu określono biomasę obsad, średnią masę ryb, specyficzne tempo wzrostu (SGR),współczynnik wykorzystania paszy (FCR) oraz przeżywalność. Analizy wykonano oddzielnie dla osobników młodocianych, dorastających i dorosłych, z wyłączeniem okresów zahamowanego wzrostu wynikającego ze skrajnych temperatur wody. Drugi eksperyment, skupiający się na osobnikach 28-miesięcznych, był realizowany od września 2022 r. do września 2023 r. oceniano w nim wzrost i przeżywalność ryb utrzymywanych we wspólnych obsadach, analizując dodatkowo konkurencję pokarmową. Podczas sześciu okresów podchowu określano długość, masę, współczynnik kondycji Fultona (K) oraz SGR (Publikacja 3). Wszystkie parametry obliczano zgodnie z metodyką opisaną przez Zakęś i in. (2022).

3.6. Ocena wartości wędkarskiej diploidalnych i triploidalnych lipieni

Ocenę wartości wędkarskiej diploidalnych i triploidalnych lipieni dokonano metodą muchową, analizując ich łowność i waleczność w 8 sesjach przeprowadzonych w październiku i listopadzie 2023 r. W eksperymencie wykorzystano oznakowane znacznikami PIT ryby w wieku około 40 miesięcy (średnio 30 cm i 250 g), które wpuszczano do betonowego stawu doświadczalnego po 10 osobników (5 diploidów i 5 triploidów). Badania przeprowadzono w 45-minutowych sesjach połowowych poprzedzonych 48-godzinną aklimatyzacją bez karmienia. Łowność oceniano punktowo na podstawie kolejności złowienia ryb, natomiast waleczność określano przy użyciu wskaźnika uwzględniającego czas oraz atrakcyjność holu ryb biorąc pod uwagę masę złowionego osobnika. Szczegółowe informacje w publikacji 3.

3.7. Opracowanie metody indukcji gynogenezy u lipienia europejskiego

Doświadczenia mające na celu wywołanie gynogenetycznego rozwoju przeprowadzono w kwietniu 2020 r. W celu opracowania metody indukcji gynogenezy

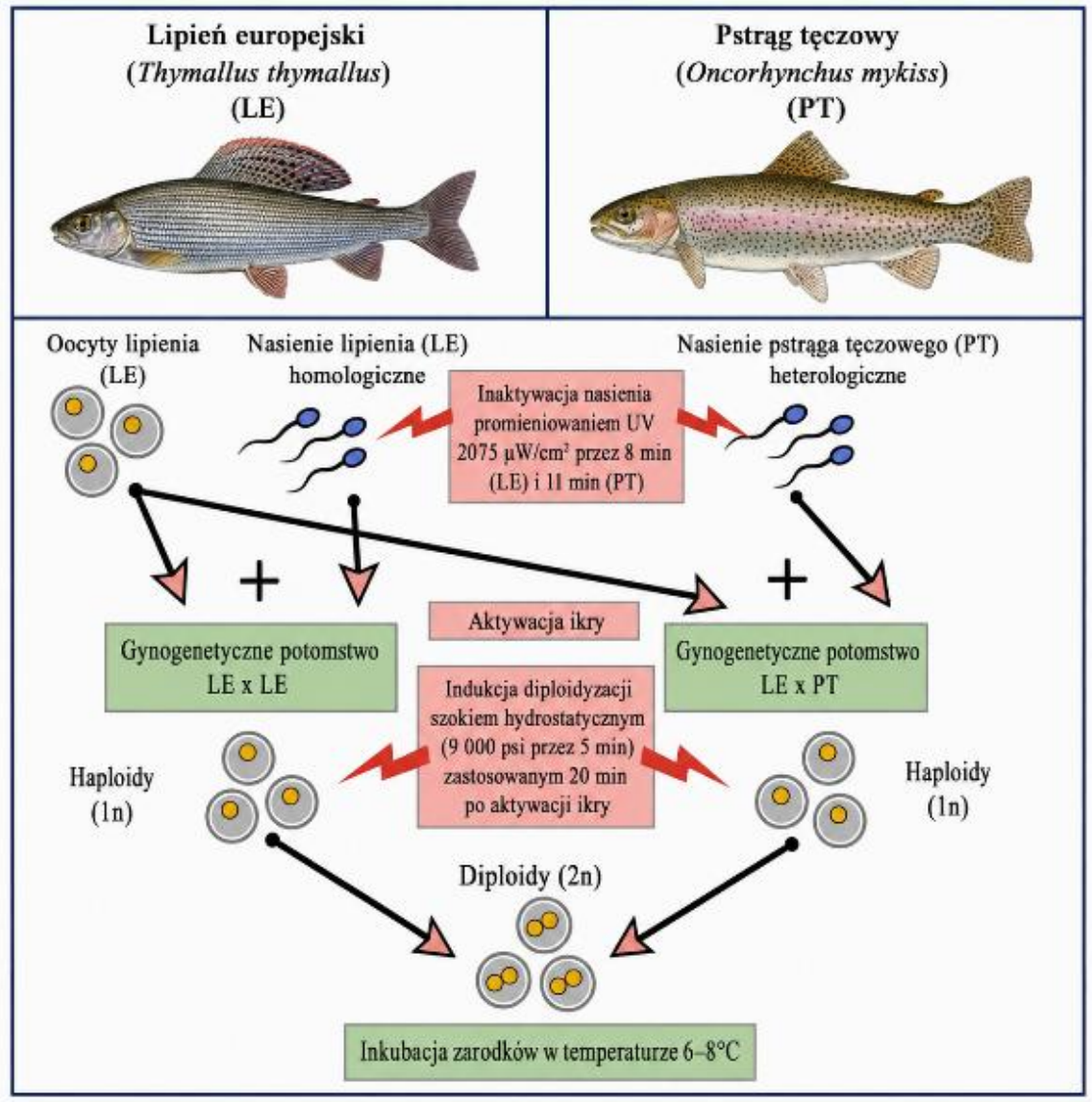
u lipienia wykorzystano nasienie homologiczne (lipienia europejskiego) oraz heterologiczne (pstrąga tęczowego), którego materiał genetyczny poddano inaktywacji poprzez ekspozycję na promieniowanie UV. Uzyskane nasienie wykorzystano następnie do aktywacji jajek lipienia. W celu diploidyzacji gynogenetycznej zygoty poddano ją ekspozycji na działanie szoku ciśnieniowego (rys. 3). W badaniach wykorzystano gamety pochodzące od ośmiu samic lipienia (♀1–♀8), od których pozyskano od 1009 do 3119 ziaren ikry. Ikrę od każdej samicy podzielono na trzy (dla samic ♀2–♀5) lub cztery partie (dla samic ♀1, ♀6–♀8), liczące od 291 do 1006 jaj w każdej grupie. Eksperyment obejmował cztery główne warianty: grupy kontrolne zapłodnione nasieniem homologicznym i heterologicznym oraz grupy gynogenetyczne aktywowane napromieniowanym nasieniem lipienia i pstrąga tęczowego. Kompletny opis procesu gynogenezy znajduje się w publikacji 1.

3.8.Weryfikacja skuteczności indukowanej gynogenezy

Diagnostyka cytogenetyczna służyła do potwierdzenia diploidalnej liczby chromosomów (2n = 100) u uzyskanych gynogenotów oraz do identyfikacji ewentualnych pozostałości chromosomów ojcowskich. Aby wykluczyć udział genomu samca i potwierdzić wyłącznie matczyne dziedziczenie, przeprowadzono genotypowanie z wykorzystaniem loci mikrosatelitarnych DNA (m.in. Ogo-2, One-2, BFRO-018). Dodatkowo, płeć genetyczną osobników gynogenetycznych weryfikowano za pomocą techniki PCR z użyciem markera sdY, sprzężonego z chromosomem Y. Szczegółowe dane dotyczące weryfikacji genetycznej potomstwa znajdują się w publikacji 1.

3.9.Maskulinizacja hormonalna lipienia przy zastosowaniu 11β-hydroksyandrostendionu (OHA) i 17α-metylotestosteronu (MT)

Hormonalną maskulinizację lipienia prowadzono poprzez podanie rybom w paszy 11β-hydroksyandrostendionu (10 i 20 mg/kg paszy) oraz 17α-metylotestosteron (3 i 6 mg/kg paszy). Wspomniane androgeny podawano rybom w wieku około 20 dni po wykluciu (o masie około 80–85 mg) przez 80–83 dni. Weryfikacja skuteczności maskulinizacji obejmowała analizę cech dymorfizmu płciowego, morfologii gonad, analizę histologiczną oraz molekularną. Szczegółowe informacje w publikacji 4.



Rysunek 3. Graficzne podsumowanie procesu gynogenezy u lipienia europejskiego.

4. Wyniki realizacji zadań badawczych

Zadanie 1.

Analiza makroskopowa i porównawcza struktury oraz stopnia rozwoju gonad u diploidalnych i triploidalnych lipieni europejskich, obejmująca ocenę ich funkcjonalności rozrodczej (Publikacja 2).

Analiza makroskopowa gonad u osobników w wieku 2+ wykazała, że dodatkowy zestaw chromosomów powoduje u tego gatunku istotne zaburzenia w rozwoju układu rozrodczego, co przejawia się drastycznymi różnicami w morfologii i funkcjonalności gonad w porównaniu do osobników diploidalnych. Podczas gdy ryby diploidalne w badanym wieku posiadały w pełni rozwinięte gonady wypełnione gametami, u triploidów stwierdzono poważne upośledzenie różnicowania tkanki rozrodczej.

I tak, w grupie triploidalnych samców proces dojrzewania płciowego został niemal całkowicie zahamowany na poziomie funkcjonalnym. Żaden z badanych samców triploidalnych nie wykazywał cech spermacji, co stanowi wyraźny kontrast wobec grupy kontrolnej (2n), w której 100% samców aktywnie oddawało nasienie.

Jądra triploidów opisano jako struktury zwarte i twarde, wykazujące przy tym znaczną zmienność morfologiczną. Na podstawie szczegółowej klasyfikacji makroskopowej wyodrębniono cztery typy jąder u triploidów: najliczniejszą grupę stanowiły jądra o różowym zabarwieniu, niesymetryczne lub segmentowane (36,0%), a kolejne 32,0% osobników posiadało jądra o podobnym kolorze lecz symetryczne. Pozostałe ryby charakteryzowały się jądrami o mlecznobiałym kolorze (32,0%), przy czym jedynie u 4,0% badanych triploidów jądra były białe i symetryczne. Mimo białego zabarwienia sugerującego obecność gamet, w żadnym przypadku nie stwierdzono obecności płynnego nasienia.

Jajniki triploidalnych samic były silnie zredukowane i miały formę cienkich, najczęściej przezroczystych pasm tkanki łącznej, w których makroskopowo nie stwierdzono obecności ziaren ikry. Jest to stan radykalnie odmienny od obserwowanego u diploidalnych samic, których jajniki zawierały dojrzałe ziarna ikry, a u 33,3% samic stwierdzono obecność w jamie ciała owulowanych jaj.

Zadanie 2.

Ocena przeżywalności, tempa wzrostu oraz konkurencji pokarmowej diploidalnych i triploidalnych lipieni (Publikacja 3).

Analiza tempa wzrostu lipienia europejskiego wykazała, że specyficzne tempo wzrostu (SGR) wykazuje zróżnicowaną dynamikę w zależności od stadium rozwojowego ryb. W stadium juwenalnym (do 15. miesiąca życia) oraz w stadium dorosłym nie odnotowano istotnych statystycznie różnic w wartościach SGR między osobnikami diploidalnymi i triploidalnymi. Kluczowe różnice zaobserwowano jednak w stadium dorastania (między 15 a 24 miesiącem życia), w którym lipienie triploidalne charakteryzowały się istotnie wyższym tempem wzrostu (SGR = 0,89) w porównaniu do osobników diploidalnych (SGR = 0,81). Jednocześnie przeżywalność ryb w obu grupach pozostawała na zbliżonym poziomie na wszystkich etapów rozwoju. W stadium narybku wyniosła ona 85,8% u diploidów i 85,1% u triploidów, a w okresie dorastania odpowiednio 99,2% i 99,0%. W stadium dojrzałości odnotowano spadek przeżywalności w obu grupach do 65,0% u osobników diploidalnych oraz 56,8% u triploidalnych.

W eksperymencie obejmującym chów wspólny ogólne wartości specyficznego tempa wzrostu (SGR) nie wykazały istotnych różnic statystycznych i wyniosły 0,28 dla osobników diploidalnych oraz 0,26 dla triploidalnych. W okresie trwania doświadczenia długość ciała ryb zwiększyła się średnio o 29,4% w grupie diploidalnej oraz o 27,3% w grupie triploidalnej. Wyniki te wskazują, że przyrost długości ciała był zbliżony w obu badanych grupach, choć nieznacznie wyższe wartości odnotowano u ryb diploidalnych. Średni współczynnik kondycji Fultona (K) na zakończenie doświadczenia wyniósł 0,89 dla ryb diploidalnych oraz 0,84 dla triploidalnych. Wartości te wskazują, że obie grupy charakteryzowały się zbliżoną kondycją somatyczną, przy czym ryby diploidalne były nieznacznie lepiej odżywione niż triploidalne. Odnotowane niższe parametry (SGR i K) u triploidów w warunkach chowu wspólnego wskazują na ich mniejszą skuteczność w zdobywaniu pokarmu w stosunku do diploidalnych rówieśników. Podobną tendencję zaobserwowano w zakresie przeżywalności – w całym okresie doświadczenia była ona wyższa u osobników diploidalnych (70,7%) niż triploidalnych (58,7%), jednak różnice między grupami nie osiągnęły poziomu istotności statystycznej.

Zadanie 3.

Porównanie łowności oraz waleczności diploidalnych i triploidalnych lipieni w standaryzowanych warunkach imitujących środowisko rzeczne (Publikacja 3).

W przeprowadzonej serii standaryzowanych połowów wędkarskich metodą muchową, lipienie triploidalne w 8 sesjach wędkarskich zgromadziły łącznie 194 punkty, natomiast osobniki diploidalne uzyskały 167 punktów. Oznacza to, że w poszczególnych sesjach ryby triploidalne były łowione nieco częściej lub osiągały wyższe wyniki punktowe niż ryby diploidalne. Średnia liczba punktów uzyskanych w pojedynczej sesji wędkarskiej ukształtowała się na poziomie 24,3 dla triploidów oraz 20,9 dla diploidów, a odnotowane różnice nie były istotne statystycznie. Analiza kolejności złowienia wykazała, że osobniki diploidalne częściej stanowiły pierwszą rybę w sesji, co miało miejsce w pięciu z ośmiu prób. Z kolei lipienie triploidalne najczęściej zajmowały drugą pozycję w kolejności brań i dominowały w tym zakresie również w pięciu na osiem przeprowadzonych sesji. Jako trzecia złowiona ryba triploidy wystąpiły trzy razy, natomiast diploidy pięć razy. W badaniach nad walecznością ryb podczas holu średnia punktacja dla osobników triploidalnych wyniosła 10,6, a dla ryb diploidalnych 9,3. Podobnie jak w przypadku łowności te parametry nie wykazały istotnych statystycznie różnic między badanymi grupami.

Zadanie 4.

Opracowanie i optymalizacja procedury indukcji gynogenetycznego rozwoju u lipienia europejskiego w celu uzyskania wyłącznie samiczego potomstwa (Publikacja 1).

W eksperymencie badano ryby z ośmiu rodzin otrzymanych w wyniku gynogenezy (potomstwo 8 samic) oraz ich rodzeństwo z grup kontrolnych (klasyczne zapłodnienie). Analiza przeżywalności wykazała znaczną zmienność już w grupie kontrolnej, gdzie przeżywalność do stadium wylęgu pływającego (swim-up) wynosiła od 20,3% do 77,6%. W grupach gynogenetycznych w których jajka aktywowano nasieniem homologicznym (samice ♀1, ♀6–♀8) wartości te mieściły się w zakresie od 1,1% do 14,2%, natomiast w grupie, gdzie użyto nasienie heterologiczne (samice ♀1–♀8) od 1,0 % do 60,6%. W przypadku jajek od jednej z samic przeżywalność gynogenetycznych embrionów w stadium zaoczkowania była zbliżona do wyników uzyskanych w grupie kontrolnej. Po dwóch latach hodowli przeżywalność dojrzałych płciowo gynogenotów nie przekraczała 6 procent.

Wśród gynogenotów zaobserwowano ponad trzykrotnie wyższy odsetek osobników z deformacjami ciała (31,2% i 37,3%) w porównaniu do grupy kontrolnej, w której odsetek ryb z nieprawidłowościami rozwoju wynosił 9,3%. Najczęściej obserwowanymi anomaliami były powiększony woreczek żółtkowy oraz skolioza. Stwierdzano również lordozę, kifozę, deformacje spiralne oraz larwy o kształcie litery C. Nie wykazano istotnych różnic w częstości występowania deformacji pomiędzy grupami gynogenotów uzyskanych w wyniku aktywacji jaj nasieniem homologicznym i heterologicznym

Weryfikacja molekularna potwierdziła pełne dziedziczenie matczyne u wszystkich gynogenotów uzyskanych przy użyciu nasienia pstrąga tęczowego. W grupie aktywowanej nasieniem homologicznym stwierdzono obecność fragmentów genomu ojcowskiego u części analizowanych osobników.

Wszystkie osobniki, które się wykluły z jajek aktywowanych nasieniem heterologicznym były genetycznymi samicami. U potomstwa samicy 8, wyprodukowanego przy użyciu nasienia homologicznego, u którego wykryto domieszkę ojcowskiego DNA, zidentyfikowano cztery osobniki o genotypie samczym. Makroskopowa analiza gonad przeprowadzona u dwóch z tych samców wykazała, że jeden posiadał prawidłowo wykształcone jądra, natomiast u drugiego gonady były niedorozwinięte.

Analizy cytogenetyczne potwierdziły diploidalny stan (2n= 100) wszystkich badanych ryb, odpowiadających kariotypowi charakterystycznemu dla lipienia europejskiego (Ocalewicz i in., 2013; Sävilammi i in., 2019). U części osobników z grupy aktywowanej nasieniem lipienia, u których wykryto obecność ojcowskiego DNA, obserwowano dodatkowe niewielkie fragmenty chromosomów obecne w pojedynczych komórkach. Liczba tych fragmentów wynosiła od jednego do trzech.

W jednej z analizowanych rodzin wyprodukowanych przy użyciu nasienia homologicznego odsetek osobników wykazujących obecność fragmentów genomu ojcowskiego wynosił 33%, natomiast udział markerów pochodzenia ojcowskiego w całkowitej puli analizowanych loci osiągał maksymalnie około 8% u wybranych osobników. W grupie wyprodukowanej z użyciem nasienia heterologicznego nie wykryto obecności genomu ojcowskiego ani osobników o genotypie samczym.

Zadanie 5.

Opracowanie i ocena skuteczności hormonalnej maskulinizacji lipienia europejskiego z zastosowaniem 11β-hydroksyandrostendionu (OHA) oraz 17α-metylotestosteronu (MT) w celu uzyskania funkcjonalnych neosamców (Publikacja 4).

W eksperymencie badano konsekwencje podawania narybkowi lipienia paszy wzbogaconej o 11β-hydroksyandrostendion (OHA) lub 17α-metylotestosteron (MT). W przypadku grup ryb, którym podawano 11β-hydroksyandrostendion (OHA), wykazano znaczną skuteczność hormonu w indukowaniu zewnętrznych cech samczych, przy jednoczesnym braku pełnej inwersji płci na poziomie gonadalnym. Najwyższy odsetek ryb wykazujących zewnętrzny dymorfizm samczy, taki jak ciemne ubarwienie ciała, duża płetwa grzbietowa i obecność brodawek tarłowych, odnotowano w grupie otrzymującej dawkę 20 mg/kg (OHA_{20ppm}), gdzie wyniósł on 76,6%. W grupie z niższą dawką (10 mg/kg) wskaźnik ten osiągnął 66,7%. Kluczowym spostrzeżeniem była jednak wyraźna dysocjacja między dymorfizmem zewnętrznym, a faktycznym rozwojem gonad. Analizy morfologiczne potwierdzone testami PCR wykazały, że część ryb wyglądających na samce w rzeczywistości posiadała jajniki – zjawisko to dotyczyło 4,5% osobników w grupie OHA_{10ppm} oraz aż 28,8% w grupie OHA_{20ppm}. Wszystkie te ryby były genetycznymi samicami, co dowodzi, że OHA wpłynął głównie na tkanki wrażliwe na androgeny odpowiedzialne za cechy zewnętrzne, nie powodując trwałej zmiany kierunku rozwoju gonad u wszystkich osobników.

Zastosowanie 17α-metylotestosteronu (MT) spowodowało istotne zaburzenia procesu różnicowania gonad u genetycznych samic, choć nie udało się uzyskać w pełni funkcjonalnych neosamców. MT okazał się silniejszym czynnikiem maskulinizującym w odniesieniu do gonad niż OHA, jednak w testowanych dawkach u genetycznych samic prowadził głównie do powstawania osobników interseksualnych. W badanych gonadach obserwowano obecność oocytów przemieszanych z rozproszonymi spermatogoniami oraz plemnikami (spermatozoa). Częstość występowania cech interpłciowych wśród lipieni wzrastała wraz z dawką hormonu: w grupie MT_{3ppm} wynosiła 28,6%, natomiast w grupie MT_{6ppm} objęła 57,1% genetycznych samic. Pozostałe samice w tych grupach wykształciły prawidłowe jajniki, co sugeruje, że zastosowane dawki lub czas ekspozycji były niewystarczające do wywołania całkowitej i stabilnej inwersji płci.

5. Podsumowanie

Celem niniejszej rozprawy doktorskiej było określenie wpływu trzech zestawów chromosomów (3n) na rozwój gonad, tempo wzrostu i atrakcyjność wędkarską lipienia europejskiego oraz próba opracowania warunków produkcji wyłącznie samiczych triploidalnych osobników tego gatunku.

Uzyskane wyniki wykazały istotne zaburzenia rozwoju gonad u triploidalnych samic jak i samców lipienia, wskazując, że indukcja triploidalnego rozwoju u tego gatunku stanowi efektywną metodę produkcji materiału zarybieniowego o ograniczonej zdolności reprodukcyjnej, a tym samym może ograniczać ryzyko zanieczyszczenia genetycznego dzikich populacji. Badania potwierdziły porównywalne tempo wzrostu, przeżywalność oraz kondycję diploidalnych i triploidalnych lipieni. Ponadto doświadczenia dotyczące konkurencji pokarmowej pokazały, że osobniki triploidalne charakteryzują się nieco niższą efektywnością zdobywania pokarmu oraz mniej agresywnym zachowaniem podczas żerowania niż osobniki diploidalne. Jednocześnie nie stwierdzono istotnych różnic pod względem łowności i waleczności, co potwierdza przydatność triploidalnych lipieni do zarybień wód otwartych ukierunkowanych na cele wędkarskie.

W ramach niniejszej rozprawy doktorskiej opracowano skuteczną metodę indukcji gynogenezy u lipienia europejskiego z wykorzystaniem nasienia heterologicznego, co stanowi podstawę do dalszego rozwoju technologii produkcji stad złożonych wyłącznie z triploidalnych samic. Przeprowadzone eksperymenty z zakresu hormonalnej maskulinizacji pozwoliły stwierdzić, że zarówno 11β-hydroksyandrostendion, jak i 17α-metylotestosteron wpływają na proces różnicowania płci u lipienia europejskiego, jednak uzyskane wyniki nie pozwalają jeszcze na efektywną produkcję funkcjonalnych neosamców tego gatunku.

Przeprowadzone badania dostarczają nowych i istotnych informacji na temat możliwości wykorzystania biotechnik rozrodu w ochronie i gospodarowaniu populacjami lipienia europejskiego. Uzyskane wyniki mają zarówno znaczenie poznawcze, poszerzając wiedzę na temat biologii i fizjologii tego gatunku, jak i aplikacyjne, stanowiąc podstawę do wdrożenia innowacyjnych rozwiązań w praktyce hodowlanej oraz w zarządzaniu zasobami ryb.

6. Wnioski

1. Triploidalne lipienie europejskie, dzięki potwierdzonej u dorosłych osobników (2+) sterylności wynikającej z trwałej dysfunkcji jajników i jąder (brak oocytów i spermacji) mogą stanowić bezpieczny materiał zarybieniowy. Niemniej jednak zaleca się przeprowadzenie badań stanu rozwoju gonad u triploidalnych lipieni w starszym wieku.
2. W warunkach imitujących naturalne, triploidalne lipienie zachowują pełne walory sportowe oraz użytkowe i nie odbiegają pod względem aktywności żerowania oraz dynamiki walki od form diploidalnych tego gatunku, co umożliwia utrzymanie łowisk na wysokim poziomie atrakcyjności wędkarskiej.
3. W celu uzyskania pełniejszej oceny przydatności triploidalnych lipieni do celów zarybieniowych należy prowadzić badania dotyczących przeżywalności, zachowania i łowności triploidalnych lipieni w warunkach naturalnych.
4. Gynogeneza z wykorzystaniem inaktywowanego promieniowaniem UV nasienia pstrąga tęczowego jest skuteczną metodą produkcji jednopłciowych samiczych stad lipienia europejskiego.
5. Zaproponowane warunki maskulinizacji narybku lipienia europejskiego przy użyciu androgenów (OHA i MT) okazały się nieskuteczne i nie doprowadziły do uzyskania neosamców (XX). Uniemożliwiło to opracowanie warunków produkcji potomstwa lipienia złożonego wyłącznie z triploidalnych samic.

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Successful application of UV-irradiated rainbow trout (*Oncorhynchus mykiss*) spermatozoa to induce gynogenetic development of the European grayling (*Thymallus thymallus*)

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ABSTRACT

In fish with the XX/XY sex determination system, the production of all-female offspring within one generation was achieved by gynogenesis. Successful induction of gynogenesis in the European grayling (*Thymallus thymallus*) (Salmonidae) included egg activation with UV-irradiated homologous (996 J/m^2) and heterologous (rainbow trout) sperm (1370 J/m^2) followed by exposure of the inseminated eggs to a high hydrostatic pressure (HHP) shock (9000 psi for 5 min) 20 min after their activation. The survival rates of the gynogenotic larvae at the swim-up stage exhibited a huge inter-clutch variability that ranged from 1.12 ± 0.53% to 14.15 ± 1.27% and from 0.96 ± 0.33% to 60.57 ± 1.85% when the eggs were activated by UV-irradiated homologous and heterologous sperm, respectively. Maternal inheritance was only confirmed in the gynogenotes that hatched from eggs inseminated by inactivated heterologous sperm, whereas an admixture of the paternal DNA was detected among screened individuals that originated from eggs activated by homologous sperm. All the examined gynogenotes produced using heterologous sperm were confirmed to be genetically females. In the case of fish with an admixture of the paternal DNA, male genotypes and phenotypes were observed. One of the genetic males had well developed testes. Differences in the ratio of deformed larvae that hatched from eggs activated by UV-irradiated homologous and heterologous sperm were nonsignificant. Based on the obtained results, it has to be stated that the activation of grayling eggs with UV-irradiated rainbow trout sperm followed by HHP shock is a reliable and fast method for the production of all-female grayling stocks.

1. Introduction

The European grayling (*Thymallus thymallus*), hereinafter referred to as grayling, is a non-anadromous salmonid fish (order Salmoniformes, family Salmonidae, subfamily Thymallinae) native to Europe. The species is considered as an important bioindicator of the quality of lotic ecosystems and one of the most attractive species for fly fishing in European rivers and streams. As a result of environmental degradation, the pressure of predation and overfishing, many of the local grayling populations have experienced a significant decline in their distribution range and size during recent decades (Susnik et al., 2004; Maric et al., 2012; Weiss et al., 2013). Due to the decline of the grayling populations, numerous conservation programmes have been undertaken, including fishing restrictions and stocking activities (Lyach and Remr, 2019, 2020). Unfortunately, however, the use of hatchery-reared fish and individuals from allochthonous populations has resulted in the observed genetic contamination of many local grayling populations with non-native specimens (Koskinen et al., 2002; Susnik et al., 2004; Duftner et al., 2005) that has sometimes resulted in reduced fitness and a decline in or even complete disappearance of the native grayling populations (Koskinen et al., 2002; Susnik et al., 2004; Geist et al., 2009; Turek et al., 2010). Although, rearing of grayling is difficult due to the high fragility of the fish to handling and manipulations (netting, stripping), high mortality of embryos and larvae and lack of standardized conditions for culture (Turek et al., 2014; Kodela et al., 2023), in some European countries, grayling is farmed under aquaculture conditions for consumption and for restocking programmes (Paisley et al., 2010; Turek et al., 2010; Kodela et al., 2023). The grayling has been also used as

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model in evolutionary studies and its genome sequence assembly has been produced to examine chromosomal rearrangements (Sävilammi et al., 2019) and evolution of regulation of gene expression (Varadharajan et al., 2018) following the Whole Genome Duplication (WGD) event that occurred in salmonids between 80 and 100 million years ago (Macqueen and Johnston, 2014).

Gynogenesis is a chromosome set manipulation leading to the production of fish that inherit only maternal genetic information. Induction of the gynogenetic development is accomplished by the activation of eggs with UV-irradiated homologous or heterologous sperm. The application of heterologous sperm for gynogenesis has been justified in fish and amphibian species due to the fact that the radiation-induced foreign chromosome fragments that sometimes appear after an incomplete UV inactivation of the paternal chromosomes may be more easily eliminated from the nuclei of gynogenetic individuals than fragments from the homologous sperm (Jaylet and Ferrier, 1978; Chourrout, 1986; Polonis et al., 2018). This is connected with the conflict between the egg nucleus and the sperm genome that in some salmonid hybrids results in the preferential elimination of the paternal chromosomes from the nuclei (Fujiwara et al., 1997). As such, the application of heterologous sperm may assure the production of individuals that contain exclusively maternal genetic material. Subsequent exposition of gynogenetically activated eggs to high hydrostatic pressure (HHP) shock or thermal shock enables the diploidization of the gynogenetic haploid zygotes. The shock can be applied early after egg activation to suppress the anaphase stage of the second meiotic division (meiogynogesis) or later to inhibit the first cleavage at the mitotic division (mitogynogenesis) (Pandian and Koteeswaran, 1998). Gynogenesis is an important breeding technology applied to produce genetically improved fish stocks, to investigate sex determination mechanisms and to produce monosex populations of fish for the aquaculture. Moreover, exclusive maternal inheritance and increased homozygosity make gynogenotes useful in studies related to the developmental biology (Araki et al., 2001). Increased homozygosity facilitates the *de novo* assembly of the genomes sequenced using the next-generation sequencing approach (Xu et al., 2021). Gynogenetic all-female specimens may be utilized for the generation of neomales (XX) and the production of sterile triploid all-females (XXX) (Luo et al., 2011) but their application is limited rather to the laboratory research than aquaculture production. Apart from aquaculture, triploid all-females can be also used as safe stocking material whose application excludes the risk of detrimental genetic introgression into the native fish populations.

Taking into account role of the European grayling in the natural environment, aquaculture and studies related to genomics and evolution, production of gynogenetic specimens would be of a broad interest. However, to our knowledge, no information concerning the induction of gynogenetic development in the European grayling has been published to date thus, the main goal of the present study was production of the grayling that inherit exclusively maternal genetic material due to gynogenesis. For this purpose, grayling eggs were activated by UV-inactivated homologous and heterologous (rainbow trout) sperm and exposed to HHP shock using conditions that have been already established for the production of gynogenetic rainbow trout using grayling and rainbow trout sperm (Dobosz et al., 2015; Jagiełło et al., 2018) and triploid grayling (Hliwa et al., 2022). Molecular examination confirmed that the graylings that hatched from eggs activated by UV-irradiated rainbow trout sperm were genetically all-females with only maternal inheritance. As application of the UV-irradiated homologous sperm resulted in the production of some percentage of individuals with residues of the paternal nuclear DNA, the use of UV-inactivated heterologous spermatozoa is recommended for efficient gynogenesis in the European grayling.

2. Materials and methods

2.1. Ethics

This study was carried out in strict accordance with the Polish legal regulations (The Act of the Polish Parliament of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes, Journal of Laws 2015, item 266). The protocol was approved by the Local Ethics Committee for Animal Experiments in Bydgoszcz, Poland (no.14/2022).

2.2. Gamete donors and experiment design

European grayling and rainbow trout (*Oncorhynchus mykiss*) gamete donors originated from the broodstocks kept in the Department of Salmonid Research in Rutki, Inland Fisheries Institute in Olsztyn. The basic characteristics of the gamete donors used are included in the supplementary Table S1. Three- and four-year-old spawners were reared in a pond of 3000 dm^3 with a water flow of 5 dm^3/s, powered by water from the Radunia River (mean temperature 10 °C and oxygenation level above 80%). The fish were fed with commercial broodstock feed (Aller Bronze 3 mm and Aller Silver 3 mm, Aller Aqua, Poland) 3 or 4 times per day in amounts of between 0.5 and 1.5% fish biomass, depending on the water temperature. Five gynogenesis induction trials were carried out in April 2020 (Table 1).

2.3. Gamete collection

Two weeks before the spawning period, the fish feeding was stopped. Prior to the gamete stripping, selected specimens were anesthetized using ms-222 (50 mg/dm^3, Sigma-Aldrich, Spain). Eggs from eight grayling females were collected in separate 2-l plastic bowls. The total number of eggs from each female varied from 1009 to 3119 (Supplementary Table S1). The eggs collected from each female were divided into three (females ♀EG2–5) or four (females ♀EG 1, 6–8) batches that comprised 291–1006 eggs per each batch. The eggs were kept at a temperature of 10 °C until further procedures. Sperm from four grayling (♂EG1–4) and five rainbow trout (♂RT1–5) males were collected using a 5 ml syringe and stored in separate 100 ml plastic containers. To assess the motility of the spermatozoa, 1 μl of sperm from each male was mixed with 199 μl of the sperm activating medium (SAM) (1 mM $CaCl_2$, 20 mM Tris, 30 mM glycine, 125 mM NaCl pH 9.0) (Billard, 1977) and examined under a light microscope (Nikon Eclipse E 2000). The sperm from all males displayed satisfactory quality and was kept at 4 °C until their further use.

2.4. Semen inactivation

Before the UV irradiation procedure, portions of sperm (0.375 ml) were diluted in the rainbow trout seminal plasma (ratio 1:40) according to the procedure described by Jagiełło et al. (2021). A total volume of 15.375 ml of the diluted semen was transferred to a 60 ml glass beaker (50 mm diameter, 30 mm height, depth of diluted sperm 7.8 mm), placed onto a magnetic stirrer (1400 G) 20 cm under a UV-C lamp (30 W) and irradiated (2075 $\mu W/cm^2$) for 8 min (European grayling) (Jagiełło et al., 2018) and 11 min (rainbow trout), respectively (Dobosz et al., 2015; Jagiełło et al., 2018; Polonis et al., 2018). During the irradiation, the motility of the spermatozoa was microscopically checked at 30 s intervals. Longer exposure of the sperm to UV resulted in complete immobility of the irradiated spermatozoa and an inability to activate eggs for gynogenetic development.

2.5. Egg activation and diploidization

Immediately after irradiation, the sperm was used for activation of the eggs. To induce meiotic gynogenesis, selected batches of eggs from

Table 1
Number and survival rates of two-year-old sexually matured gynogenetic European graylings.

	Egg donors							
	♀EG1	♀EG2	♀EG3	♀EG4	♀EG5	♀EG6	♀EG7	♀EG8
homologous sperm	19 (2.03%)	N/P	N/P	N/P	N/P	0	9 (1.34%)	19 (4.32%)
heterologous sperm	34 (3.68%)	42 (5.18%)	3 (0.77%)	1 (0.1%)	0	0	4 (0.54%)	2 (0.46%)

N/P – variant of the experiment that was not performed.

each female were activated with the UV-irradiated semen of the European grayling (Gyno $_{EG\times EG}$) and rainbow trout (Gyno $_{EG\times RT}$) in the presence of sperm activating medium (SAM). Three minutes after activation, the eggs were rinsed with hatchery water. Activated eggs were then incubated for further treatment in a water bath at 10 °C. About 100 activated eggs from each gynogenetic experimental group were left to develop as the haploid control. To restore the diploid state in gynogenetically activated eggs, a high hydrostatic pressure (HHP) shock (9000 psi for 5 min) was applied 20 min after activation of the eggs, using the TRC-APV electric/hydraulic device (Hliwa et al., 2022). To provide control groups, the remaining batches of eggs from each female were fertilized with the non-irradiated sperm of the European grayling (Cont $_{EG\times EG}$) and rainbow trout (Cont $_{EG\times RT}$). All the batches of eggs were placed into separate compartments of the incubation apparatus (Supplementary Table S2). The eggs from each female and experimental group were incubated in three separate replicates at 6–8 °C. After the swim-up stage, fish from the replicates were pooled and the gynogenetic offspring of each female produced by homologous and heterologous sperm were reared in separate tanks until sexual maturation and longer. The gynogenetic grayling were reared in the plastic aquaculture tanks (300 dm^3) powered by water from the Radunia River (mean temperature 10 °C and oxygenation saturation level above 80%). Depending on their size/age, the fish were fed with commercial feed (artemia, Aller Infa Ex 0.4 mm, Aller Futura Ex GR 0.5–1.0 mm, 0.9–1.6 mm, Aller Bronze 1.5 and 3 mm) (Aller Aqua, Poland).

2.6. Survivability, body abnormalities and statistical analysis

The survival rates in each group were analysed at the eyed-egg stage (14 days post fertilization, dpf), at hatching (26 dpf), and at the swim-up stage (35 dpf). Moreover, the gynogenetic specimens were counted after two years of rearing when they had attained sexual maturation. All the larvae that had died were collected and preserved in 95% ethanol for assessment of their morphology and to evaluate any body deformations. Differences in the survivability and frequency of deformations between all the experimental groups were compared using Statistica software v. 3.0 (StatSoft Company). Prior statistical test selection, data distribution normality and homogeneity of variance were tested by the Shapiro–Wilk and the Levene's test, respectively. The HSD Tukey test was used to determine if there were any significant differences in the recorded data between all the experimental groups. Significance was established at the $P < 0.05$ level.

2.7. Molecular verification of gynogenesis

Microsatellite DNA genotyping was performed to confirm maternal inheritance in the gynogenetic progeny produced. For this purpose, all gamete donors, grayling specimens from the control groups and ten randomly chosen larvae that hatched from the gynogenetically activated eggs using homologous and heterologous sperm originated from each grayling female were genotyped (Supplementary Table S3). In the case of gynogenotes from group Gyno $_{♀EG8\times♂EG4}$, eight specimens that were sampled for the cytogenetic purpose were also genotyped. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the protocol provided by the vendor. The quality and amount of the isolated genomic DNA was checked by the spectrophotometer NanoDrop™ One (Thermo Scientific).

Five markers were selected for the genetic screening of the European grayling (*Ogo-2*, *BFRO-005*, *One-2*, *BFRO-018*, *BFRO-015*, *OMM-1007*), and another five markers were used for the rainbow trout (*OMM-1077*, *OMM-1036*, *OMM-1008*, *OMM-1051*, *OMM-1012*) (Koskinen et al., 2002; Kaczmarczyk and Kaczor, 2013). Reaction mixtures were prepared in a total volume of 25 μl composed by the 1× Green Reaction Buffer, 1.5–3.5 mM of $MgCl_2$, 0.2 mM dNTP mix, 0.1–1.0 μM of each primer, 0.55 U of GoTaq® Polymerase and around 10 ng of the DNA template. PCR amplifications were performed with a Thermal Cycler SimpliAMP (Applied Biosystems, Foster City, California, USA) under the following conditions: an initial denaturation at 94 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, annealing at 55–61 °C for 45 s, elongation at 72 °C for 45 s and a final elongation step at 72 °C for 10 min. Detailed conditions of the PCR reactions have been described by Kaczmarczyk and Kaczor (2013). The resulting products of the PCR amplifications were separated in a 1.5% agarose gel (Sigma-Aldrich, St. Louis, USA) stained with ethidium bromide (0.05 mg/ml) and then visualized by a UV trans-illuminator (Vilber Laurmat ECX-20.M, Eberhardzell, Germany).

The genetic sex of the graylings that hatched from eggs activated by UV-irradiated sperm was verified by the PCR duplex amplification of the Y-chromosome linked DNA marker (*sdY*) and 18S rDNA as a positive control (Yano et al., 2013). The PCR amplifications were carried out using GoTaq® DNA Polymerase (Promega GmbH, Germany) and the resulting PCR products were visualized by the agarose gel electrophoresis as described above.

2.8. Cytogenetic analysis and gonadal development

Cytogenetic studies were carried out in September/October 2022 on randomly selected two-year-old individuals that had hatched from eggs originating from one female (♀EG1) that had been activated by UV-irradiated homologous ($n = 4$) (Gyno $_{♀EG1\times♂EG1}$) and heterologous (n = 4) (Gyno $_{♀EG1\times♂RT1}$) sperm. Furthermore, eight graylings that had hatched from eggs activated by UV-irradiated grayling sperm from the group Gyno $_{♀EG8\times♂EG4}$ were also cytogenetically examined (Table 1). Somatic metaphase chromosomes were prepared from cephalic kidney cells according to the method described by Ocalewicz et al. (2013). Chromosomes were stained with 4′, 6-diamidino-2-phenylindole DAPI (Vector, Burlingame, USA) and analysed under a BX53 Olympus microscope (Olympus, Japan) equipped with epifluorescence, an appropriate filter set and a dedicated 5 M CMOS camera (Applied Spectral Imaging, Israel). Only high-quality metaphase cells from each individual specimen were obtained and examined using the GenASIs 8.1.1 software (Applied Spectral Imaging, Israel). The gonadal development of each cytogenetically examined fish was assessed macroscopically and the genetic sex assessed using PCR and Y-linked DNA marker genotyping (Yano et al., 2013) as described above.

3. Results

3.1. Survival rates of embryos and larvae

Eggs of the European grayling obtained from different females and used in the experiment exhibited inter-clutch differences in the quality expressed as the variable survivability of the grayling offspring from the control groups (Cont) and ranged from 20.3 ± 3.08% (♀EG8) to 77.6 ±

1.25% (♀EG7) at the swim-up stage (Fig. 1). Interestingly, the eggs with the highest survival rate originating from normal fertilization (♀EG7) did not exhibit increased potential for the gynogenesis development (Fig. 1). Survival rates of the grayling swimming larvae that hatched from eggs activated by UV-irradiated grayling sperm showed also a significant inter-clutch variability, ranging from 1.4 ± 0.53% (♀EG6) to 14.1 ± 1.27% (♀EG1). In turn, the survivability of larvae developing in eggs inseminated with the inactivated heterologous sperm varied from 1.0 ± 0.15% (♀EG5) to 60.6 ± 1.85% (♀EG2) (Fig. 1).

In the case of eggs that originated from female ♀EG2, the survival of eyed embryos from gynogenetic and control groups did not differ significantly ($P < 0.05$). Survival of the gynogenetic offspring that hatched from eggs originating from the same donor was usually slightly higher when heterologous sperm was applied. However, apart from the gynogenetic offspring of the female ♀EG8, observed differences were not significant ($P > 0.05$) (Fig. 1). The European grayling and rainbow trout hybrid embryos were not viable and all died before the eyed stage. A similar situation was observed for the embryos that were developed in eggs activated by UV-irradiated sperm but that were not exposed to HHP shock. After two years of rearing, the survival rates of sexually matured gynogenetic graylings varied from 0 to 5.18% and the highest number of matured gynogenotes were those that hatched from the eggs that originated from females ♀EG1, ♀EG2 and ♀EG8 (Table 1).

3.2. Larval deformities

The average percentage of deformed grayling larvae from the control groups (9.31% ± 9.96%) was significantly ($P < 0.05$) lower than the gynogenetic groups: Gyno $_{EG\times EG}$ (31.21 ± 19.42%) and Gyno $_{EG\times RT}$ (37.27 ± 10.08%). Differences in the observed ratio of deformed larvae between both gynogenetic groups were not significant ($P > 0.05$). Varied body deformations have been reported in the examined grayling specimens (Fig. S1) and the most frequently observed abnormalities were an enlarged yolk sac and scoliosis, which often co-occurred (Fig. 2, Fig. S1).

3.3. Molecular validation of the performed gynogenesis

All the screened gamete donors from the experiment were heterozygous for at least one of five microsatellite DNA markers used for genotyping (Supplementary Table S3). Five microsatellite DNA markers (*Ogo-2*, *One-2*, *BFRO-018*, *OMM-1007* and *OMM-1008*) were successfully amplified in both gamete donor species, *i.e.*, the European grayling and rainbow trout, displaying species-specific patterns of the genetic profiles. The genetic verification confirmed full maternal inheritance in all gynogenetic progeny except six out of eighteen (33%) examined offspring from the Gyno $_{♀EG8\times♂EG4}$ variant produced using homologous sperm, where the admixture of the paternal DNA was detected (Fig. S2). The genetic profiling of the grayling gamete donors and their offspring from the control variants confirmed a co-dominant form of inheritance of the screened microsatellite DNAs. The observed rate of heterozygosity differed between experimental groups and ranged from 52% (controls) to 19% and 16% reported among specimens that hatched from eggs activated by the irradiated homologous and heterologous sperm, respectively.

Apart from four individuals from the Gyno $_{♀EG8\times♂EG4}$ group that were proved to be genetically males, all the other examined European graylings that hatched from eggs activated by the UV-irradiated spermatozoa were confirmed to be genetically females (Fig. S3).

3.4. Cytogenetic examination and gonadal development

All the cytogenetically studied graylings had 100 chromosomes (Fig. 3), a number characteristic for the diploid state of the individuals

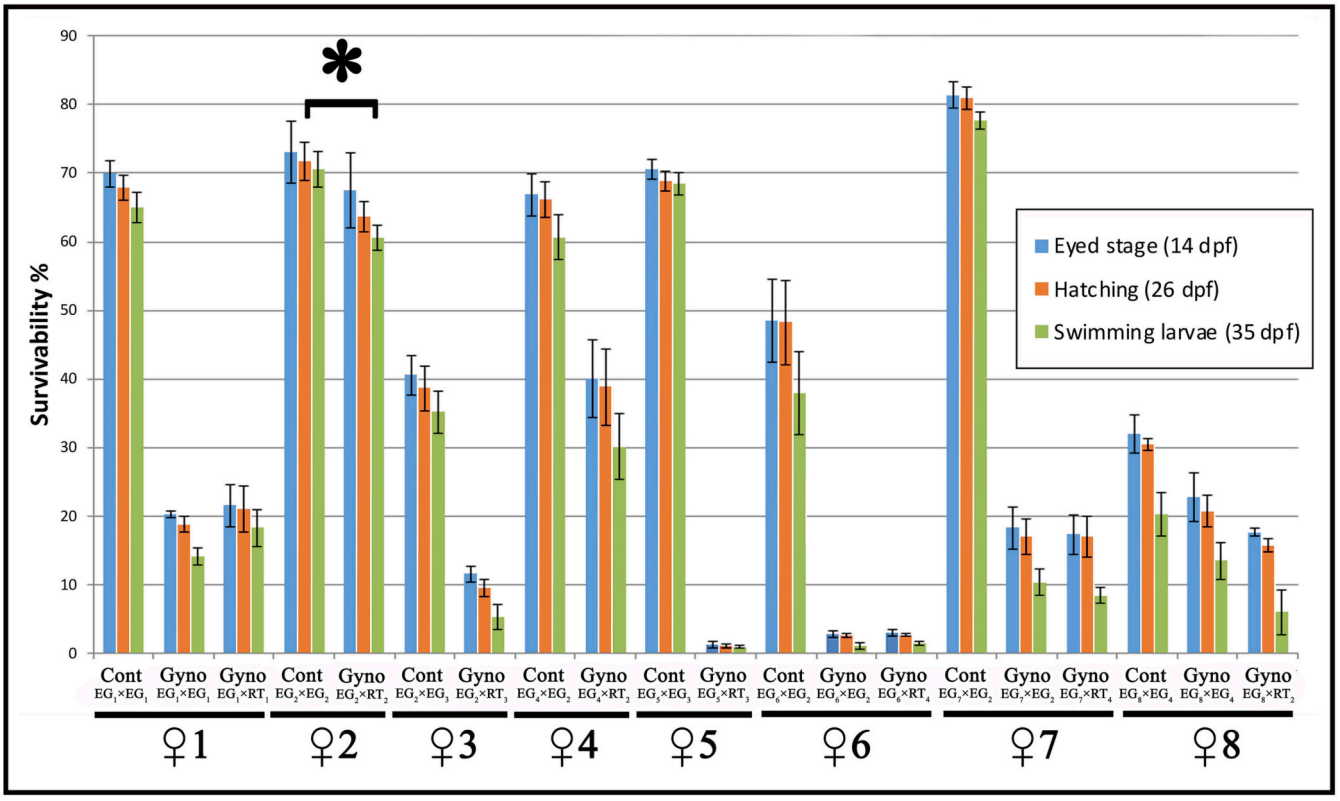

Fig. 1. Survival rates of the European grayling (*Thymallus thymallus*) offspring that hatched from eggs inseminated with non-manipulated grayling sperm (Cont) and from eggs that were activated by UV-irradiated grayling (Gyno $_{EG\times EG}$) and rainbow trout (Gyno $_{EG\times RT}$) sperm. An asterisk indicates insignificant differences ($P > 0.05$).

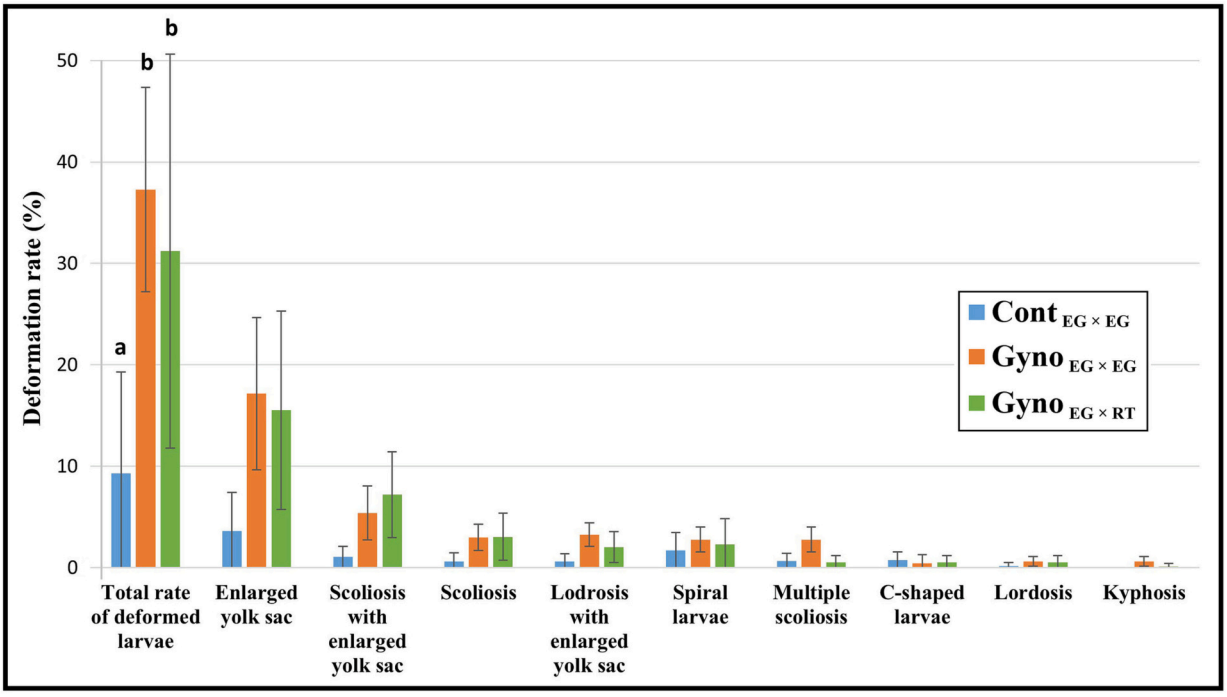

Fig. 2. Type and ratio of observed body abnormalities in the European grayling (*Thymallus thymallus*) that hatched from eggs activated with the non-manipulated sperm (Cont) and UV-irradiated sperm of grayling (Gyno $_{EG\times EG}$) and rainbow trout (Gyno $_{EG\times RT}$) followed by HHP (± SD). Values noted by different letters are significantly different from each other ($P < 0.05$).

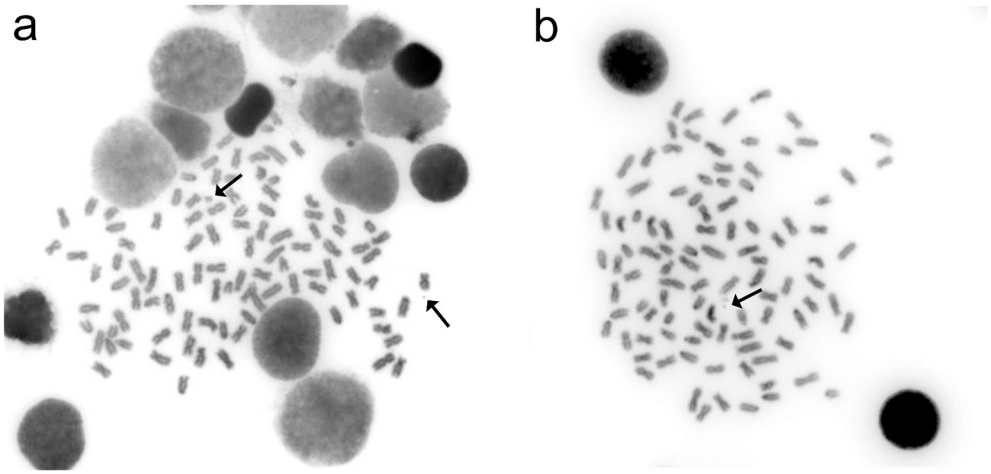

Fig. 3. Metaphase spreads of the European grayling (*Thymallus thymallus*) gynogenetic male from group Gyno $_{♀EG8\times♂EG4}$ (2n = 100) (a) and gynogenetic female 2n = 100 from group Gyno $_{♀EG1\times♂EG1}$ (b). Arrows indicate chromosome fragments.

from the stock used in the experiment (Hliwa et al., 2022). Apart from staining intact grayling chromosomes, the DAPI that strongly binds to DNA also exhibited small particles, presumed fragments of chromatin in a few cells of one of the gynogenetic fish from the Gyno $_{♀EG1\times♂EG1}$ group and in some cells from four gynogenotes from the Gyno $_{♀EG8\times♂EG4}$ group. The number of such fragments varied intra-individually from one to three (Fig. 3). Two out of eight cytogenetically studied individuals from the Gyno $_{♀EG8\times♂EG4}$ group were found to be genetically males. Well-developed testes were found in one of them (Fig. S4), while the other one had underdeveloped gonads. In one of these two fish, the presence of chromatin fragments has been microscopically confirmed.

4. Discussion

The efficient procedures of paternal genome inactivation and diploidization of the maternal genome play a pivotal role in the induction of the gynogenetic development. Too low a dose of UV irradiation applied to sperm may only partially inactivate the paternal chromosomes, which leads to a contamination of the maternal nuclear genome of the gynogenotes with residues of the paternal chromosomes (Michalik et al., 2015; Polonis et al., 2018). Radiation-induced chromosome fragments are unstable genomic elements that may delay or even block the cell cleavages and interfere with the development of gonads in the gynogenotes (Krisfalusi et al., 2000). The application of heterologous sperm originating from species that form non-viable hybrids if crossed with the egg donor species excludes the risk of the contamination of the

gynogenotes with the radiation-induced fragments of the paternal chromosomes and has been successfully applied during gynogenesis in several salmonid fish species (Chourrout and Quillet, 1982; Chourrout, 1984, 1986; Kobayashi et al., 1993; Dobosz et al., 2015; Michalik et al., 2015; Jagiełło et al., 2017; Polonis et al., 2018). Here, the utility of UV-irradiated rainbow trout sperm for induction of gynogenetic development in the European grayling was confirmed. Reciprocal hybrids of rainbow trout and grayling are not viable (Dobosz et al., 2015, present study) presumably due to the chromosomal incompatibility and the cytonuclear conflict. Cytogenetically, both species differ substantially; the European grayling individuals from the stock used in this research have 100 chromosomes (Hliwa et al., 2022), while the rainbow trout from the Rutki strain show 59–62 chromosomes (Ocalewicz, 2002). Therefore, even if the trout chromosomes were not fully inactivated by the UV, their fragments would be eliminated from the grayling eggs presumably by a similar molecular mechanism as that responsible for the paternal chromosomes elimination observed in the other non-viable salmonid fish hybrids (Fujiwara et al., 1997).

Analysis of the microsatellite DNA exhibited residues of the paternal genome in about 33% of the examined graylings that hatched from eggs originating from the female ♀EG8 and activated by the UV-irradiated homologous sperm. Moreover, in some fish from this group, small fragments of chromatin that might be radiation-induced chromosome fragments of the male origin were observed. Some of the individuals with a confirmed contribution from the paternal genome appeared to be genetically males and one of them exhibited well developed testes. Thus, a hypothesis suggesting the transfer of the sex-related loci from partially UV-inactivated paternal chromosomes to the gynogenetic zygotes needs to be considered even though microscopic analysis confirmed the presence of only a few and tiny chromosome fragments in a few gynogenotes. It is not excluded that there were many more residues of the irradiated chromosomes in cells of the gynogenotes but some of them could have fused with the intact chromosomes, lost during subsequent cell cleavages during embryogenesis or after hatching, or they may simply have been too small for microscopic detection (Ocalewicz et al., 2004; Ocalewicz et al., 2012). Conditions of UV irradiation of sperm were the same for all males used in the experiment. The 'leak' of genetic information to the gynogenetic offspring from only one male suggests that the efficiency of UV-induced genetic inactivation of spermatozoa also depends on the inter-individual variation in the sperm quality, including sperm density, initial motility of the spermatozoa, and their velocity, among others.

An unexpected sex ratio among gynogenotes was also observed in other fish species (including rainbow trout and *Astyanax altiparanea)* and environmentally-induced sex reversal or mutation within sex determination gene(s) have been proposed to explain such phenomenon (Quillet et al., 2002; Nascimento et al., 2019). Here, the female sex genotype of the ♀EG8 egg donor and male and female sex genotypes of its gynogenetic offspring were confirmed molecularly (Yano et al., 2013) so, hypotheses related to environmentally induced sex reversion must be excluded. If it was an issue of an incomplete irradiation of the homologous sperm, then it is highly recommended to use UV-irradiated rainbow trout sperm for the gynogenetic activation of the grayling eggs to avoid any contamination with the irradiated genome. This is especially since the survival of gynogenotes that hatched from eggs activated by homologous and heterologous did not differ substantially (Fig. 1).

Inter-clutch differences in the survival rates of the gynogenotes that were hatched from eggs originating from different females recorded in our study and in the other research (Ocalewicz et al., 2013; Fopp-Bayat and Ocalewicz, 2015; Jagiełło et al., 2018, 2021; Ocalewicz et al., 2020) confirm that the quality of eggs and their maternal origin are important factors determining the efficiency of gynogenesis. As the exposure of eggs activated with UV-irradiated sperm to sub-lethal temperatures or high hydrostatic pressure for diploidization may decrease their developmental competence, it is crucial to use only eggs of the highest quality for the gynogenesis. Eggs originating from different females may show varied sensitivity to temperature and pressure shock (Yamaha et al., 2002) and eggs with a higher developmental competence for gynogenesis may also exhibit altered levels of transcripts of the particular maternal genes (Ocalewicz et al., 2020).

An increased ratio of individuals with body deformations is observed quite frequently among gynogenetic progeny including the graylings examined here. The types of body disorders in the gynogenetic graylings and their siblings from the control groups were the same, but the gynogenetic individuals exhibited an increased prevalence for malformations. Disturbances in the physical development in the gynogenotes are considered to be caused by the increased homozygosity, low gamete quality, side effects of applied physical shocks, nutritional factors and their interactions (Yamaha et al., 2002; Fjelldal et al., 2016; Jagiełło et al., 2021). Most of the malformed gynogenetic grayling showed an enlarged yolk-sac while the prevailing physical deformations in the brown trout, rainbow trout and brook trout were scoliosis and lordosis, emaciation and scoliosis, or scoliosis and kyphosis, respectively (Jagiełło et al., 2017, 2018, 2021). Species specific types of deformations in the genome-manipulated fish indicates the genetic-based etiology of the malformations.

5. Conclusions

Gynogenetic development in the European grayling has been induced by activating eggs with UV-irradiated homologous (996 J/m^2) and heterologous (rainbow trout) sperm (1370 J/m^2) and exposure of the inseminated eggs to high hydrostatic pressure shock (9000 psi for 5 min) for 20 min after the eggs' activation. Some of the graylings that hatched from the eggs activated by the UV-irradiated homologous sperm exhibited an admixture of the paternal genome. The maternal inheritance, only, has been proven when UV-irradiated heterologous (rainbow trout) sperm was used for the induction of gynogenesis. No significant differences in the survival of gynogenetic graylings that hatched from eggs activated by UV-irradiated homologous and heterologous sperm were reported.

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CRediT authorship contribution statement

Rafał Rożyński: Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **Marcin Kuciński:** Methodology, Writing – original draft, Visualization. **Stefan Dobosz:** Conceptualization, Methodology. **Konrad Ocalewicz:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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The Effect of Triploidy on Gonadal Development, Hematology and Biochemistry in the European Grayling (*Thymallus thymallus*)

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Simple Summary: Two-year-old triploid European graylings (*Thymmallus thymallus*) produced by the application of the high hydrostatic pressure (HHP) shock exhibited an increased white blood cell count (WBC), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) when compared to their diploid siblings. On the other hand, the red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit and mean corpuscular volume (MCV) were significantly lower in the triploid individuals. While diploid graylings had well-developed gonads filled with gametes, triploids were found to have significant problems with gonadal differentiation; ovaries were reduced without any oocytes, and testes were solid and non-spermiating.

Abstract: Sterile triploid European graylings (*Thymallus thymallus*) could serve as an alternative to allochthonous stocking, potentially protecting native populations from genetic introgression. In this study, two-year-old triploid and diploid graylings were examined to assess their hematological and biochemical characteristics and to evaluate the development of their gonads. When compared to diploids, triploids exhibited elevated white blood cell counts, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) by 5.66%, 162.68% and 207.57%, respectively. Moreover, the diameters of red blood cells and their nuclei were significantly higher in triploid graylings. In contrast, the red blood cell count, hemoglobin concentration (Hb), hematocrit and mean corpuscular volume (MCV) were lower in triploids by 64.82%, 5.80%, 70.16% and 14.49%, respectively. Most blood plasma biochemical indices showed no significant differences between specimens of different ploidies; however, triploids had a 21.96% higher level of triglycerides, while diploids had 3.74% more albumin. Additionally, the chloride concentration was 4.74% lower in triploids. Examined diploid males exhibited well-developed, sometimes asymmetrical testes and were actively spermiating. Triploid males were non-spermiating, and their testes were solid with varying morphology. Ovaries in diploid females contained mature oocytes, and in about 30% of the females, the body cavities were filled with ovulated eggs. In turn, the ovaries in triploid females were significantly reduced, usually transparent and lacked any oocytes. However, a longer study over a period exceeding two years needs to be performed to state unequivocally that triploid grayling females are sterile.

Keywords: biochemical indices; European grayling; genetic introgression; hematology; protecting native populations; triploidization; sterility; stocking

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1. Introduction

The presence of three sets of chromosomes in somatic cells is defined as triploidy [1]. Spontaneous triploid fish arise during meiotic alterations within a species (autotriploids) and in interspecies hybrids (allotriploids) [1]. Disturbances in the gametogenesis process, caused by cytogenetic changes during meiosis including the pre-meiotic endoduplication of the chromosome set and the suppression of the first or second meiotic division [2], result in the development of autotriploids and have been observed in several fish species, including California roach (*Hesperoleucus symmetricus* Baird & Girard, 1854) [3], rainbow trout (*Oncorhynchus mykiss*) [4,5], nurse shark (*Ginglymostoma cirratum* Bonnaterre, 1788) [6] and pond loach (*Misgus anguillicaudatus* Cantor, 1842) [7], among others. An increased ratio of triploids is observed among fish embryos developing in post-ovulatory (aged) eggs, which are characterized by cytoskeletal alterations that lead to the suppression of the second meiotic division [8,9]. In contrast, allotriploids possess three sets of chromosomes derived from different species [1]. Such situations have been described, among others, in hybrid complexes of *Cobitis* (loaches) [10–13], Prussian carp (*Carassius gibelio* Bloch, 1782) [10,13], hybrid minnow *Squalius alburnoides* [14] and pond loach [7,15]. In these cases, triploid females are fertile and can reproduce gynogenetically, producing non-reduced triploid eggs [1]. Triploid fish can also be generated intentionally by mating diploid and tetraploid individuals [10,16–20] or by the dispermic fertilization of a haploid egg [21,22]. Furthermore, chemical or physical shock applied to fertilized eggs disrupts the microtubules of the meiotic spindle, preventing the extrusion of the second polar body and resulting in the development of triploid embryos [23,24]. The additional set of chromosomes in triploid specimens causes cytogenetic incompatibility that impairs proper gonadal development and gamete production [25]. Triploid females usually exhibit highly reduced ovaries that are unable to produce eggs. In contrast, triploid males develop testes comparable in size to those observed in diploid individuals; however, such fish are generally infertile due to the aneuploidy of their spermatozoa [1,25]. Triploid fish do not experience a decline in growth, survival or meat quality, which typically accompanies sexual maturation in normal diploid specimens. Thus, artificial triploidization is a promising method for cultivating fish species that exhibit early sexual maturation [1,26]. Moreover, the sterility of triploid individuals inhibits interactions between fish from wild stocks and those that have escaped from fish farms or are deliberately released into rivers or lakes as part of stocking programs for recreational fishing purposes.

The European grayling (*Thymallus thymallus* L.) is considered one of the most attractive species for fly fishing in Europe; however, it is also one of the salmonids that is endangered or even critically endangered [27–31]. The distribution range and size of specific populations of the European grayling have decreased due to water pollution, habitat destruction, river engineering, predation by birds and intensive fishing [27–31]. Unfortunately, restocking with farmed specimens has resulted in the contamination of many local European grayling populations with non-native individuals [32,33]. To produce safe stocking material, a protocol for the production of triploid graylings has recently been developed [34].

European graylings mature sexually in the second year of life [5]. The only available data on gonadal development in triploid graylings were collected from much younger specimens; gonads of one-year-old diploid and triploid graylings were macroscopically similar in most of the examined individuals, and only histological analysis confirmed that triploids had problems with gonadal development [34]. As only sterile triploids may be considered safe stocking material, the main purpose of the present research was to evaluate gonadal development in sexually matured two-year-old diploid graylings and their triploid siblings. We also took advantage of the fact that the sampled individuals were of a size

enabling blood collection and estimated hematological and plasma biochemical indices that may help to monitor fish health and physiological status [35–37]. The hematology examination and assessment of welfare-related blood parameters (concentration of glucose and triglycerides, among others) are particularly important in triploids as fishes with an additional set of chromosomes usually have higher environmental requirements than diploids and are less tolerant than diploids to nonoptimal rearing conditions [1].

2. Materials and Methods

2.1. Fish, Origin and Rearing Conditions

Diploid (n = 40) and triploid (n = 40) European graylings from the families established and kept in the Department of Salmonid Research (DSR) of the Inland Fisheries Institute (IFI) in Olsztyn, Rutki, Poland, were sampled (13–14 June 2022) and studied. Triploid families of the European grayling were generated by the application of a recently elaborated protocol that involves the application of a 5 min high hydrostatic pressure (HHP) shock (9000 psi) 20 min after egg insemination using a TRC-APV electric/hydraulic device (TRC Hydraulics Inc. in Dieppe, NB, Canada) [34]. Examined individuals were the offspring of five females, and eight diploid and eight triploid siblings from each family were randomly selected for the experiment. The body length and body weight of diploids ranged from 22.7 to 27.7 cm [(25.1 ± 1.2 cm (mean ± SD)] and from 87.3 to 162.2 g [(118.4 ± 15.9 g (mean ± SD)], respectively. In the case of triploids, body length varied from 23.0 to 28.7 cm (25.8 ± 1.5 cm) and body weight varied from 91.7 to 174.2 g (124.5 ± 22.6 g). Both diploid and triploid grayling individuals were reared separately under the same husbandry conditions in hatchery plastic tanks of 0.3 m^3 (first year) and 1.2 m^3 (second year) in size. The fish were fed daily, and feeding rates were adjusted to their growth and diurnal temperature. European graylings from both stocks were fed with the feed formulated for rainbow trout. For the first month, the fish were fed with Aller Infa EX GR 0.4 mm granulated feed and then Aller Futura EX GR 0.5–1.0 mm and Aller Futura EX GR 0.9–1.6 mm. Juvenile and adult individuals were fed with Aller Futura EX GR 1.3–2.0 mm pellets (Aller Aqua A/S, Christiansfeld, Denmark) containing the following: 58% crude protein, 17% crude fat, 6.1% nitrogen-free extract (NFE), 12.2% ash and 0.7% fiber, with a total energy of 21.6 MJ kg^{-1} of feed (manufacturer data).

2.2. Blood Sampling

Prior to blood collection, the fish were anesthetized using an aqueous solution of MS-222 at a dose of 100 mg L^{-1} buffered with sodium bicarbonate (2 min, Sigma-Aldrich, St. Louis, MO, USA) [34,38]. Blood was collected with heparinized syringes (Smiths Medical International ASD, Inc., St. Paul, MN, USA) directly from the caudal vein (approximately 1 mL of blood from each individual). Immediately after blood collection, the fish were euthanized without regaining consciousness using an aqueous solution of MS-222 at a dose of 200 mg L^{-1}. Preparations for the erythrocyte measurements were made by the smearing of the heparinized whole blood on the microscopic slides. The preparations of blood were air-dried, then fixed in methanol for 20 min and finally stained with 10% Giemsa solution for 15 min.

2.3. The Macroscopic Morphology of the Gonads

After the blood collection procedures, the euthanized specimens were subjected to a macroscopic analysis of the gonads. For this purpose, the abdominal integuments of each individual were cut open, and the gonads were observed to determine their morphology.

The testes of each male were assigned to one of the following categories:
(A) Milky white, symmetrical, spermiating;
(B) Milky white, symmetrical, non-spermiating;
(C) Milky white, asymmetrical or segmented, spermiating;
(D) Milky white, asymmetrical or segmented, non-spermiating;
(E) Pinkish, symmetrical, non-spermiating;
(F) Pinkish, asymmetrical or segmented, non-spermiating.
The ovaries were assigned to the following categories:
(A) Yellow–orange, symmetrical with observed non-ovulated oocytes;
(B) Yellow–orange, symmetrical with ovulated oocytes;
(C) Yellow–orange, asymmetrical with observed non-ovulated oocytes;
(D) Yellow–orange, asymmetrical with ovulated oocytes;
(E) Pinkish milky, reduced, symmetrical, no oocytes;
(F) Pinkish transparent, reduced, symmetrical, no oocytes;
(G) Pinkish transparent, reduced, asymmetrical, no oocytes.

2.4. Erythrocyte Measurements

The cell (C) and nucleus (N) diameters of fifty erythrocytes from each sampled specimen were measured using the microscope Eclipse E600 (NIKON, Tokio, Japan) equipped with the camera and software NIS-Elements BR 3.2 (NIKON, Tokio, Japan). Then, the nucleocytoplasmic index (NCI) was calculated ($NCI = N \times C^{-1}$) [39].

2.5. Automatic Measurement of Hematological and Biochemical Indices

Blood samples (approximately 1 mL) were collected from the caudal vein of the anesthetized individuals using heparinized syringes (Smiths Medical International ASD, Inc., St. Paul, MN, USA). Hematological parameters including white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined. Biochemical indices including glucose (GLU), triglycerides (TGs), cholesterol (CHOL), total protein (TP), albumin (ALB), globulin (GLB), total bilirubin (BIL-T), creatinine (KREA), lactate (LACT), ammonia (AMON), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), amylase (AMYL), potassium (K^+), sodium (Na^+), chloride (Cl^-), phosphorus (P), calcium (Ca) and magnesium (Mg^{2+}) ions were measured in the blood plasma provided by the centrifugation of the blood at 4000 rpm for 3 min (Fresco 17, Thermo Scientific, Waltham, MA, USA). Hematological tests were performed using a semi-automated hematology analyzer, the BC-2800 VET (Mindray, Shenzhen, China). The analyzer readings were calibrated for several fish species, including the grayling, based on the hematological results obtained by traditional methods [40] by its distributor, Stamar (Dąbrowa Górnicza, Poland). Plasma biochemical tests were performed using the BS-120 automated biochemical analyzer (Mindray, Shenzhen, China).

2.6. Statistical Analysis

The obtained values were subjected to statistical analysis in the Statistica 12 program (StatSoft, Inc., Tulsa, OK, USA). The normality of variances was tested using the Shapiro–Wilk W test. The obtained values for diploid and triploid graylings were compared using Student's *t*-test. Differences were recorded as significant at $p \leq 0.05$.

3. Results

3.1. Macroscopic Analysis of Gonads

European graylings from the broodstock used in the present research sexually mature usually at the age of two. The examined diploid males had well-developed testes (with some asymmetry observed in less than 30% of males), and all were spermiating (Figure 1 and Table 1). In turn, triploid males were not spermiating at all, and their testes were solid, showing large variation in morphology (Table 1). The ovaries of most diploid females contained matured oocytes. Ovulated eggs were observed in about 30% of the females. Ovaries in triploid females were highly reduced and were transparent without oocytes (Figure 2 and Table 2).

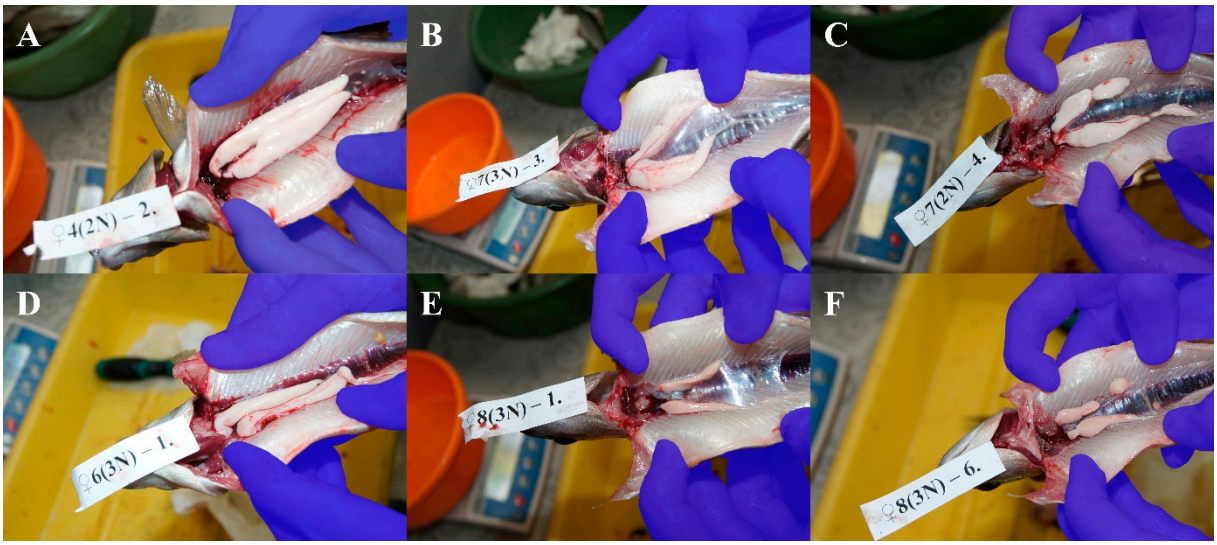

Figure 1. Development of gonads in diploid and triploid males: (**A**) milky white, symmetrical, spermiating; (**B**) milky white, symmetrical, non-spermiating; (**C**) milky white, asymmetrical or segmented, spermiating; (**D**) milky white, asymmetrical or segmented, non-spermiating; (**E**) pinkish, symmetrical, non-spermiating; (**F**) pinkish, asymmetrical or segmented, non-spermiating.

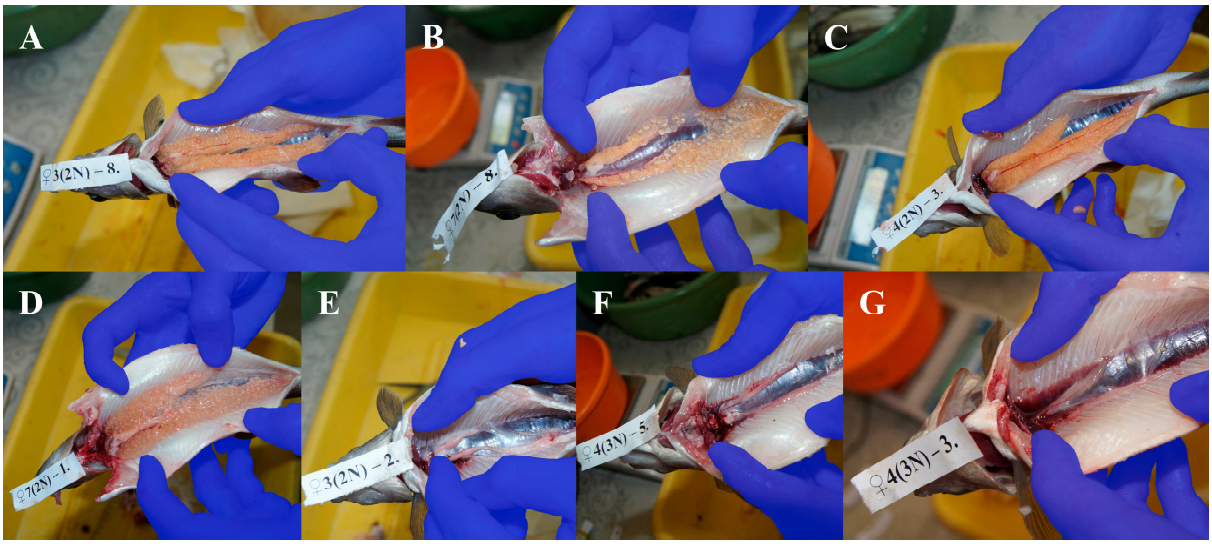

Figure 2. Development of gonads in diploid and triploid females: (**A**) yellow–orange, symmetrical with oocytes; (**B**) yellow–orange, symmetrical, ovulation; (**C**) yellow–orange, asymmetrical with oocytes; (**D**) yellow–orange, asymmetrical, ovulation; (**E**) pinkish milky, reduced, symmetrical, no oocytes; (**F**) pinkish transparent, reduced, symmetrical, no oocytes; (**G**) pinkish transparent, reduced, asymmetrical, no oocytes.

Table 1. Characteristics of the testes in diploid and triploid European grayling males and percentage of individuals with each particular testicle type.

Classification of Testes	Diploid Males (n = 25)	Triploid Males (n = 25)
(A) milky white, symmetrical, spermiating	72.00%	-
(B) milky white, symmetrical, non-spermiating	-	4.00%
(C) milky white, asymmetrical or segmented, spermiating	28.00%	-
(D) milky white, asymmetrical or segmented, non-spermiating	-	28.00%
(E) pinkish, symmetrical, non-spermiating	-	32.00%
(F) pinkish, asymmetrical or segmented, non-spermiating	-	36.00%

Table 2. Characteristics of the ovaries in diploid and triploid European grayling females and percentage of individuals with particular types of ovaries.

Classification of Ovaries	Diploid Females (n = 15)	Triploid Females (n = 15)
(A) yellow–orange, symmetrical with oocytes	33.33%	-
(B) yellow–orange, symmetrical, ovulation	26.67%	-
(C) yellow–orange, asymmetrical with oocytes	20.00%	-
(D) yellow–orange, asymmetrical, ovulation	6.67%	-
(E) pinkish milky, reduced, symmetrical, no oocytes	13.33%	-
(F) pinkish transparent, reduced, symmetrical, no oocytes	-	47.06%
(G) pinkish transparent, reduced, asymmetrical, no oocytes	-	52.94%

3.2. Hematological Indices

The hematological indices of triploid and diploid European graylings are presented in Figure 3. In triploid individuals, the white blood cell count (WBC), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were 5.66%, 162.68% and 207.57% (respectively), higher than in diploids ($p \leq 0.05$). In turn, the red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT) and mean corpuscular volume (MCV) in triploids were 64.82%, 5.80%, 70.16% and 14.49% lower, respectively (Figure 3).

3.3. Biochemical Indices

Most of the biochemical indices measured in the blood plasma did not show significant differences between diploid and triploid graylings (Tables 3 and 4); however, the concentration of triglycerides was higher by 21.96% in triploids, while the concentration of albumins was higher by 3.74% in diploids ($p \leq 0.05$) (Table 3). Moreover, the chloride concentration was lower by 4.74% in the triploid specimens ($p \leq 0.05$) (Table 4).

Table 3. Mean and standard deviation of plasma metabolites in diploid and triploid European graylings (*Thymallus thymallus*).

	Diploid Specimens (n = 40)	Triploid Specimens (n = 40)	Significant Differences ($p \leq 0.05$)
Glucose (mg/dL)	79.55 ± 12.95	85.55 ± 15.93	0.068
Triglycerides (mg/dL)	298.04 ± 116.15	363.49 ± 142.53	0.027
Cholesterol (mg/dL)	515.05 ± 118.05	478.53 ± 108.00	0.153
Total protein (g/dL)	3.82 ± 0.32	3.74 ± 0.30	0.301
Albumin (g/dL)	2.06 ± 0.20	1.98 ± 0.12	0.038
Globulin (g/dL)	1.75 ± 0.21	1.76 ± 0.21	0.320
Total bilirubin (mg/dL)	0.25 ± 0.22	0.19 ± 0.18	0.191
Creatynine (mg/dL)	0.25 ± 0.09	0.25 ± 0.08	0.901
Lactate (mg/dL)	58.60 ± 22.32	68.76 ± 24.94	0.059
Ammonia (µg/dL)	534.30 ± 174.58	593.36 ± 170.37	0.130
Alanine transaminase (U/L)	51.83 ± 28.88	55.09 ± 29.95	0.622
Aspartate transaminase (U/L)	1890.07 ± 1235.95	2417.49 ± 1534.91	0.095
Alkaline phosphatase (U/L)	360.68 ± 65.26	347.40 ± 87.55	0.444
Amylase (U/L)	45.15 ± 18.32	42.50 ± 14.12	0.470

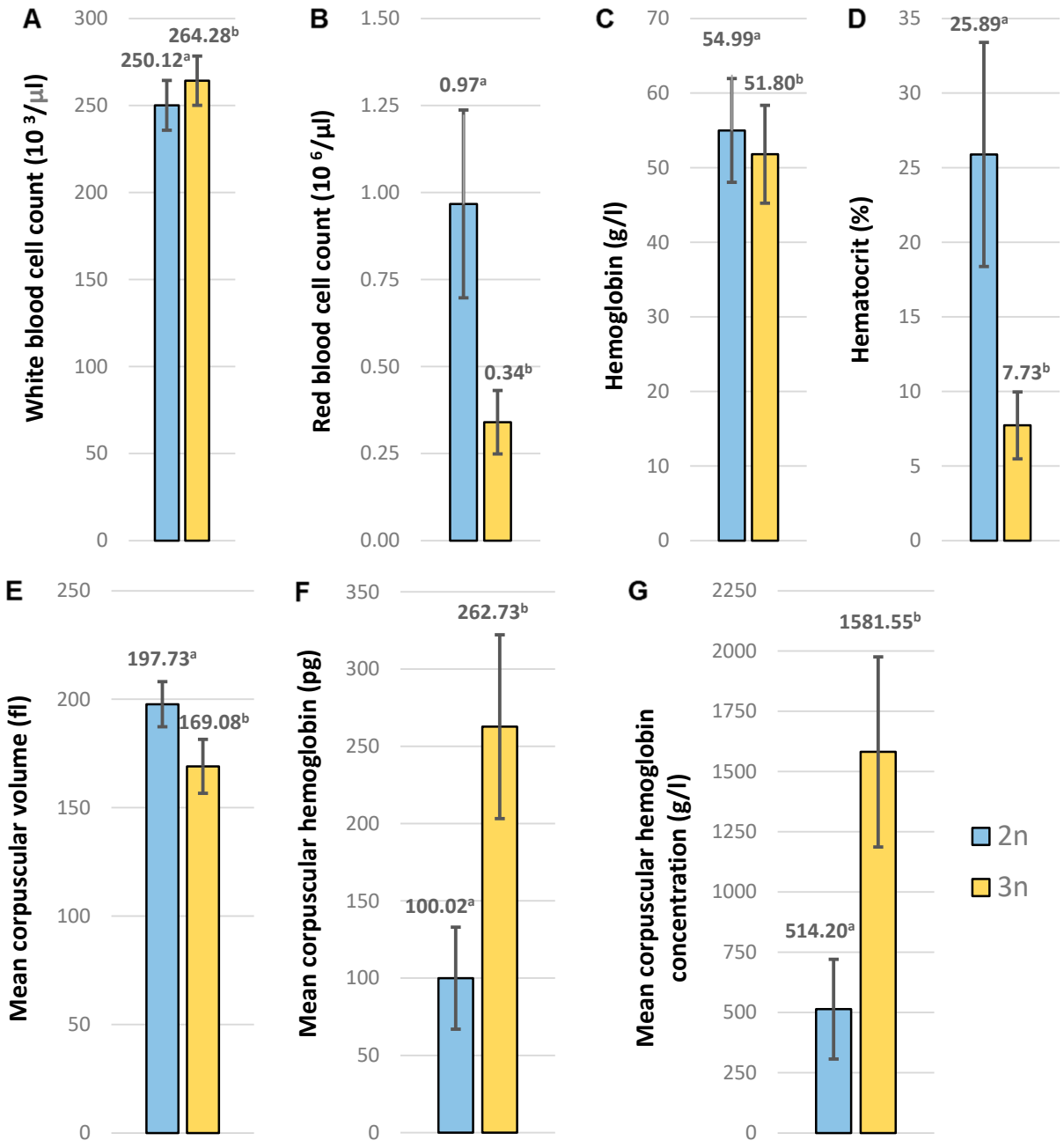

Figure 3. Hematological indicators of diploid (2n) and triploid (3n) European graylings: (**A**) white blood cell count; (**B**) red blood count; (**C**) hemoglobin; (**D**) hematocrit; (**E**) mean corpuscular volume; (**F**) mean corpuscular hemoglobin; (**G**) mean corpuscular hemoglobin concentration. Distinct letters indicate statistically significant ($p \leq 0.05$) differences in the recorded values.

Table 4. Mean and standard deviation of ion concentrations in diploid and triploid European graylings (*Thymallus thymallus*).

	Diploid Specimens (n = 40)	**Triploid Specimens (n = 40)**	**Significant Differences ($p \leq 0.05$)**
Potassium (mmol/L)	1.73 ± 0.68	1.62 ± 0.79	0.504
Sodium (mmol/L)	159.04 ± 5.60	158.34 ± 5.17	0.562
Chloride (mmol/L)	299.84 ± 29.05	285.64 ± 34.31	0.049
Phosphorus (mg/dL)	8.19 ± 1.45	8.34 ± 1.29	0.641
Calcium (mg/dL)	12.65 ± 3.19	11.76 ± 1.11	0.999
Magnesium (mg/dL)	2.73 ±0.33	2.73 ± 0.25	0.942

3.4. Erythrocyte Measurements

The lengths of erythrocytes for diploid and triploid fish ranged from 10.99 µm to 17.97 µm and from 15.43 µm to 24.59 µm, respectively, while the lengths of the erythrocyte nuclei ranged from 4.46 µm to 9.58 µm (diploids) and from 7.32 µm to 12.59 µm (triploids). The diameters of the erythrocytes and erythrocyte nuclei of graylings from the triploid group were greater than those in fishes from the diploid group by 37.62% and 33.81%, respectively, and the differences were significant ($p \leq 0.05$). In turn, no significant differences in the NCI between individuals from diploid and triploid groups were observed (Figures 4 and 5).

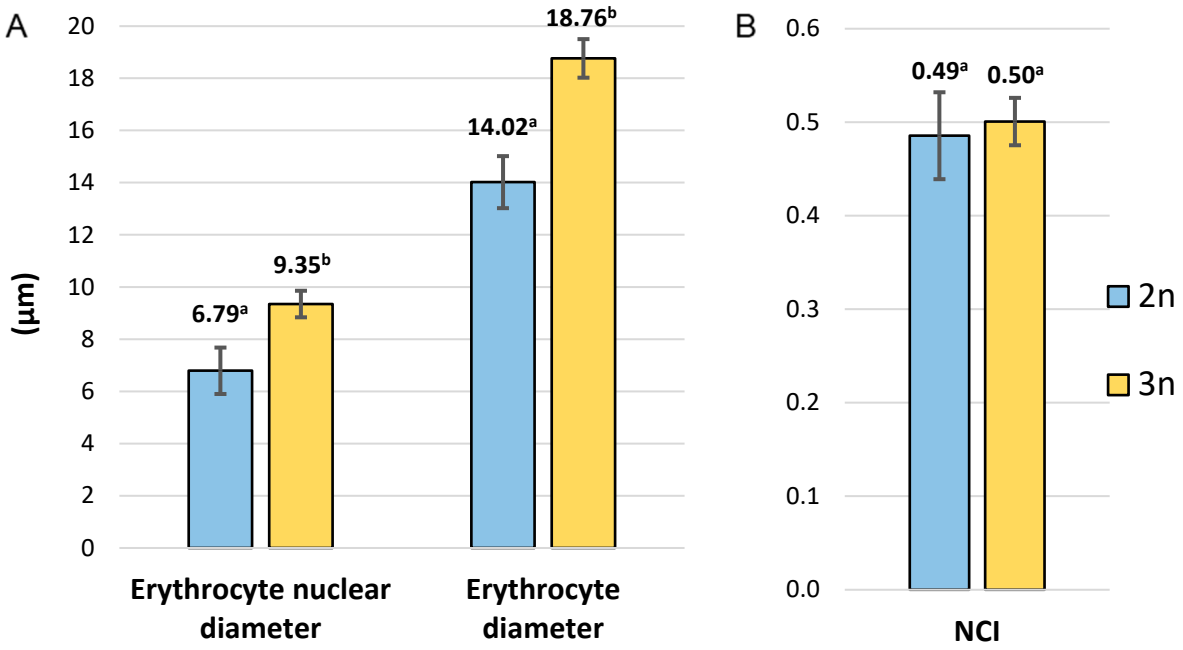

Figure 4. The diameters of erythrocyte nuclei and erythrocytes (**A**) and NCI (**B**) of diploid and triploid European graylings (mean (±SD)). Distinct letters indicate statistically significant ($p \leq 0.05$) differences in the recorded values.

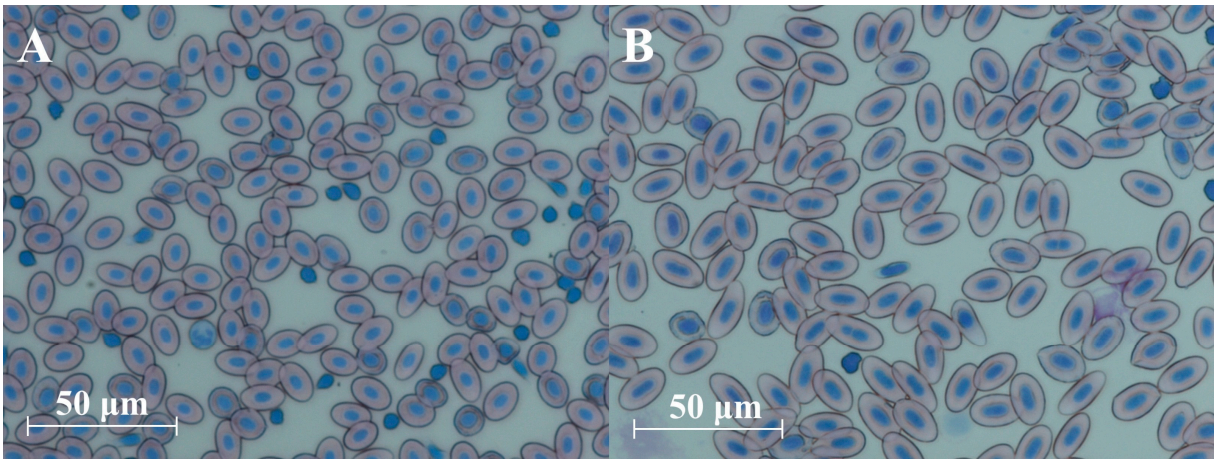

Figure 5. Erythrocytes of diploid (**A**) and triploid (**B**) European graylings (*Thymallus thymallus*).

4. Discussion

In triploids, problems with the segregation of three sets of chromosomes during meiosis result in improper gonadal development and disturbed gametogenesis, and triploid

females are considered sterile [1,25,41,42]. In one-year-old triploid graylings, macroscopically visible pairs of gonads are morphologically like those of diploid specimens [34]. Only histological examination exhibited that gonadal strands in the triploid specimens were composed of connective tissues including fibroblasts, adipocytes and degenerated epithelial structures, while the gonads of their diploid counterparts contained properly formed gametes [34]. Moreover, in the case of one-year-old triploid grayling individuals, it was not possible to distinguish males and females based on macroscopic and histological analyses of their gonads. European graylings from the studied broodstock reach sexual maturity in the second year after hatching [43]; thus, it was reasonable to examine the status of gonadal development in diploids and triploids two years after hatching. The two-year-old diploid European grayling individuals analyzed in the present study were matured, with well-developed testes reported in males and ovulated oocytes observed in females (Figures 1 and 2, Tables 1 and 2). Among their triploid siblings, a simple macroscopic analysis enabled the identification of males that usually had asymmetrical and segmented testes (Figure 1) and females showing gonadal strands similar to those observed in the one-year-old triploids (Figure 2). The testes of the triploid males were well developed and, in some cases, similar to testes from the diploids. Triploid male salmonids usually develop testes with germ cells at the different stages of development/maturation [44], and in at least some of such males, spermiation has been observed [45]. In the brown trout, three-year-old and older triploids produce high-quality sperm [46]. The lack of oocytes in the ovaries of two-year-old triploid grayling females suggests that such individuals might be sterile. Nonetheless, taking into account that in triploid brown trout, gonadal differentiation is only delayed and five-year-old triploid females develop functional ovaries [46], research over a period exceeding two years needs to be performed to ascertain if triploid grayling females are sterile.

In general, triploid individuals, when compared to diploids, show increased heterozygosity and have larger but fewer cells, and the development of their gonads is disturbed [25]. Concerning the number and size of the cells in the adult European grayling, an additional haploid set of chromosomes caused a significant increase in the diameters/dimensions of the erythrocytes and their nuclei in triploids; however, the nucleocytoplasmic index (NCI) did not differ between triploids and diploids (Figure 4). A similar pattern has also been observed in one-year-old cytogenetically verified diploid and triploid graylings [34]. The substantial differences between diploid and triploid red cell dimensions observed in the present study make erythrocyte measurement a reliable, less laborious and inexpensive approach to verifying the ploidy level in the graylings. Although it has been widely observed that the ploidy level affects cell size [25], this is not the case here, where the cell volumes (MCV) in the diploid and triploid grayling individuals do not differ significantly (Figure 3). A similar trend has been observed in triploid sturgeons, where the MCV in triploids was only slightly higher than the MCV observed in diploid specimens [47]. The relationship between the ploidy level and cell size might be much more complex than the simple assumption that increased numbers of copy genes increase the amount of proteins, which eventually results in the increased cell volume. In *Arabidopsis thaliana*, experimental data suggest that the ploidy level is not directly linked to the cell volume [48].

The number of erythrocytes was substantially lower in the triploid graylings when compared to diploids, which was followed by a reduction in the hematocrit (Hct), but a decreased number of red blood cells did not affect the level of total hemoglobin in the whole blood (Hb), which did not differ significantly in the specimens characterized by different ploidies (Figure 3). Although a reduced number of red cells is a common feature of triploids, the total hemoglobin level in specimens with an additional set of chromosomes showed large interspecies variation and may be comparable to that observed in diploids [49–51],

lower [52–54] or higher [46]. In turn, the hemoglobin content per cell assessed in the erythrocytes (MCA) and in the volume of erythrocytes (MCHC) was substantially increased in the triploid graylings (Figure 3). A similar pattern has been observed in triploids of other species [52,55,56].

Contrary to red blood cells, no significant differences in the leucocyte counts between triploid and diploid graylings were observed. A lack of substantial differences in the white blood cells has also been described in tench [57] and Caspian trout [58]; nevertheless, other studies show decreased leucocyte counts in triploids [59]. The similar number of leucocytes in graylings of different ploidies suggests that diploid and triploid specimens have similar non-specific defense activities and comparable susceptibility to the disease.

Many studies have demonstrated that under optimal environmental conditions, the aerobic and anaerobic capacity of diploid and triploid salmonids are comparable [50,51,60,61]. However, under less favorable conditions including a higher water temperature and lower oxygen level, triploid salmonid individuals exhibit reduced performance [62,63]. Therefore, it can be inferred that triploid specimens are less resistant to the stress caused by suboptimal circumstances. In our studies, however, glucose and lactate concentrations (secondary stress markers) did not differ substantially between diploid and triploid graylings. The lack of differences in these markers may indicate that the examined fish were reared under optimal rearing conditions.

Measurements of triglyceride levels, in combination with other lipid markers, are useful in the diagnosis of hyperlipoproteinemia, dyslipidemia and triglyceridemia. Assessments of triglyceride concentrations are also used in the diagnosis and treatment of diabetes, nephrosis, liver obstruction and other diseases related to lipid metabolism or various endocrine disorders [64,65]. The levels of these parameters, along with other biochemical indicators such as total protein, albumins, globulins and ALP in blood plasma/serum, generally reflect endocrine function and the integrity of key organs, especially the liver and kidneys, and are often used to evaluate the nutritional status of fish [66,67]. In the present research, the levels of triglycerides, albumins and chloride ions differed significantly between diploids and triploids. The elevated triglyceride level observed in triploids may indicate disturbances in body lipid metabolism or even impaired liver function. In turn, the significantly reduced albumin level could be linked with malnutrition in the individuals with higher ploidy. However, the levels of the above-mentioned parameters, even if they differ between 2n and 3n, are still within the ranges that are found in healthy salmonid fish species [63], which undoubtedly suggests the proper performance of the triploid graylings.

5. Conclusions

An additional set of chromosomes has a large impact on gonadal development in the European grayling. Two-year-old diploid individuals have properly developed ovaries and testes filled with gametes, while triploid ovaries are highly reduced without any oocytes and triploid testes are solid and non-spermiating. Diploid females were ovulating, and males were spermiating. In turn, hematological analysis exhibited that triploids have bigger red blood cells, an elevated white blood cell count and increased mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) than diploids, but the red blood cell count, hemoglobin concentration (Hb) and hematocrit were higher in diploids. Smaller differences between specimens of different ploidies were observed when plasma biochemical indices were assessed. Two-year-old triploid grayling females were shown to have underdeveloped ovaries lacking oocytes; however, research over a longer period exceeding two years needs to be undertaken to state if such individuals are sterile.

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Growth performance, survival rates and catchability of diploid and triploid European grayling (*Thymallus thymallus*)

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ABSTRACT

European grayling (*Thymallus thymallus* L.) is an important bioindicator of the health and quality of riverine ecosystems and one of the most prized trophies in the sport of fly fishing in Europe. The extensive use of hatchery-reared and non-native individuals for stocking of open waters has caused genetic introgression in many indigenous grayling populations, potentially impairing their local adaptations and fitness. The reduced fertility of triploid fishes possibly offers a viable opportunity for stocking open waters with lessened genetic risk to the native populations, thereby permitting fisheries managers to provide high-quality, accessible fishing experiences for anglers. The aim of the present study was to compare growth rate, survival and food competition between mixed–sex diploid and triploid European graylings from the hatching to the adulthood under aquaculture settings. The catchability and prowess of two-year-old diploid and triploid grayling individuals were also examined under conditions simulating the natural environment. Mixed–stock diploid and triploid graylings in our experiment showed comparable growth rates and survival. Food competition trials revealed slightly lower effectiveness in food acquittance and less aggressive feeding behaviors among triploids when compared to diploid graylings. The catchability and prowess trials did not reveal significant differences between diploids and triploids. Our preliminary study indicated that hatchery-reared triploid graylings offer a viable opportunity to stock open waters enabling fisheries managers to meet the growing demand for the recreational fishing. However, further research is required to confirm the degree of sterility in triploid graylings, as well as to evaluate their performance and catchability in open waters.

1. Introduction

Triploidization is a reproductive biotechnique that allows for the production of fish with genomes composed of three sets of chromosomes (3 n). The odd chromosome number in the triploid fishes causes disturbances in their gonadal development and gamete production (Benfey, 1999; Maxime, 2008). In salmonids, triploid females show highly reduced ovaries, usually containing a few aneuploid and immature oocytes, which suggests such females might be sterile. Though, in the triploid brown trout, ovarian differentiation is only delayed and five-year-old triploid females can produce a small number of eggs that are able to be fertilized (Lahnsteiner and Dünser, 2024). In turn, triploid males develop testes with similar morphology to these observed in the diploid specimens but with a limited number of aneuploid spermatozoa capable for the egg fertilization (Benfey, 1999; Maxime, 2008). Progeny resulting from the interploid crossing typically experience early mortality during embryonic development (Benfey, 1999; Maxime, 2008), although in some cases, they can develop normally and hatch (Lahnsteiner and Dünser, 2024). Due to reduced fertility, triploid fishes invest energy acquired from food in the somatic growth instead of the gonadal development and gamete production what in diploids cause decreased growth and deterioration of the meat quality (Piferrer et al., 2009). Thus, mono-sex female triploid (XXX) stocks of certain salmonid species have found application in the aquaculture production (Piferrer et al., 2009 Fraser et al., 2012; Zhou and Gui, 2017). Apart from the food production, triploid fishes with confirmed sterility offers potential applications as safe stocking material that minimizes the risk of detrimental genetic introgression into native fish populations (Kozfkay et al.,

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2006). Triploid salmonids have been released into open waters across various countries, particularly in North America and the British Isles, with their use for stocking purposes mainly involving rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), sockeye salmon (*Oncorhynchus nerka*), Atlantic salmon (*Salmo salar*), and brown trout (*Salmo trutta*) (Brock et al., 1994; Warrillow et al., 1997; Cotter et al., 2000; Dillon et al., 2000; Wilkins et al., 2001; Chatterji et al., 2007; Environment Agency, 2009; Koenig et al., 2011; Simon et al., 2011; Pease et al., 2023).

Despite the triploid fishes are expected to display faster growth rates, larger ultimate body sizes and increased longevity (Benfey, 1999; Maxime, 2008), the available observations have shown that the growth rates and survival of triploids are frequently the same or even lower than those of diploids (Quillet and Gaignon, 1990; Brock et al., 1994; Ojolick et al., 1995; Galbreath and Thorgaard, 1995; McCarthy et al., 1996; Teuscher et al., 2003; Simon et. al., 2011). Moreover, triploid individuals may be more sensitive than diploids to the sub-lethal environmental conditions, such as hypoxia and suboptimal temperatures (Rahel and Kolar, 1990; Benfey, 1999; Maxime, 2008; Piferrer et al., 2009). It has been observed that the overall fitness and performance of triploid fishes when compared to their diploid counterparts appear to be highly variable and frequently depend on the fish species or even strain (Ihssen et al., 1990; Brock et al., 1994; Rutz and Baer, 1996; Warrillow et al., 1997; Cotter et al., 2000; Dillon et al., 2000; Teuscher et al., 2003). Although, some studies suggest that hatchery-reared triploid trout when stocked at the catchable size have rather limited ecological impact due to their short post-stocking lifespan (*e.g.,* Cotter et al., 2000; Meyer and Schill, 2007; Fraser et al., 2012), concerns remain that under certain conditions, triploids may still compete with the native diploid fish for food and habitat, especially when stocked in the large numbers or in the sensitive ecosystems (Solar et al., 1986; Teuscher et al., 2003). Hence, evaluating both the performance and the value of trophy angling for the triploid fish, along with their potential competition with diploids, is crucial for developing sustainable stocking strategies utilizing triploids that maximize the recreational potential of open waters while minimizing adverse impacts on the wild genetic resources.

European grayling (*Thymallus thymallus* L.), hereafter grayling, is a non-anadromous member of the family Salmonidae (order Salmoniformes) and the only representative of grayling species endemic to Europe. It inhabits cold, pure, well-oxygenated riverine waters, lakes and the brackish waters of the Baltic Sea. Apart from its ecological significance as an important bioindicator of the health and quality of riverine ecosystems, grayling is also one of the most prized trophies in the fly-fishing (FishBase, 2024). Several grayling populations have substantially declined, both in the distribution range and in the abundance over the past few decades, mainly because of the environmental degradation, water pollution, overfishing and poaching (Susnik et al., 2004; Maric et al., 2012; Weiss et al., 2013). As a result, grayling is currently classified as critically endangered or endangered in many areas of Europe (HELCOM.,2013; IUCN, 2024). Various conservation measures including fishing restrictions and stocking of open waters have been, thus, undertaken to support local populations of the species (Lyach and Remr, 2019, 2020). Unfortunately, the widespread use of the hatchery-reared fish and individuals from the allochthonous populations has led to the genetic introgression of numerous local grayling populations with the non-native specimens (Koskinen et al., 2002; Susnik et al., 2004; Duftner et al., 2005). Due to the genetic introgression, several indigenous grayling populations have suffered from the reduced fitness and lost their local adaptations (Avise and Hamrick, 1996; Geist et al., 2009; Turek et al., 2010; Huff et al., 2011).

Efficient protocol for induction of the triploid development in the grayling have already been established (Hliwa et al., 2022). Moreover, histological analysis showed that one-year-old triploid grayling females (XXX) and males (XXY) had sterile gonads composed of connective tissue including fibroblasts, adipocytes and degenerated epithelial structures without any differentiated germ cells (Hliwa et al., 2022). In two-year-old grayling males, solid, non-spermiating testes with varying morphology were reported, while triploid females of the same age had significantly reduced, usually transparent ovaries lacking any oocytes (Rożyński et al., 2025). Despite the promising potential of triploids to mitigate the issue of the genetic contamination of the autochthonous populations and to meet the high angling demand, triploid graylings have not yet been considered for stocking into open waters across Europe.

As the suitability of the triploid graylings for stocking open waters remains largely unknown, this study aimed to compare survival, growth rate and food competition between diploid and triploid graylings from hatching to the moment when the diploids mature sexually (two-year-old) under the aquaculture conditions. Additionally, the catchability of diploid and triploid adult grayling individuals was examined under conditions simulating the natural environment.

2. Materials and methods

2.1. European grayling stock production and ploidy level verification

Diploid and triploid stocks of grayling specimens examined in the present study were produced using gametes originating from five females and two males from the stock kept at the Department of Salmonid Research (DSR), National Inland Fisheries Research Institute in Olsztyn, located in Rutki, Poland (54°19'51.1"N 18°20'15.1"E). Three- and four-year-old spawners were reared in a pond with a capacity of 3000 m^3 and a water flow 5 dm^3/s that was supplied with water from the Radunia River (mean temperature 10°C and oxygenation level above 80 %). The fish were fed commercial broodstock feed (Aller Bronze 3 mm and Aller Silver 3 mm, Aller Aqua, Poland) three or four times daily in amounts ranging from 0.5 % to 1.5 % fish biomass depending on water temperature.

Feeding ceased two weeks before the spawning period and two experimental crossings were carried out on 23.04.2020 and 22.04.2021. Prior to gamete stripping, selected specimens were anesthetized with MS-222 (50 mg/dm^3, Sigma-Aldrich, Spain). Eggs stripped from five grayling females were pooled in a single container and thoroughly mixed before the milt was added to fertilize the entire batch. Before fertilization, spermatozoa quality was assessed under the microscope after activation with the sperm activating medium (SAM) (1 mM $CaCl_2$, 20 mM Tris, 30 mM glycine, 125 mM NaCl pH 9.0) under an optical microscope (Nikon Eclipse E 2000, Tokio, Japan) at a total magnification of 100 × .

Eggs were inseminated in the presence of SAM and thoroughly washed with the hatchery water. To induce triploid development, half of the activated eggs incubated in water at 10°C were subjected to the high hydrostatic pressure (HHP) shock of 9000 psi that was applied 20 min after egg fertilization at 10°C (Hliwa et al., 2022). The HHP shock was generated with a TRC-APV electric/hydraulic device (TRC Hydraulics Inc., Dieppe, Canada). All eggs were incubated at a temperature of 7.0 ± 0.5°C, with oxygen levels maintained at 10.8 ± 0.5 mg/L and the pH at 7.5 ± 0.1, in the separate egg tray vertical incubators under the routine salmonid hatchery sanitary conditions (Cios et al., 2018).

The triploidization rate of the fish stock produced on 22.04.2021 was assessed using 30 randomly selected fish at the age of 6 months old with a CyFlow® Ploidy Analyzer (Sysmex Partec GmbH, Germany) equipped with CyViewTM software v1.8.0.82. For this purpose, adipose fins were sampled from each fish intended to be used in further experiments and then immediately processed with a CyStainTM UV Precise T kit (Sysmex Partec GmbH, Germany). Before analysis, about 10 mg of fin material was minced, incubated for 5 min in Extraction Buffer solution and then stained with DAPI Staining Buffer. The triploidization rate of the fish stock produced on 23.04.2020 was assessed by measuring the cell (C) and nucleus (N) diameters of fifty erythrocytes from each sampled specimen. For this purpose, 40 randomly selected fish at two years of age were sacrificed, and measurements were taken using an Eclipse E600

microscope (NIKON) equipped with a camera and NIS-Elements BR 3.2 software (NIKON) (Zakęś et al. 2016). The total estimated triploidization rate in both experiments was 97,2 %.

The diploid and triploid graylings after hatching were reared under the same conditions in tanks and ponds supplied with water from the Radunia River (mean temperature 10°C and oxygenation level above 80 %). In total, three experiments were conducted between 2021 and 2023 to assess and compare the survival, growth (experiment 1 and 2), food competition (experiment 2) and catchability (experiment 3) of diploid and triploid graylings.

2.2. Experiment 1

In the first experiment, the growth rate and survival of diploid and triploid grayling specimens were tested until adulthood (up to 28 months old). The experiment ran from June 2021 to September 2023 and included 14 rearing periods that lasted from 14 to 86 days, depending on the water temperature and fish biomass growth rate (Table 1). For experimental rearing, diploid and triploid grayling fry (mean body weight of 0.15 g per individual) produced on 22.04.2021 were used. For each ploidy level, a total of 570 randomly selected individuals were divided into three replicate groups of 190 individuals and stocked into separate plastic tanks, each with a volume of 300 dm^3 and a flow rate of 1 l/s. After reaching a mean body weight of 10 g, the fish were transferred to 1200 dm^3 tanks with a flow rate of 4 l/s. All fish were reared under the same conditions in tanks supplied with water from the Radunia River, following the natural photoperiod. Mean water temperature across all rearing periods ranged from 8.2 to 20.5°C, the total ammonia nitrogen (TAN) below 0.2 mg/L, the pH at 7.5 ± 0.1 (Table 1). During the experiment, the fish were fed a commercial feed formulated for salmonids (Aller Aqua Company, Poland) with a 20 % reduction in feed rations according to recommendations for rainbow trout at a specific temperature (From and Rasmussen, 1984) (Table 1). The fish were fed manually four-six times per day during the rearing phase until they attained a mean body weight of 10 g, after which automatic timer feeders (FIAP 1520, Fiap Company, Germany) were used to provide feed continuously 8–12 hours per day. Feeding ceased during periods when the water temperature exceeded 20°C.

At the end of each rearing period, total fish biomass (± 1 g), specific growth rate (SGR), feed conversion ratio (FCR), and survival (S;%) were determined for diploids and triploids following the procedure in Zakęś et al. (2022). For biomass measurement, all fish from each tank were anesthetized with MS-222 (100 mg/l, Sigma-Aldrich, Spain) and weighed on-site using a Radwag C315.60.C2.M warehouse scale (± 1 g). The average individual body mass of fish in each group and replicate tank was calculated based on the total recorded fish number and biomass. Before further analysis, the data obtained were grouped into three stages: (1) juveniles (up to 15 months old, periods 1–9), (2) sub-adults (between 15 and 24 months old, periods 10–12) and (3) adults (between 24 and 26 months old, periods 13–14). Winter (11.11.22–19.04.22 and 03.11.22–12.04.23) and summer (14.07.22–05.09.22) months were excluded from the analysis, as fish do not grow during these periods due to low and high temperatures, respectively.

2.3. Experiment 2

In the second experiment, survival, growth rate and food competition between 28-month-old diploid and triploid graylings were examined. The experiment ran from September 2022 to September 2023 and included six rearing periods (Table 2). For the experimental rearing, a total of 150 randomly selected individuals, consisting of 75 diploids (2 n) and 75 triploids (3 n) produced on 23.04.2020, were used. All fish were tagged intraperitoneally with 12 mm Passive Integrated Transponder (PIT) tags according to the procedure described by Zakęś et al. (2022). Subsequently, randomly mixed stocks of 25 triploid (87.6 g ± 34.1 [mean ± SD]; 23.8 cm ± 2.6 [mean ± SD]) and 25 diploid (82.5 g ± 21.9; 22.7 cm ± 2.4) fish were stocked into three separate 1200 dm^3 plastic tanks as replicates. All fish were reared under identical conditions in tanks supplied with water from the Radunia River, following the natural photoperiod. The mean water temperature across all rearing periods ranged from 3.5 to 17.1°C, with the total ammonia nitrogen (TAN) remaining below 0.2 mg/L and pH at 7.5 ± 0.1 (Table 2). The fish were fed commercial feed designed for salmonids (Aller Aqua Gold 2 mm), using automatic timer feeders. Feed rations were calculated in the same way as described in the first experiment. Feeding ceased when water temperature exceeded 20°C.

At the end of each rearing period, all fish were individually weighed, as well as fish survival (S;%) and specific growth rate (SGR) were determined for both ploidy groups. Moreover, total body length (± 1 mm) was measured at the beginning and end of the experiment to calculate the initial and final Fulton's condition factor (*K*; [total weight × total length^{-3}] × 100) along with total body length gain (%) for both ploidy groups throughout the experimental rearing. All parameters were calculated following the methodology described by Zakęś et al. (2022). For measurements, all fish from each tank were anesthetized with MS-222 (100 mg/l, Sigma-Aldrich, Spain) and weighed on site, using a Radwag WTB 2000 laboratory scale (± 0.01 g).

2.4. Experiment 3

The third experiment was conducted in 2023 to assess the fly–fishing catchability and prowess of diploid and triploid graylings. For this purpose, the individually tagged fish (40-months-old) were randomly selected from the fish obtained at the end of Experiment 2. A mixed stock of five triploids (mean body weight: 249.2 g (± 95.2) and mean body

Table 1
Rearing periods, including start dates and end dates, total duration (in days), mean water temperature during each rearing period, as well as fish age, initial average body mass and the type of used feed in Experiment 1.

Period	Dates of each rearing period	Duration (days)	Mean temperature (°C)	Initial fish age (months)	Initial mean weight (g)	Feed (Aller Aqua)
1	16.06.21–29.06.21	14	19.5	1	0.15	Infa EX GR 0.4 mm
2	30.06.21–20.07.21	21	20.5	1	0.40	Futura EX GR 0.5–1.0 mm
3	21.07.21–18.08.21	28	17.7	2	1.23	Futura EX GR 0.5–1.0 mm
4	19.08.21–15.09.21	28	14.6	3	4.05	Futura EX GR 0.9–1.6 mm
5	16.09.21–13.10.21	28	11.7	4	7.19	Futura EX GR 0.9–1.6 mm
6	14.10.21–10.11.21	28	10.0	5	10.51	Futura EX GR 0.9–1.6 mm
7	20.04.22–18.05.22	29	10.8	11	16.82	Futura EX GR 0.9–1.6 mm
8	19.05.22–15.06.22	28	15.0	12	23.36	Futura EX GR 1.3–2.0 mm
9	16.06.22–13.07.22	28	17.3	13	39.21	Futura EX GR 1.3–2.0 mm
10	06.09.22–04.10.22	29	15.7	15	63.18	Futura EX GR 1.3–2.0 mm
11	05.10.22–02.11.22	29	8.2	16	90.88	Futura EX GR 1.3–2.0 mm
12	13.04.23–17.05.23	35	10.3	22	114.96	Gold 2 mm
13	18.05.23–11.08.23	86	16.3	24	138.63	Gold 2 mm
14	12.08.23–24.09.23	44	17.1	26	203.42	Gold 2 mm

Table 2
Rearing periods, including start and end dates, total duration (in days), mean water temperature during each period, fish age, initial average body mass and the type of feed used in Experiment 2.

Period	Dates of each rearing period	Duration (days)	Mean temperature (°C)	Initial fish age (months)	Initial mean weight (g)	Feed (Aller Aqua)
1	19.09.22–16.10.22	28	11.1	28	85.1	Gold 2 mm
2	17.10.22–15.11.22	30	9.9	29	111.7	Gold 2 mm
3	16.11.22–11.04.23	147	3.5	30	134.3	Gold 2 mm
4	12.04.23–17.05.23	36	10.3	34	124.7	Gold 2 mm
5	18.05.23–10.08.23	85	16.3	36	140.5	Gold 2 mm
6	11.08.23–23.09.23	44	17.1	37	223.2	Gold 2 mm

length: 30.6 cm (± 3.0)) and five diploids (mean body weight: 252.2 g (± 87.2) and mean body length: 30.2 cm (± 3.3)) was released into a concrete pond (10.0 m × 6.0 m × 1.5 m). To simulate natural conditions, the pond was filled to a depth of 0.8 m and allowed to flow through at a rate of 20 l/second. The pond was supplied with water from the Radunia River, with a mean water temperature ranging from 5.2 to 12.4°C and an oxygenation saturation level exceeding 80 %. The fly–fishing catchability tests were conducted following a 48-hour acclimatization period without feeding after the fish were stocked into the pond. The trial fly fishing was performed by a qualified member of the Polish fly–fishing team, who is a multiple world championship medalist (Michał Greszta, Greszta Fishing). Each 45–minute fishing session comprised a total of eight fly fishing trials conducted on various dates in October–November 2023, with new fish used for each trial. The ploidy level of each grayling was determined by interrogating the individual PIT tags immediately after fish capture and the fish were not returned to the pond.

To assess the catchability of diploids and triploids, each fish caught received points based on its catch probability. The scoring system assigned 10 points to the first fish, nine points to the second and decreased progressively to 1 point for the tenth fish. For each catch session, the points scored by diploid and triploid fish were summed up and an average score was calculated for each fishing session. Moreover, the catch frequency distribution of each ploidy for the first three fish caught during the fishing sessions was determined, as this reflected the European daily bag limit (FIFS, 2004). The prowess of each fish was assessed using a point scale that considered the following factors: total time of fish landing in seconds (Ti); the number of fish turns during landing (Tu); the number of fish hits on the bottom (B); the number of fish jumps above the water (J); the total body weight of each fish in grams (W). Points were awarded according to the formula $(Ti + Tu + B + J) \times W^{-1} \times 100$ and the average number of points was determined for both diploid and triploid individuals.

2.5. Statistical analysis

Statistical analysis was performed with Statistica software v. 13.1 (StatSoft, Inc., Tulsa, Oklahoma, USA). Before selecting statistical tests, data distribution normality and homogeneity of variance were tested with the Shapiro-Wilk and Levene's tests, respectively. Obtained values for diploid and triploid graylings were compared using student's *t*-test. Differences were recorded as significant at $P \leq 0.05$.

3. Results

3.1. Experiment 1

There was not difference ($P > 0.05$) in the specific growth rates between diploid and triploid graylings among juveniles and adults (Fig. 1), while triploid sub-adults displayed significantly ($P < 0.05$) higher values of SGR (SGR=0.89 (± 0.01)) compared to diploids (SGR=0.81 (± 0.02)) (Fig. 1).

The average survival (S;%) observed in the juveniles, sub-adults and adults were comparable ($P > 0.05$) between diploid and triploid graylings (Fig. 1). However, significant differences ($P < 0.05$) were recorded during period 1 of experimental rearing, where triploids exhibited lower survival rates (S=71.4 % (± 7.3)) than did diploids (S=87.5 % (± 0.3)) (supplementary Figure S1).

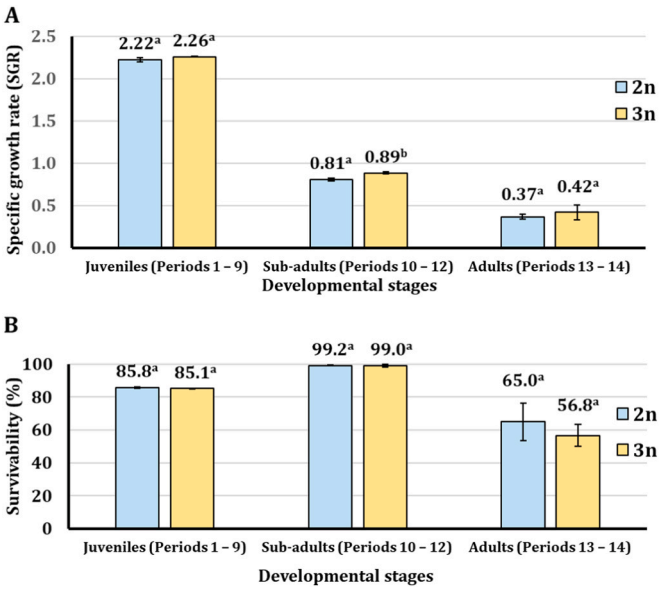

Fig. 1. Specific growth rates (SGR) (A) and survival (S;%) (B) recorded for diploid and triploid graylings, averaged across all periods within each of three developmental stages during experimental rearing in the present study. Standard deviations indicate the magnitude of differences in the averaged values between the three replicated tanks of each ploidy.

3.2. Experiment 2

During the second rearing experiment there was no difference ($P > 0.05$) in overall SGR values between diploids (SGR=0.28 (± 0.02)) and triploids (SGR=0.26 (± 0.01)). Throughout the third period of experimental rearing, both ploidy level fish groups exhibited noticeable declines in SGR because of the winter-induced cessation of feeding that led to the loss of body mass (Fig. 2).

Throughout experimental rearing, total body length (Lt) increased similarly in diploid (Lt gain=29.4 % (± 1.2)) and triploid graylings (Lt gain=27.3 % (± 2.9)), with no significant differences ($P > 0.05$). Likewise, no significant differences were recorded in Fulton's condition index (*K*) between diploid (*K*=0.89 (± 0.03)) and triploid graylings (*K*=0.84 (± 0.05)) (Fig. 3). The overall survival throughout the entire experiment was higher in diploids (S=70.7 % (± 12.9)) than in triploids (S=58.7 % (± 22.0)), although the differences were also not statistically significant ($P > 0.05$) (Fig. 2).

3.3. Experiment 3

Over the course of eight fly fishing catchability tests, diploid and triploid graylings accumulated 167 and 194 points, respectively (Fig. 4). The mean number of points per catch for triploids (24.3 points) was 3.4

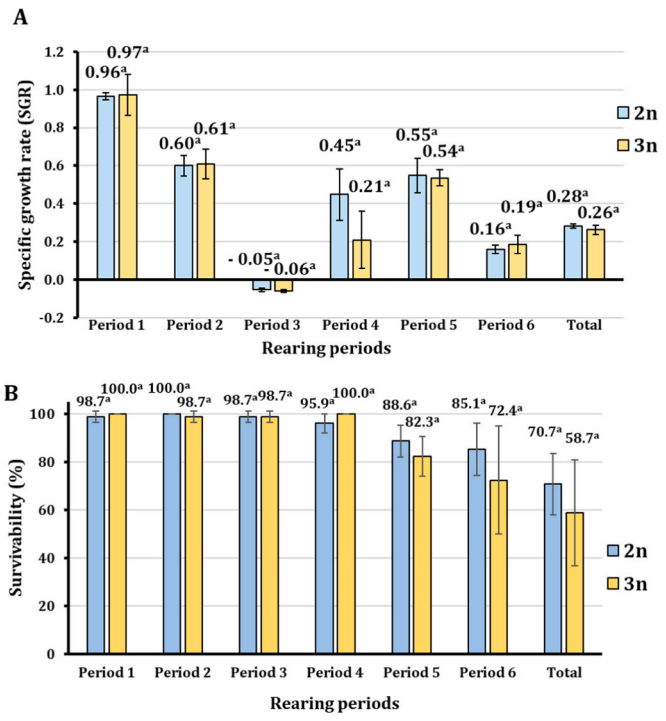

Fig. 2. Specific growth rate (SGR) values (A) and fish survival (S;%) (B) recorded in diploid and triploid graylings during the second experimental rearing in the present study. Standard deviations indicate the magnitude of differences in the recorded values for diploids and triploids between the three replicated tanks.

points higher than for diploids (20.9 points); however, the recorded differences were insignificant ($P > 0.05$). Diploids were caught as the first fish during five fishing sessions, while triploids were caught as the first fish during three fishing sessions. Triploids were the second fish caught in five sessions while in three sessions diploids were. In turn, triploids were the third fish caught in three sessions and diploids were the third fish caught in five sessions (Fig. 5). The mean fighting prowess score of triploid graylings was slightly higher (10.6 points (± 3.1)) than that of diploids (9.3 points (± 1.7)); however, the differences were not statistically significant ($P > 0.05$) (Fig. 5).

4. Discussion

Research on the feasibility and potential application of triploid salmonids for stocking of open waters, particularly in the context of put-and-take fisheries, has focused mainly on the rainbow trout (Teuscher et al., 2003; Koenig and Meyer, 2011; Koenig et al., 2011; Pease et al., 2023), Atlantic salmon (Cotter et al., 2000; Wilkins et al., 2001) and brown trout (Chatterji et al., 2007). Investigations on the growth rates, survival, individual movements and the catchability of diploid and triploid individuals of these species have been conducted on specimens stocked into open waters across North America and the British Isles (Environment Agency, 2009; Koenig and Meyer, 2011; Koenig et al., 2011; Pease et al., 2023). Grayling is another ecologically and recreationally important representative of salmonid fishes for which the use of triploid individuals represents an attractive opportunity for fisheries managers to satisfy increasing demand from anglers for suitable recreational fishing opportunities while minimizing negative impacts on native populations. Our study is the first attempt to evaluate the potential use of triploid grayling for stocking in the European waters.

Numerous studies on rainbow trout, brown trout and Atlantic salmon have documented growth advantages of triploid individuals over diploids after sexual maturation (Boulanger, 1991; Galbreath et al., 1994; Sheehan et al., 1999; Oppedal et al., 2002; Teuscher et al., 2003). Nevertheless, the growth superiority of triploids often diminishes or vanishes when they are reared together with diploid conspecifics under the controlled conditions (Galbreath and Thorgaard, 1995; Ojolick et al., 1995; McCarthy et al., 1996; Simon et. al., 2011), or in the natural environment (Brock et al., 1994; Chatterji et al., 2007; Koenig et al., 2011; Koenig and Meyer, 2011). Similar was observed during our study, particularly with regard to diploids and triploids reared in the mixed stocks. Slightly lower Fulton's condition factor (*K*) values were recorded in triploid graylings that were reared with diploids, which seemed to indicate their diminished competitive abilities for the food intake. Indeed, several studies report that while triploids allocate most of their consumed energy to the somatic growth, they display less aggressive feeding behaviors (Carter et al., 1994; Galbreath et al., 1994; O'Keefe et al., 1999; Garner et al., 2008). Thus, our research indicated that

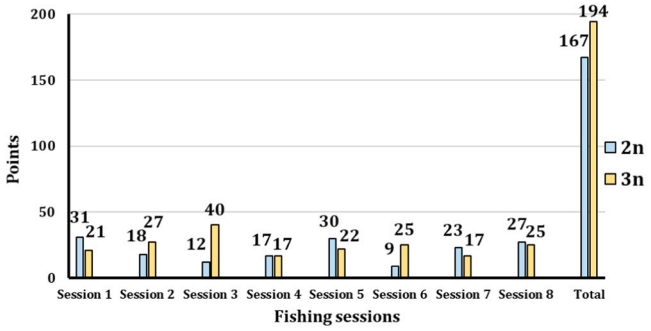

Fig. 4. Results of catchability tests for diploid and triploid graylings for all eight fly fishing sessions.

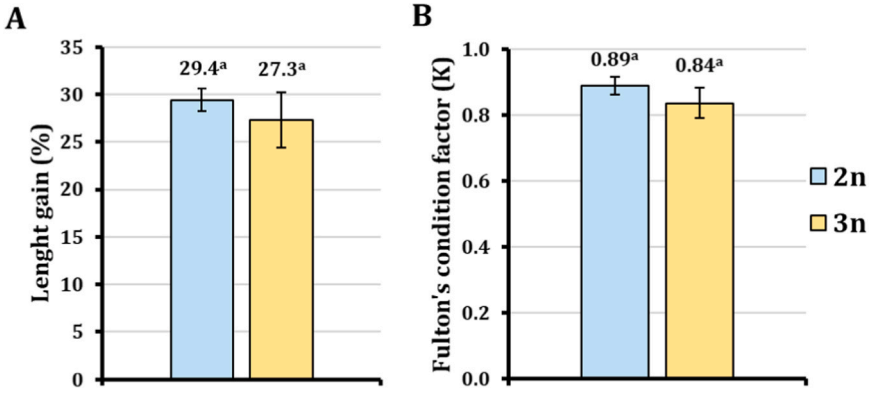

Fig. 3. Relative total body length (Lt) gain (A) and Fulton's condition factor (K) (B) recorded for diploid and triploid graylings during the second experiment of the present study. Standard deviations indicate the magnitude of differences in the recorded values for diploids and triploids between the three replicated tanks.

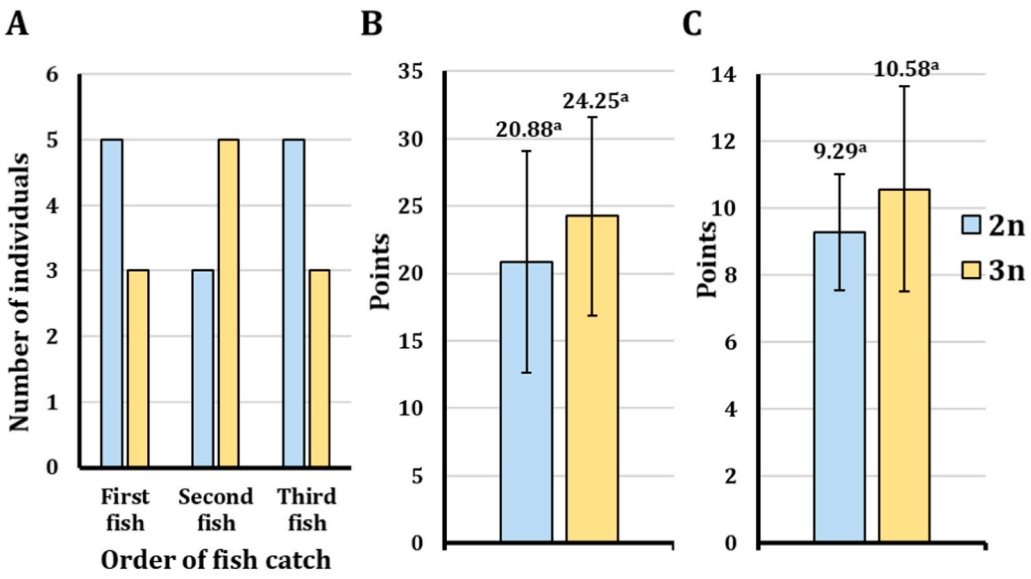

Fig. 5. Results of catchability tests and prowess conducted on diploid and triploid graylings in the present study. Catch frequencies within the first three fish caught during all the fishing sessions (A), mean points awarded for each ploidy level per fly fishing session (B) and average prowess points assigned per fish of each ploidy level (C).

triploid graylings could be used for stocking open waters without significantly impacting native populations, as they exhibit less competitive feeding behavior when compared to diploids. A similar conclusion was drawn by Chatterji et al. (2007) regarding triploid brown trout stocked in UK waters, where stocking regimes showed no adverse effects on the diets of wild fishes and minimal evidence of predation on the wild brown trout eggs and juveniles by triploids. Moreover, there was no evidence that stocked female triploid trout interfered with the spawning activity of wild diploid brown trout. However, in Atlantic salmon triploid males can interfere with the diploid males during the spawning period in the wild (Fjelldal and Wennevik, 2014).

Although triploid salmonids can display similar survival as diploids when raised separately under optimal conditions (Piferrer et al., 2009), their performance both in co-culture with the diploids under aquaculture settings (Galbreath et al., 1994; Bonnet et al., 1999) and in natural environments when stocked into the open waters (Havens and Sonnichsen, 1993; Rutz and Baer, 1996; Cotter, 2000; Wilkins et al., 2001; Chatterji et al., 2007; Koenig et al., 2011) is usually lower. The challenging habitat conditions arising from the variations in water level, temperature, oxygenation, the presence of pathogens, predation, limited food resources and high fishery exploitation rates are commonly implicated as key factors contributing to the low survival and returns of stocked fishes (Wiley et al., 1993; Dillon and Alexander, 1996). Because of their specific physiology, triploid salmonids are particularly vulnerable to deteriorated water quality conditions, such as low dissolved oxygen, elevated water temperatures and chronic environmental stress (Maxime, 2008; Piferrer et al., 2009; Fraser et al., 2012). Available data on rainbow trout and brown trout indicate that triploids, compared to diploids, experience higher mortality (measured as catch return levels) in reservoirs with low water levels, higher summer temperatures, lower dissolved oxygen, high predation and where multiple fish species compete for food resources (Teuscher, 2003; Chatterji et al., 2007; Koenig and Meyer, 2011; Koenig et al., 2011; Simon et al., 2011). Some studies have reported that triploid rainbow trout experience the highest mortality rates within the first year after stocking, raising concerns about their suitability for the long-term fishing opportunities (Dillon et al., 2000; Koenig and Meyer, 2011; Simon et al., 2011). However, other research indicates that in water bodies with favorable habitat conditions, triploids can achieve lifespans comparable to or even exceeding those of diploids (Dillon et al., 2000; Teuscher et al., 2003; Wagner et al., 2006; Koenig and Meyer, 2011). Elevated mortality was also observed in the triploid Atlantic salmon and rainbow trout when fish at the early stages of ontogenetic development were used for stocking, particularly in waters characterized by the poor habitat conditions. These findings suggest that it might be necessary to produce older graylings for stocking and release them near fishing access points shortly before the fishing season begins, ensuring sufficient triploid catch returns for anglers. Nevertheless, to formulate specific recommendations regarding the optimal age and size grayling that may be released, more research is needed to better understand the connection between the survival rates of triploid graylings, their age or size at stocking and environmental conditions in open waters.

The catchability of triploids is another important factor determining their utility as stocking material intended for the enhancement of put-and-take fisheries. Differences in catchability between diploid and triploid salmonids are difficult to assess in open waters, as they are influenced by numerous factors such as survival rates, stocking densities, location and time, food availability, as well as migration rates and their pattern (Miko et al., 1995; Yule et al., 2000; Haddix and Budy, 2005; Losee and Phillips, 2017; Harmon et al., 2018). The mixed-sex triploid rainbow trout stocked into the open waters generally exhibit lower overall catchability than diploids (Brock et al., 1994; Rutz and Baer, 1996; Dillon et al., 2000; Koenig and Meyer, 2011; Koenig et al., 2011; Pease et al., 2023), however similar performance of the mixed-sex triploids and the diploids has been also reported (Simon et al., 2011). Chatterji et al. (2007) and Pease et al. (2023) found that triploid rainbow trout in the lowland lakes of western Washington and triploid brown trout in the United Kingdom had reduced migration rates, similar survival, comparable daily movement patterns and less variability in the home range distribution when compared to the diploids. Furthermore, the mentioned authors attributed the lower catch rates of triploids to their reduced movement variability and increased susceptibility to displacement by the high flows. Interestingly, Goryczko and Dobosz (1997) reported that the catchability of triploid rainbow and brook trout hybrids was approximately two to five times higher than that of diploid rainbow trout, brook trout, and brown trout. Moreover, they reported clear differences in catchability among the species examined what suggests that the catchability potential of salmonid fishes is more likely species-specific than dependent on the ploidy level. Thus, differences observed in the catch rates between diploid and triploid fishes in the open waters seem to be influenced more by the environment-dependent

factors like survival, migration rates and daily movement patterns than differences in feeding behavior and activity.

It has been reported that all-female triploid rainbow and brown trout exhibit superior growth performance, survival and comparable or even higher cumulative catchability than mixed-sex diploid and triploid specimens, especially in waters with the optimal environmental conditions, likely due to their lower vulnerability to predation and reduced migration rates linked to absence of the spawning-related behaviors (Josephson et al., 2001; Solomon, 2003; Chatterji et al., 2007; Koenig et al., 2011; Pease et al., 2023). Such characteristics show the potential advantage of using all-female triploid grayling as a stocking material, particularly in reservoirs facing drawdowns, high summer temperatures, intensive predation and limited food resources, where higher catch rates are prioritized, potentially allowing fisheries managers to enhance their trophy attractiveness for anglers with minimal genetic impacts on wild populations. The sex verification of examined triploid grayling was not, however, performed in the present study as it was a preliminary research. A potential superiority of the triploid grayling females over the mixed-sex triploids makes that our further research needs to be focused on the development of grayling neo-males and generation of exclusively female triploid (XXX) individuals.

In conclusion, our experimental rearing revealed comparable growth rates and survival between diploid and triploid graylings. Additionally, food competition trials exhibited that triploid graylings have slightly lower food acquisition efficiency and less aggressive feeding behaviors than diploids, while catchability and prowess trials showed no significant differences between individuals of both ploidy level. Overall, our preliminary study suggests that stocking triploid graylings may offer a viable opportunity to enhance open water fisheries without posing genetic risks to the native populations, thereby enabling fisheries managers to meet the growing demand for recreational fishing opportunities. However, additional investigation on three-year-old triploid graylings is necessary to confirm their sterility. It should also be emphasized that research on the performance and catchability of triploid grayling in open waters, using traditional stock assessment tools such as tagging, creel surveys and telemetry, is needed to further evaluate their suitability for the stocking purposes and to provide the data necessary for their future integration into the stocking plans.

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CRediT authorship contribution statement

Marcin Kuciński: Writing – original draft, Visualization, Methodology. **Rafał Rożyński:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Michał Greszta:** Investigation. **Stefan Dobosz:** Methodology, Conceptualization. **Konrad Ocalewicz:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.102908.

Data availability

Data will be made available on request.

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animals

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Article

Hormonal Masculinization of the European Grayling (*Thymallus thymallus*) Using 11β-Hydroxyandrostenedione (OHA) and 17α-Methyltestosterone (MT)

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Simple Summary

The European grayling is an important salmonid species whose natural populations have declined due to anthropogenic pressure on riverine ecosystems. Conservation efforts that involve stocking open waters with hatchery-reared fish can lead to genetic pollution in native grayling populations. Production of sterile triploid females might offer a solution to this problem. To produce such stocks, efficient masculinization methods for the species are needed to provide neo-males. This study preliminary assessed the potential of 11β-hydroxyandrostenedione (OHA) and 17α-methyltestosterone (MT) for masculinization of the grayling. OHA only induced the development of external male traits in up to 77% of fish, while MT frequently caused intersex differentiation. These findings indicate that hormonal masculinization in the grayling is feasible but requires further studies focused on dosage, timing and administration methods.

Abstract

The European grayling is an ecologically and recreationally important salmonid fish species. However, its wild populations have declined in recent years across Europe due to habitat degradation, predation and overexploitation. Unfortunately, conservation measures such as stocking with hatchery-reared fish may threaten the genetic integrity of native populations. The use of triploid all-females, which display markedly reduced fertility, offers a potential solution to this problem. While protocols for inducing triploid and gynogenetic development of the species exist, an effective method for producing neo-males, essential for large-scale triploid female stock production, is still lacking. In the present study, the potential suitability of 11β-hydroxyandrostenedione (OHA) and 17α-methyltestosterone (MT) for masculinization of the European grayling was investigated, aiming to provide preliminary data to support the future development of a reliable biotechnique for neo-male production in this species. Pilot trials of hormonal masculinization were conducted by feeding 20-day post-hatch fry with diets supplemented with OHA (10 mg/kg—OHA_{10ppm}, 20 mg/kg—OHA_{20ppm}) or MT (3 mg/kg—MT_{3ppm}, 6 mg/kg—MT_{6ppm}) for ~80 days. In the OHA-treated groups, the proportion of externally male-like individuals ranged from 66.7% (OHA_{10ppm}) to 76.6% (OHA_{20ppm}). However, some of these specimens were found to be genetically female with ovaries (4.5% and 28.8%, respectively), which indicated a dissociation between external dimorphism and gonadal development. In turn, MT treatments

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resulted in strong disruption of the female gonads with the intersex individuals comprising 28.6% (MT_{3ppm}) and 57.1% (MT_{6ppm}), indicating that the applied hormonal treatment was insufficient for complete masculinization. The results indicate that androgen-mediated neo-male induction by OHA and MT is possible in the species but requires optimization of dose, timing and delivery, potentially combining embryonic immersion with prolonged dietary administration.

Keywords: neo-males; sex reversal; androgen exposure; gonadal differentiation; sterility; intersex

1. Introduction

The European grayling (*Thymallus thymallus*), hereafter referred to as the grayling, is a freshwater salmonid fish (order Salmoniformes, family Salmonidae, subfamily Thymallinae) native to European running waters. The grayling plays an important ecological role as a bioindicator of river ecosystem health, being particularly sensitive to elevated water temperature (>20 °C), low dissolved oxygen (<6 mg/L) and increased loads of nutrients and pollutants such as nitrates and heavy metals [1–3]. The grayling is also a highly valued species for recreational angling, especially in rivers in Central and Northern Europe, where thousands of licensed fly-fishers participate annually in mostly catch-and-release fisheries, contributing substantial local economic benefits through tourism and fishing license revenues [4,5].

Unfortunately, over recent decades, habitat degradation, predation pressure and excessive fishing have led to a marked decline in grayling abundance and distribution across Europe [6–8]. Specifically, electric-fishing surveys in southern Belgium showed a ~42.8% decline in the individual grayling abundance and ~52.6% loss in its biomass in 2000–2022, while in Bavarian streams, the number of grayling individuals dropped by about 41.7% between the early 1990s and mid-2000s [3]. To counteract this decline, conservation management measures, such as size limits (from 28 to 45 cm, depending on the country), closed seasons for spawning periods, catch quotas and restocking initiatives using hatchery-reared individuals from regional broodstock, have been implemented in recent years [1,5,9,10].

Multiple observations have revealed that the widespread introduction of graylings from non-native populations and hatchery-raised specimens raises a serious threat to the genetic integrity of many local populations of this species [7,11,12]. In several cases, the resulting loss of genetic identity has led to the complete disappearance of many local grayling populations due to reduced fitness [7,11,13]. To address this issue, the use of functionally sterile fish for stocking has been proposed as a strategy to prevent unwanted gene flow into wild populations.

In salmonid fish species, triploid females typically exhibit impaired gametogenesis, which makes them safe for wild fish populations if stocked [14]. Triploid all-female stocks can be obtained through a three-step process, including (1) the generation of all-female diploids via gynogenesis, (2) the production of neo-males (XX) through hormonal sex reversal of the genetic females, and (3) the triploidization of eggs fertilized with the sperm from the neo-males. While gynogenesis and triploidization protocols have already been developed for graylings [15,16], methods enabling the reliable production of grayling neo-males are still missing.

Hormonal masculinization in salmonids is usually induced through the treatment of the fish with androgens such as 11β-hydroxyandrostenedione (OHA) or 17α-methyltestosterone (MT) [17–21]. These compounds act by binding to androgen receptors and inhibiting aro-

matase, thereby redirecting undifferentiated gonads toward testicular development [22,23]. Effective masculinization depends on the correct hormone dose and application during the critical developmental window prior to gonadal differentiation [24,25].

In this study, the potential of OHA and MT for the masculinization of the grayling was assessed to provide preliminary data supporting the development of a reliable protocol enabling the production of neo-males.

2. Materials and Methods

2.1. Fish Stock Origin and Maintenance

The experiments were performed using graylings from the broodstock kept at the Department of Salmonid Research (DSR), National Inland Fisheries Research Institute, in Olsztyn, Poland (Rutki; 54°19′51.1″ N 18°20′15.1″ E). Graylings from the broodstock used in the research reached sexual maturity in their second year of life. The spawning fish, aged three to four years, were raised in a 3000 m^3 flow-through pond system supplied continuously with water from the Radunia River at a rate of 5 L/s. The inflowing river water exhibited a seasonal temperature range of 2–17 °C, with daily fluctuations not exceeding ±0.5 °C and dissolved oxygen levels consistently above 80% saturation (8.5–11.5 mg/L) throughout the year. They were fed a commercial broodstock diet (Aller Bronze 3 mm and Aller Silver 3 mm, Aller Aqua, Golub-Dobrzyń, Poland) three to four times daily, with feed amounts ranging from 0.5% to 1.5% of their biomass depending on water temperature. Feeding was stopped two weeks before spawning, and experimental crossings took place on 29 April 2021 and 24 April 2023.

Gametes were collected from ten females and five males, which measured 26.3–35.5 cm in body length and 141–393 g in body weight. This female/male ratio is commonly used in aquaculture to ensure a sufficient number of eggs, while keeping the number of males manageable. Before gamete collection, fish selected for stripping were anesthetized with MS-222 (50 mg/L, Sigma-Aldrich, Madrid, Spain). Eggs stripped from all females were pooled and thoroughly mixed before fertilization with pooled milt. Sperm quality was evaluated using a computer-assisted sperm analysis (CASA) system after activation in sperm activation medium (SAM; 1 mM $CaCl_2$, 20 mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0). The quantitative parameters measured included total motility (%), progressive motility (%), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and average path velocity (VAP, µm/s), recorded 10 s post-activation. Only ejaculates exhibiting ≥80% total motility were used for fertilization.

Eggs were incubated at a temperature of 7.0 ± 0.5 °C, with oxygen levels maintained at 10.8 ± 0.5 mg/L and pH at 7.5 ± 0.1, in separate egg trays in vertical incubators under the salmonid hatchery's routine sanitary conditions [26]. The system was continuously supplied with fresh, oxygenated river water, and metabolic waste (ammonia and nitrite) levels were monitored weekly using colorimetric tests (JBL GmbH, Neuhofen, Germany), remaining below 0.05 mg/L for NH_3 and 0.01 mg/L for NO_2^- throughout incubation. Following complete yolk sac resorption (at 70°D post-hatching, i.e., 70 accumulated degree-days calculated as the product of days and water temperature in °C), the grayling hatchlings were fed *Artemia nauplii* for 10 days. In this species, the active swim-up fry are large enough, with sufficiently developed jaws, to be directly fed on Artemia, as commonly practiced in aquaculture.

The produced fry were used to conduct two experiments that focused on the hormonal sex reversion and production of neo-males (genetic females). For this purpose, 11β-hydroxyandrostenedione (OHA) (Sigma-Aldrich, Madrid, Spain) was used in experiment 1 and 17α- methyltestosterone (MT) (Sigma-Aldrich, Madrid, Spain) was used in

experiment 2. Both hormones were administered in variable doses by feeding the fish with specially prepared hormone-enriched feed.

2.2. Diet Preparation

Two types of commercial feed were used depending on the fish size, namely Aller Aqua Infa EX GR 0.4 mm for fish up to 2 g (approximately 15–50 mm in body length) and Aller Aqua Futura EX GR 0.5–1.0 mm for fish of 2–5 g (approximately 50–80 mm in body length) (Aller Aqua, Golub-Dobrzyń, Poland). For masculinization experiment 1, OHA feed with doses of 10 mg (OHA_{10ppm}) and 20 mg (OHA_{20ppm}) per kg was used. For experiment 2, MT feed with doses of 3 mg (MT_{3ppm}) and 6 mg (MT_{6ppm}) was utilized. For both experiments, non-enriched feeds were also used as the control diets (OHA_C and MT_C). Hormone supplementation of the feeds was carried out using the sprinkle method. For this purpose, each hormone supplied as dry powder (Sigma Aldrich, Madrid, Spain) was accurately weighed, dissolved in ethanol and evenly applied to the commercial feed by hand spraying. The feeds were then dried at room temperature for 24 h to ensure complete ethanol evaporation and subsequently stored at 4 °C until use. To eliminate the potential effect of ethanol on the feed palatability, the control diet underwent the same spraying process but without the addition of the active compounds.

2.3. Experimental Design

The hormonal sex reversal experiments were carried out on 18 June 2021 (experiment 1) and on 13 June 2023 (experiment 2) using grayling fry 20 days post-hatching, weighing, on average, 82–85 mg, with fully resorbed yolk sacs and exhibiting active feeding behavior. For each experiment, a total of 450 fish were divided into three equal groups (150 indiv. per group) and then stored in nine separate 300 L flow-through tanks (1 L/s water flow), with 50 fish per tank, corresponding to three treatments (two hormonal doses and one control), each replicated in triplicate. Each setup included two experimental tanks, i.e., OHA_{10ppm} and OHA_{20ppm} (for experiment 1) or MT_{3ppm} and MT_{6ppm} (for experiment 2), along with control tanks, i.e., OHA_C and MT_C, respectively. Although immersion treatments can be used in salmonids, in this study we focused on oral administration throughout the critical period of gonadal differentiation to achieve continuous hormone exposure, ensuring sufficient uptake while minimizing handling stress.

The hormone administration phase lasted 80 days (experiment 2) and 83 days (experiment 1). Further, the fish were fed with Aller Aqua Infa EX GR 0.4 mm, until their average weight exceeded 5 g per individual. After this period, the fish were transferred to larger tanks with a capacity of 1200 L and a water flow of 4 L/s, where the second phase of each experiment commenced. For the next 22 (experiment 1) and 12 (experiment 2) months, fish were fed Aller Futura GR 0.5–2.0 feed without hormone supplementation. The fish were hand-fed eight times per day, with the daily feed ration reduced by 20% compared to the recommended amount for rainbow trout at a specific temperature, following From's and Rasmussen's [27] guidelines. During the hormone administration phase, the feed consumption was strictly monitored to ensure that >95% of the rations were taken. Feed was replaced twice daily to prevent hormone degradation and ensure continuous availability.

2.4. Assessment of Masculinization Rate

Masculinization effectiveness in fish from experiment 1 was assessed by analyzing dimorphic traits and gonadal morphology, combined with PCR-based molecular genetic sex verification. Taking into account the fact that a reliable assessment of the gonadal differentiation and masculinization effect in graylings from the Rutki broodstock may be performed as early as 24 months after hatching, 25-month-old fish were examined, including 66 and 77 individuals from the experimental groups (OHA_{10ppm} and OHA_{20ppm},

respectively) and 55 individuals from the control group (OHA_C). The unequal group sizes reflect natural survival variability during the 22-month rearing period, and all surviving individuals from each group were included to maximize statistical power and representativeness. In turn, masculinization efficacy in fish from experiment 2 was assessed using PCR-based molecular genetic sex identification and gonad histology. For molecular analysis, 20 randomly selected 15-month-old individuals each from the experimental (MT_{3ppm}, MT_{6ppm}) and control (MT_C) groups were analyzed. Gonadal histology was then performed on seven genetically verified females from each experimental group (MT_{3ppm}, MT_{6ppm}) and six from the control group (MT_C). All fish were euthanized using an overdose of MS-222 (50 mg/L) and dissected to collect the gonads for morphological or histological observations. For PCR-based molecular genetic sex verification, small pelvic or pectoral fin clips were collected from each fish, preserved in 96% ethanol and stored at a temperature of 4 °C until genetic material extraction.

The external sexual dimorphism analysis was performed following the procedure outlined by Witkowski [28]. Fish were classified as males if they displayed breeding tubercles on the posterior part of the body, dark body coloration, a large cherry-colored dorsal fin, and a small genital papilla. Females were identified by a small dorsal fin, light body coloration, and a large genital papilla. In turn, fish exhibiting a silvery body and lacking a genital papilla were classified as indeterminate. The external sexual dimorphism analysis was confirmed through the morphological observation of the dissected gonads. Males were identified by the presence of paired, elongated testes with a milky or whitish appearance, females by paired, lobulated ovaries containing visible oocytes, and sterile individuals by rudimentary, thread-like gonads lacking differentiated gametes. For data collection, all fish and their gonads were photographed with a Canon G16 camera (Canon, Tokyo, Japan). Each image was visually inspected to identify and characterize every distinct sex identified among the examined fish. Images were captured on a neutral gray background with consistent LED lighting to avoid shadows, and a scale bar was included in each frame. Camera settings were standardized with ISO 200, aperture f/5.6 and magnification adjusted to capture the entire specimen or gonad. No automated image analysis software was used. All images were visually inspected to identify and characterize each distinct external sexually dimorphic phenotype among the examined fish.

For PCR-based molecular genetic sex verification, genomic DNA was isolated from collected and preserved fin clips using the standard Chelex-100 method [29]. The quality and amount of the isolated genomic DNA were checked with the NanoDrop™ One spectrophotometer (Thermo Scientific, Waltham, MA, USA). The genetic sex of the sampled fish was verified through the PCR duplex amplification of the Y-chromosome-linked DNA marker (sdY) (F: ATGGCTGACAGAGAGGCCAGAATCCAA, R: CTGTTGAAGAGCATCACAGGGTC) and 18S rDNA (F: GTYCGAAGACGATCAGATACCGT, R: CCGCATAACTAGTTAGCATGCCG) as a positive control [30]. The PCR amplifications were carried out using 10 ng of the isolated DNA template in a reaction mixture of 12.5 µL total volume composed of 1× PCR Master Mix Green Plus (A&A Biotechnology, Gdańsk, Poland), as well as 0.4 µM of each SdY and 0.1 µM of each 18S rDNA primer. PCR amplifications were performed with a Mastercycler® X50s (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 96 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, annealing at 60 °C for 45 s, elongation at 72 °C for 45 s and a final elongation step at 72 °C for 10 min. The resulting products of the PCR amplifications were separated in a 2.0% agarose gel (Sigma-Aldrich, St. Louis, MI, USA), stained with ethidium bromide (0.05 mg/mL) and then visualized by a UV trans-illuminator (Vilber Laurmat ECX-20.M, Eberhardzell, Germany).

For gonadal histology, the sampled tissues were immediately fixed in Bouin's fluid after collection and stored at room temperature until further processing. The preserved gonad tissues were subsequently dehydrated in a series of increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin blocks using the tissue processor Leica TP 1020 (Leica Biosystems, Park, IL, USA) and a paraffin dispenser (DP500, Bio-Optica, Milano, Italy). Then, 5 µm thick sections were cut from each gonadal tissue using a Microm HM 350S microtome (Microm, Walldorf, Germany). The slides were next stained using a routine hematoxylin–eosin (HE) staining method [31,32]. Histological analysis was performed using an Axiolab light microscope (Zeiss, Oberkochen, Germany), with an Axiocam 208 color camera and Zen 3.4 software (Zeiss, Oberkochen, Germany). The classification of germ cells and gonadal cellular structures followed the nomenclature of Król et al. [33], with gonads categorized as follows:

(A) Testes—spermatogonia type A;
(B) Testes—spermatogonia type A and spermatozoa;
(C) Ovaries—oogonia and oocyte type I and II;
(D) Ovaries—oogonia and oocyte type I, II and III;
(E) Ovaries—oogonia, oocyte type I and II, and single spermatogonia;
(F) Ovaries—oogonia, oocyte type I, II, and III, single spermatogonia, and spermatozoa.

2.5. Statistical Analysis

For experiment 1, the proportions of external sexual dimorphism phenotypes (male-like, female-like, and sterile-like individuals) between the experimental (OHA_{10ppm} and OHA_{20ppm}) and control (OHA_C) groups were compared using the chi-square (χ^2) test of independence. For post hoc pairwise comparisons, adjusted residuals were examined to identify significant deviations from expected frequencies. In experiment 2, gonadal histology for all groups (MT_{3ppm}, MT_{6ppm} and MT_C) was analyzed descriptively, without statistical testing due to the small sample size, as this experiment was designed as a preliminary study. A significance threshold of $p < 0.05$ was applied for all statistical analyses. Proportional 95% confidence intervals were calculated using the Wilson score method. All calculations were performed using Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results

3.1. Masculinization Rate in Experiment 1

The effectiveness of masculinization in fish fed 11β-hydroxyandrostenedione (OHA) was assessed using external sexual dimorphism, gonadal morphology, and PCR-based molecular genetic sex verification. Examination of external sexual dimorphism revealed fish displaying male-like, sterile-like, and female-like external dimorphism in the examined graylings (Figure 1). The highest percentage (76.6%) of fish displaying external male dimorphism was observed in the OHA_{20ppm} group (59 indiv.) (Figures 1A and 2). In the OHA_{10ppm} group, this percentage was 66.7% (44 indiv.), while in the OHA_C group, it was 54.5% (30 indiv.). The highest share (12.7%) of fish classified as sterile was recorded in the OHA_C group (seven indiv.), while in the OHA_{10ppm} and OHA_{20ppm} experimental groups it varied from 7.6% (five indiv.) to 7.8% (six indiv.), respectively (Figures 1B and 2).

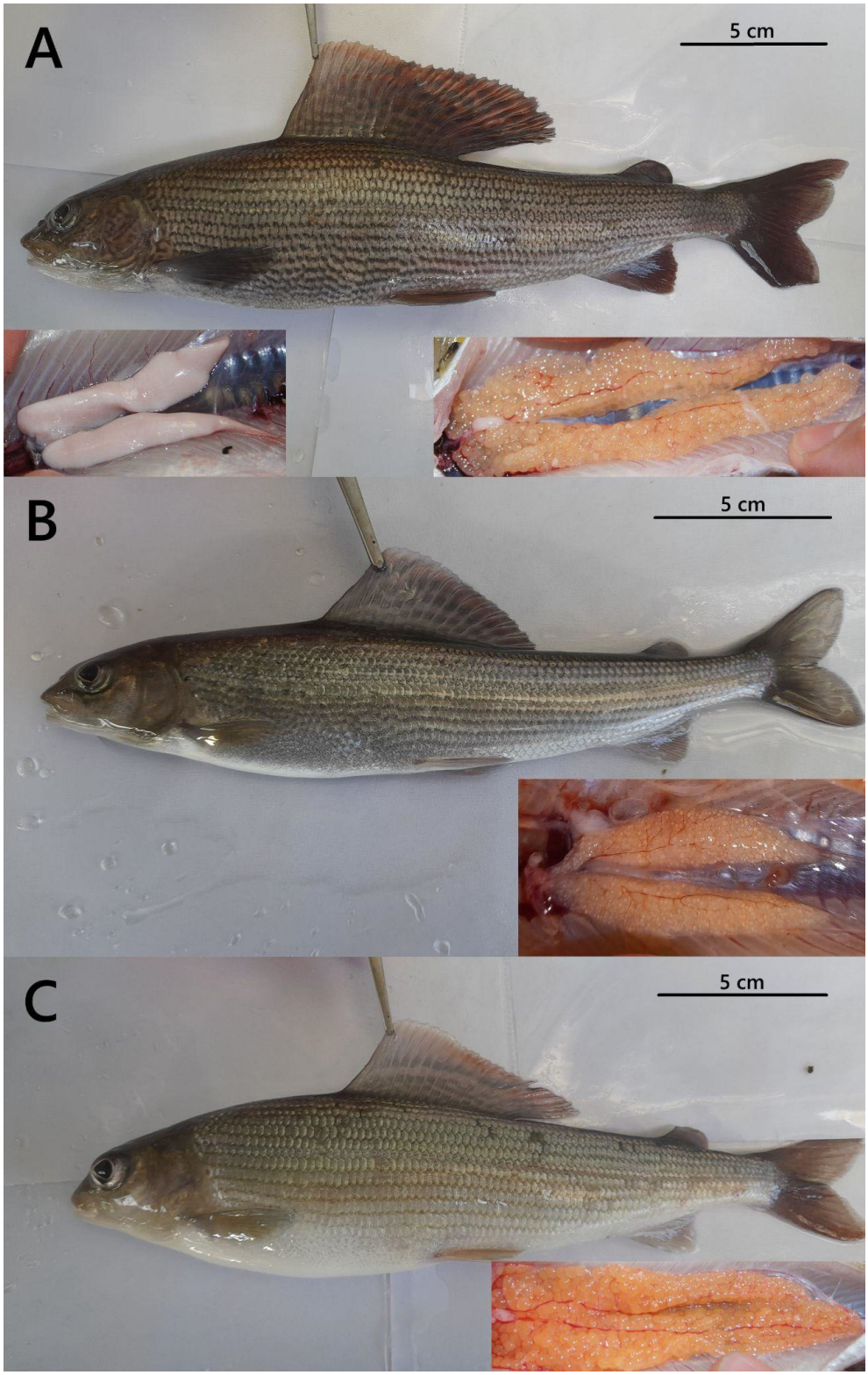

Figure 1. Types of recorded external sexual dimorphism along with gonad morphology observed in graylings fed 11β-hydroxyandrostenedione-enriched (OHA) feed: (**A**) male-like external dimorphism: fish with breeding tubercles, dark body coloration, a large cherry-colored dorsal fin and a small genital papilla; (**B**) sterile-like phenotype: fish showing a silvery body and lacking a genital papilla; and (**C**) female-like external dimorphism: fish with light body coloration, a small dorsal fin and a large genital papilla. All specimens ranged from 23.2 to 27.9 cm in total length and from 107 to 150 g in body mass.

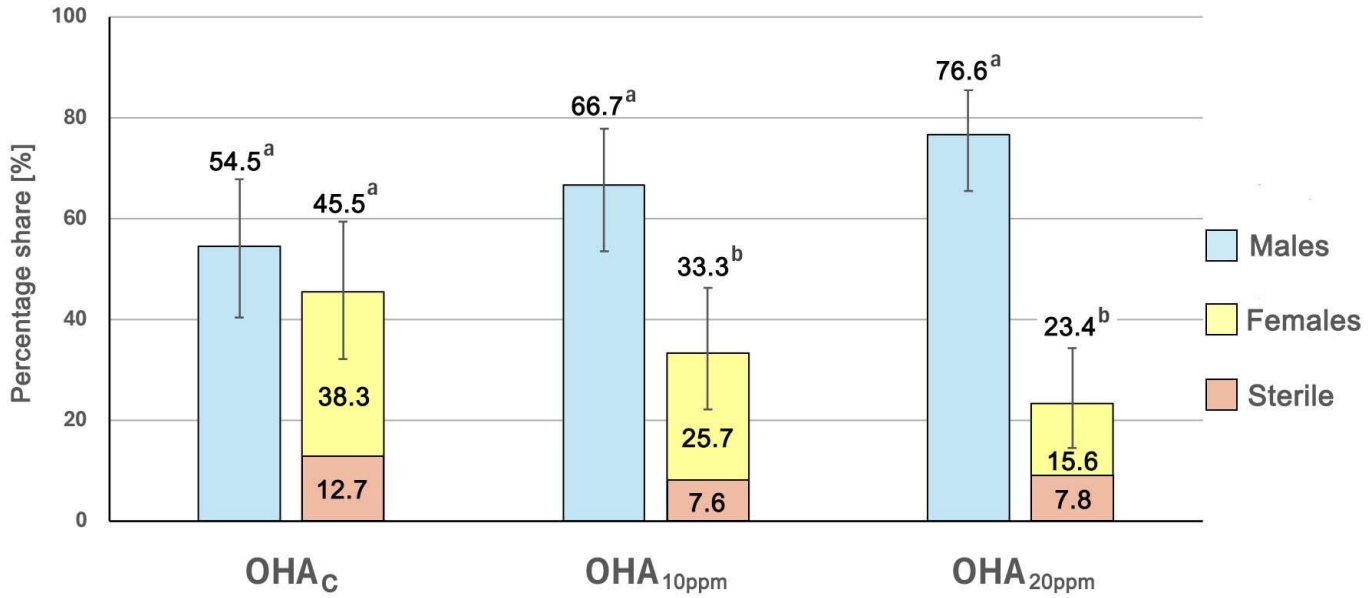

Figure 2. Percentage of fish displaying external sexual dimorphism in both experimental groups (OHA_{10ppm}, OHA_{20ppm}) and the control group (OHA_c). Different letters denote statistically significant differences ($p < 0.05$) in the proportions of external dimorphism phenotypes within groups. Error bars represent 95% confidence intervals.

Examination of gonadal morphology revealed that fish from both OHA groups and the control group classified based on external sexual dimorphism as females and sterile specimens had developed ovaries (Figure 1B,C). Among fish displaying external male dimorphism, examination of gonadal morphology revealed that 2 individuals (4.5%) in the OHA_{10ppm} group and 17 individuals (28.8%) in the OHA_{20ppm} group exhibited ovaries, while the remaining fish showed testes (Figure 1A). Genetic analysis confirmed that all fish exhibiting ovarian development were genetic females, while those with testicular development were confirmed to be genetic males.

3.2. Masculinization Rate in Experiment 2

In Experiment 2, the effectiveness of sex reversal using 17α-methyltestosterone (MT) was assessed through PCR-based molecular genetic sex verification and gonad histology. Among fish that were molecularly verified as females, five specimens (71.4%) from the MT_{3ppm} group and three specimens (42.9%) from the MT_{6ppm} group were found to have properly developed ovaries, corresponding to gonad types C and D (Table 1 and Figure 3). Intersex gonads (types E and F: ovaries with oogonia, oocytes stages I–III and scattered spermatogonia together with spermatozoa) were observed in two genetic females (28.6%) from the MT_{3ppm} group and in four molecularly confirmed females (57.1%) from the MT_{6ppm} experimental group. In the control group, both genetic males and genetic females developed well-formed testes and ovaries, respectively (gonad types A–D), consistent with their genetic sex.

Table 1. The percentage of grayling individuals with different gonad types in the control group (MT_C; both sexes) and in the two experimental groups (MT_{3ppm} and MT_{6ppm}; only individuals molecularly verified as genetic females).

	Types of Gonads	MT_C (n = 6)	MT_{3ppm} (n = 7)	MT_{6ppm} (n = 7)
(A)	Testes—spermatogonia type A	33.33%	-	-
(B)	Testes—spermatogonia type A and spermatozoa	33.33%	-	-
(C)	Ovaries—oogonia and oocyte type I and II	16.67%	57.13%	28.57%
(D)	Ovaries—oogonia and oocyte type I, II and III	16.67%	14.29%	14.29%
(E)	Ovaries—oogonia, oocyte type I and II, and single spermatogonia	-	14.29%	28.57%
(F)	Ovaries—oogonia, oocyte type I, II and III, single spermatogonia, and spermatozoa	-	14.29%	28.57%

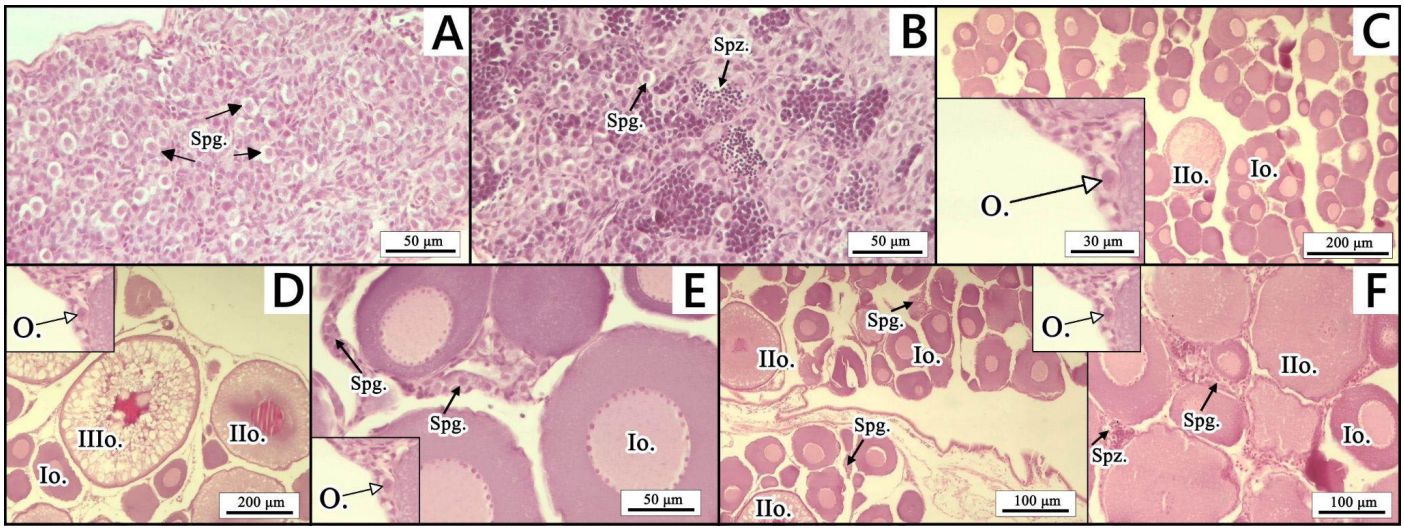

Figure 3. Gonadal development in the control group (MT_C) and two experimental groups (MT_{3ppm} and MT_{6ppm}) based on histological sections stained with hematoxylin and eosin (H&E). (**A**) Testes—spermatogonia type A; (**B**) testes—spermatogonia type A and spermatozoa; (**C**) ovaries—oogonia and oocyte type I and II; (**D**) ovaries—oogonia and oocyte type I, II and III; (**E**) ovaries—oogonia, oocyte type I and II, and single spermatogonia; (**F**) ovaries—oogonia, oocyte type I, II, and III, single spermatogonia, and spermatozoa. (Spg.—spermatogonia type A; Spz.—spermatozoa; O.—oogonia; Io.—oocyte type I; IIo.—oocyte type II; IIIo.—oocyte type III).

4. Discussion

In fishes, sex differentiation is largely controlled by steroids and gonadotropins, produced primarily by the brain during early development and later also by the gonads [23]. In the Salmoninae family, most species exhibit differentiated gonochorism, in which early development of gonads continues from the indifferent gonad directly to the testes or ovaries. This mode of gonad development has been documented in Arctic charr (*Salvelinus alpinus*), brook charr (*S. fontinalis*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*O. kisutch*) [34–38]. In contrast, representatives of the Thymallinae subfamily display undifferentiated gonochorism, where gonads initially develop along a single pathway—usually female-like—before later differentiating into testes or ovaries [23]. The European grayling represents a rare variant within this pattern, as its gonads first exhibit an all-male-like stage around the 7th week post-hatching before transitioning to ovarian differentiation from approximately the 11th week post-hatch prior to developing mature testes and ovaries [39].

The main goal of the present study was to evaluate the potential use of 11β-hydroxyandrostenedione (OHA) and 17α-methyltestosterone (MT) for masculinization in the grayling. MT is a synthetic androgen widely used in aquaculture to induce sex reversal by mimicking the action of endogenous testosterone and thus stimulating testicular differentiation during the labile period of gonadal development [23,40]. OHA, in turn, is a natural metabolite of testosterone and a potent androgen precursor, which can be enzymatically converted into active androgens within fish tissues, primarily via the 17β-hydroxysteroid dehydrogenase and 5α-reductase enzymes, thereby promoting male gonadal development [41–43]. Both OHA and MT masculinize fish primarily by activating androgen receptors in the developing gonads, while simultaneously suppressing estrogen synthesis through competitive inhibition of aromatase activity [22]. Because MT often impairs gonadal development, it is frequently replaced by OHA, which effectively induces masculinization with fewer intersex cases, less sterility, and reduced adverse effects [17,18,20].

In this study, the dietary administration of OHA slightly increased the ratio of individuals displaying male secondary sexual characteristics in graylings, including body coloration, fin shape and genital papilla, but it did not affect gonadal development. This contrasts with findings in rainbow trout, where oral OHA administration to the swim-up fry for up to 57–60 days at doses comparable to those used in the present study (10–20 ppm) resulted in masculinization rates that varied from 80 to 100% [18,20]. In turn, feeding rainbow trout fry with OHA-supplemented diets at higher doses (60 ppm) for 20–60 days after yolk sac resorption resulted in approximately 70% sex-reversed neo-males and 20% intersex individuals [19]. On the other hand, lower doses of this hormone (5 ppm) provided for 57 days from the onset of exogenous feeding resulted in a reduced masculinization rate (about 30%) [18]. Treatment of the European whitefish (*Coregonus lavaretus*) at 32 days post-hatching with feed enriched with OHA at doses of 10 and 20 ppm for 63 days resulted in gonadal sterilization and the appearance of intersexual gonads [21]. The administration of OHA through immersion (0.4 ppm for 2 h) and oral supplementation (3 ppm for 60 days) one to three times between hatching and first feeding resulted in approximately 70% sex-reversed neo-males in rainbow trout [17]. Similarly, Redding et al. [44] showed that the immersion of swim-up fry in an OHA solution (0.5 ppm for 4 h, three times per week), combined with dietary administration of OHA at doses of 5–50 ppm for 4–8 weeks, induced testis-like gonadal development in 98% of coho salmon (*Oncorhynchus kisutch*) and chum salmon (*O. keta*) individuals.

The administration of MT induced dose-dependent disruptions in gonadal development in the grayling genetic females, resulting in intersex gonads rather than developed functional testes, which suggests that in graylings MT is a more masculinizing agent than OHA. In rainbow trout, feeding fry with diets supplemented with MT (5 ppm) for 57 days from the onset of exogenous feeding resulted in the masculinization of 40% of females, while the remaining 60% of individuals developed intersex gonads [18]. A slightly higher dose of MT (6 ppm) administered in feed for 60 days to all-female rainbow trout fry resulted in approximately 60% sex-reversed neo-males and only around 2% intersex individuals [20]. In turn, Atar et al. [19] reported a sex reversal rate of approximately 90% in this species after 60 days of feeding with diets containing 3ppm of MT hormone. In contrast, feeding mixed-sex fry with higher MT doses (20 ppm) resulted in 50% sterilization, with 40% males and 10% intersex individuals [18]. A single 2 h immersion of fry in 0.4 ppm of MT before the onset of exogenous feeding (1–2 weeks post-hatch), followed by 60 days of feeding with 3 ppm of MT, resulted in the complete masculinization of rainbow trout [17].

Baker et al. [45], studying chinook salmon, achieved 82% males following fry immersion in 2–20 ppm of MT and concluded that the efficiency of hormone-induced masculiniza-

tion in salmonids strongly depends on species-specific sensitivity, as well as the dose, route and timing of administration. In general, excessive doses or prolonged exposure typically cause complete sterilization, whereas insufficient doses often result in no sex reversal or the development of intersex gonads. While exact dose thresholds for graylings remain to be established, studies in other salmonids indicate that, for 17α-methyltestosterone, dietary doses above ~20 ppm or prolonged exposure during the critical labile period can induce sterilization, whereas doses of 3–6 ppm often result in partial masculinization with frequent intersex occurrence [18,20]. It is hypothesized that the aromatization of MT or endogenous testosterone to estradiol in the gonads leads to intersex development, particularly when the administered hormone dose is too low [46,47].

Recent studies have demonstrated that sex-specific gene expression in the grayling can already be detected during embryogenesis and is particularly pronounced around hatching, but gonadal differentiation is strongly delayed [39]. At hatching and for at least three weeks thereafter (22 days post-hatching, dph), gonads remain undifferentiated, containing only a few primordial germ cells. The first signs of differentiation appear in the grayling in around the 7th week post-hatching (~50 dph), when immature testis-like structures are present in about 50% of juveniles, most of which are genetic females. This male-like phenotype in females is progressively replaced by ovarian development from about the 11th week post-hatch (~78 dph) [39]. In contrast, males often retain undifferentiated gonads for much longer, with only ~12% showing testicular tissue in the 18th week post-hatch (~130–134 dph). However, final gonadal differentiation in both sexes occurs no earlier than 29 weeks post-hatching (~208–216 dph) [39]. Taken together, these findings indicate that the period of androgen sensitivity in graylings is largely protracted and delayed, spanning well beyond the onset of exogenous feeding, i.e., from the peri-hatch period to approximately eight months post-fertilization. This suggests that the recorded discrepancy of external masculinization without gonadal reversal in the examined graylings can likely be attributed to the hormone administration period being too early and short. Therefore, hormonal treatment to produce grayling neo-males may require a later onset and/or an extended duration, potentially combined with immersion and oral administration. However, it cannot be discounted that the metabolic fate of OHA and MT hormones in graylings may differ from that in other salmonids, as they can be preferentially metabolized to weaker androgens or rapidly inactivated. As a result, the effect of both hormones may be limited to tissues that are sensitive to circulating androgens (skin, fins, secondary sexual traits), without sufficient androgenic stimulation of the gonads. A similar dissociation between external and gonadal masculinization has been described in cases where aromatase activity remains high, leading to persistent estrogen synthesis that counteracts testicular differentiation [46,47].

5. Conclusions

To conclude, the obtained results indicate that androgen-mediated neo-male induction by OHA and MT hormones is potentially possible in the grayling, but it requires substantial refinement. In particular, the efficient masculinization of this species by hormonal treatment may require higher doses of hormones, different ways of administration (a combination of immersion and oral treatment), earlier (before hatching) treatment, and prolonged exposure (up to 29 weeks post-hatch).

Author Contributions: Conceptualization, K.O. and R.R.; methodology, K.O., M.K., A.K. and R.R.; software, M.K.; validation, K.O., M.K. and R.R.; formal analysis, M.K., A.K. and R.R.; investigation, M.K., A.K. and R.R.; resources, S.D. and R.R.; data curation, R.R.; writing—original draft preparation, K.O., R.R., A.K. and M.K.; writing—review and editing, K.O.; visualization, R.R., A.K.; supervision, K.O.; project administration, S.D. and K.O.; funding acquisition, S.D. and K.O. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was carried out in strict accordance with Polish legal regulations (The Act of the Polish Parliament of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes, Journal of Laws 2015, Item 266). The protocol was approved by the Local Ethics Committee for Animal Experiments in Bydgoszcz, Poland (No. 14/2022).

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Gdynia, 28.05.2026

OŚWIADCZENIE O WSPÓŁAUTORSTWIE W PUBLIKACJI

Oświadczam, że mój udział w następującej publikacji:

Rożyński R., Kuciński M., Dobosz S., Kycko A., Ocalewicz K. 2025. Hormonal Masculinization of the European Grayling (*Thymallus thymallus*) Using 11β-Hydroxyandrostenedione (OHA) and 17α-Methyltestosterone (MT). Animals 15(20):3059. https://doi.org/10.3390/ani15203059. (IF = 3,2; 100 pkt MNiSW)

obejmował analizę histologiczną.

.....A.Kycko.....

Anna Kycko

Gdynia, 28.05.2026

OŚWIADCZENIE O WSPÓŁAUTORSTWIE W PUBLIKACJI

Oświadczam, że mój udział w następującej publikacji:

Rożyński R., Kuciński M., Dobosz S., Greszta M., Ocalewicz K. 2025. Growth performance, survival rates and catchability of diploid and triploid European grayling (*Thymallus thymallus*). Aquaculture Reports 43:102908. https://doi.org/10.1016/j.aqrep.2025.102908. (IF = 4,0; 100 pkt MNiSW)

obejmował przeprowadzenie badań w zakresie łowności poprzez realizację specjalistycznych sesji połowów muchowych.

Michał Greszta

Michał Greszta

Gdynia, 28.05.2026

OŚWIADCZENIE O WSPÓŁAUTORSTWIE W PUBLIKACJI

Oświadczam, że mój udział w następującej publikacji:

Rożyński R., Dobosz S., Rożyński M., Ocalewicz K. 2025. The Effect of Triploidy on Gonadal Development, Hematology and Biochemistry in the European Grayling (*Thymallus thymallus*). Animals 15:481. https://doi.org/10.3390/ani15030481. (IF = 3,2; 100 pkt MNiSW)

obejmował opracowanie metodologii, obsługę oprogramowania, walidację i analizę formalną wyników.

Maciej Rożyński

Gdynia, 28.05.2026

OŚWIADCZENIE O WSPÓŁAUTORSTWIE W PUBLIKACJACH

Oświadczam, że mój udział w następujących publikacjach:

Rożyński R., Kuciński M., Dobosz S., Ocalewicz K. 2023. Successful application of UV-irradiated rainbow trout (*Oncorhynchus mykiss*) spermatozoa to induce gynogenetic development of the European grayling (*Thymallus thymallus*). Aquaculture 574:739690. https://doi.org/10.1016/j.aquaculture.2023.739690. (IF = 4,4; 140 pkt MNiSW)

Rożyński R., Kuciński M., Dobosz S., Greszta M., Ocalewicz K. 2025. Growth performance, survival rates and catchability of diploid and triploid European grayling (*Thymallus thymallus*). Aquaculture Reports 43:102908. https://doi.org/10.1016/j.aqrep.2025.102908. (IF = 4,0; 100 pkt MNiSW)

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analizę molekularną, wizualizację danych, przygotowanie wstępnych wersji manuskryptów oraz pomoc techniczną przy realizacji badań.

Kuciński Marcin

Marcin Kuciński

Gdynia, 28.05.2026

OŚWIADCZENIE O WSPÓŁAUTORSTWIE W PUBLIKACJACH

Oświadczam, że mój udział w następujących publikacjach:

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obejmował przygotowanie koncepcji, zapewnienie kluczowych zasobów w postaci materiału biologicznego oraz infrastruktury badawczej.

Stefan Dobosz

Stefan Dobosz

Gdynia, 28.05.2026

OŚWIADCZENIE O WSPÓŁAUTORSTWIE W PUBLIKACJACH

Oświadczam, że mój udział w następujących publikacjach:

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obejmował opracowanie założeń doświadczalnych, opracowanie metodologii badań (triploidyzacja, gynogeneza), bezpośredni udział w badaniach (min. przeprowadzenie triploidyzacji, gynogenezy, wykonanie analiz cytogenetycznych), sprawowanie nadzoru naukowego oraz przygotowanie i redakcję manuskryptów.

Konrad Ocalewicz

Gdynia, 28.05.2026

OŚWIADCZENIE O WSPÓŁAUTORSTWIE W PUBLIKACJACH

Oświadczam, że mój udział w następujących publikacjach:

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Rożyński R., Dobosz S., Rożyński M., Ocalewicz K. 2025. The Effect of Triploidy on Gonadal Development, Hematology and Biochemistry in the European Grayling (*Thymallus thymallus*). Animals 15:481. https://doi.org/10.3390/ani15030481. (IF = 3,2; 100 pkt MNiSW)

Rożyński R., Kuciński M., Dobosz S., Greszta M., Ocalewicz K. 2025. Growth performance, survival rates and catchability of diploid and triploid European grayling (*Thymallus thymallus*). Aquaculture Reports 43:102908. https://doi.org/10.1016/j.aqrep.2025.102908. (IF = 4,0; 100 pkt MNiSW)

Rożyński R., Kuciński M., Dobosz S., Kycko A., Ocalewicz K. 2025. Hormonal Masculinization of the European Grayling (*Thymallus thymallus*) Using 11β-Hydroxyandrostenedione (OHA) and 17α-Methyltestosterone (MT). Animals 15(20):3059. https://doi.org/10.3390/ani15203059. (IF = 3,2; 100 pkt MNiSW)

obejmował pełnienie roli głównego wykonawcy kluczowych etapów prac badawczych, w tym: przygotowanie założeń naukowych oraz opracowanie metodologii, w tym procedur indukcji rozwoju gynogenetycznego i triploidyzacji oraz hormonalnego odwracania płci u lipienia europejskiego. Byłem bezpośrednio odpowiedzialny za prowadzenie prac eksperymentalnych, co obejmowało m.in. pozyskiwanie i przygotowanie gamet, obsługę urządzeń do manipulacji chromosomowych, wieloletni nadzór nad chowem ryb w warunkach akwakultury oraz realizację testów łowności. Mój wkład obejmował również gromadzenie i walidację danych, ich kompleksową analizę statystyczną oraz merytoryczną interpretację wyników badań hematologicznych, biochemicznych, histologicznych i genetycznych. Jako autor wiodący byłem odpowiedzialny za przygotowanie wszystkich czterech artykułów. Mój udział w każdej z powyższych publikacji szacuję na 60%.

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