VKM Report 2025: 02

Overview of methods for production of sterile salmonids, their applicability in aquaculture and possible implications to wild salmon populations and biodiversity in Norway

Kjetil Hindar, Johanna Bodin, Knut Tomas Dalen, Nur Duale, Åse Helen Garseth, Martin Malmstrøm, Ville Erling Sipinen, Eva B. Thorstad, Paul Ragnar Berg, Tor Atle Mo, Ingrid Olesen, Ann-Karin Hardie Olsen, Espen Rimstad and Gaute Velle

Scientific Opinion of the Norwegian Scientific Committee for Food and Environment

VKM has assessed the potential use of sterile salmonids in aquaculture, focusing on positive and negative effects on wild salmon in Norway. Triploidisation is highly efficient but raises welfare concerns such as stress and disease susceptibility. VKM has therefore assessed various measures that can amend these negative effects. Alternative sterilisation methods like gene editing, vaccination and temporal knock-down of proteins for gonadal development by antisense oligomers are promising, although remain mostly experimental and require further development. Sterility is a potentially very negative trait if spread to wild populations. Therefore, VKM find that, overall, methods that result in permanent genetic changes are more likely to have negative effects than methods that only affect the downstream effects of relevant genes.

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Norwegian Scientific Committee for Food and Environment (VKM)
Postboks 222 Skøyen
0213 Oslo
Norway

Email: vkm@vkm.no
Website: vkm.no

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Preparation of the opinion

The Norwegian Scientific Committee for Food and Environment (Vitenskapskomiteen for mat og miljø, VKM) appointed a project group to draft the opinion. The project group consisted of five VKM members, two VKM staff and one external expert. An interdisciplinary VKM approval group appointed specifically for the assignment, assessed, and approved the final opinion.

Authors of the opinion

The authors have contributed to the opinion in a way that fulfils the authorship principles of VKM (VKM, 2023). The principles reflect the collaborative nature of the work, and the authors have contributed as members of the project group or the interdisciplinary approval group.

Members of the project group

Kjetil Hindar - Chair of the project group and member of the VKM Panel on Biodiversity. Affiliation: 1) VKM; 2) Norwegian Institute for Nature Research (NINA)

Johanna Bodin – Member of the VKM Panel on Genetically Modified Organisms. Affiliation: 1) VKM; 2) Norwegian Institute of Public Health (NIPH)

Knut Tomas Dalen - Member of the VKM Panel on Nutrition, Dietetic Products, Novel Food and Allergy. Affiliation: 1) VKM; 2) University of Oslo (UIO)

Nur Duale – Member of the VKM Panel on Genetically Modified Organisms. Affiliation: 1) VKM; 2) Norwegian Institute of Public Health (NIPH)

Åse Helen Garseth – Member of the project. Affiliation: 1) Norwegian Veterinary Institute (VI)

Martin Malmstrøm – Project leader, VKM staff. Affiliation: VKM

Ville Erling Sipinen – Project member, VKM staff. Affiliation: VKM

Eva B. Thorstad - Member of the VKM Panel on Biodiversity. Affiliation: 1) VKM; 2) Norwegian Institute for Nature Research (NINA)

Members of the interdisciplinary approval group:

Gaute Velle – Chair of the approval group and chair of the VKM Panel on Biodiversity. Affiliation: 1) VKM; 2) NORCE Norwegian Research Centre; 3) University of Bergen (UiB).

Paul Ragnar Berg – Member of the VKM Panel on Biodiversity. Affiliation: 1) VKM; 2) Norwegian Institute for Water Research (NIVA)

Tor Atle Mo – Member of the VKM Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) Norwegian Institute for Nature Research (NINA)

Ingrid Olesen – Member of the VKM Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) The Norwegian food research institute NOFIMA

Ann-Karin Hardie Olsen – Member of the VKM Panel on Contaminants. Affiliation: 1) VKM; 2) Norwegian Institute for Air Research (NILU)

Espen Rimstad – Member of the VKM Panel on GMO Medicinal products. Affiliation: 1) VKM; 2) Norwegian University of Life Sciences (NMBU)

Acknowledgement

VKM would like to thank Trude Anine Muggerud (Norwegian Institute of Public Health, the library) for valuable help in designing and performing the literature searches.

VKM would also like to thank the referees Lan Xu (Queen Mary University of London) and Guro Katrine Sandvik (Norwegian University of Life Sciences), for their valuable comments through critical review of the draft opinion. VKM emphasises that the referees are not responsible for the content of the final opinion. In accordance with VKM's routines for approval of a risk assessment (VKM, 2024), VKM received their comments before evaluation and approval by the interdisciplinary VKM approval group, and before the opinion was finalised for publication.

Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third-party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Table of Contents

Summary

Background

The Norwegian Environment Agency has asked VKM to assess both the positive and the negative effects on biological diversity resulting from the potential use of sterile salmonids in aquaculture in Norway. The use of sterile salmon in aquaculture has positive effects on biodiversity as it prevents interbreeding of escaped farmed salmon with wild salmon populations. Sterility may be achieved through triploidisation and other methods. VKM has recently assessed the merits of triploidy with respect to health and welfare in production of farmed Atlantic salmon in Norway (VKM 2023:22). The Environment Agency has also requested that the current assessment include other methods for sterilising farmed fish.

Large numbers of escaped farmed Atlantic salmon have been detected in Norwegian rivers since the late 1980s, and escaped farmed salmon have been recorded in all salmon rivers in Norway. The escaped farmed salmon may interbreed with wild salmon and their offspring compete with wild juveniles for space and food. This interbreeding results in genetic changes and reduced fitness in wild salmon, thereby reducing the productivity and viability of native salmon populations. In Norway, two thirds of wild salmon populations are affected by genetic introgression from escaped farmed salmon.

Several measures to limit the negative effects of escaped farmed salmon were outlined in St. prp. No. 32 (2006-2007) *Om vern av villaksen og ferdigstilling av nasjonale laksevassdrag og laksefjorder*, including the use of sterile salmon.

The terms of reference from the Environment Agency required a summary of knowledge and an assessment of the positive and negative consequences related to:

Biological diversity with the use of salmonids sterilised by triploidisation.

- Within this category, VKM was asked to describe the effectiveness of triploidisation, the occurrence and impact of farmed and triploid farmed fish in the environment, and other positive or negative effects of triploid salmonids compared to traditional farming.

Biological diversity with the use of alternative methods for the production of sterile farmed fish.

- Within this category, VKM was asked to assess whether it is possible to advance triploid welfare in farming by breeding or other methods, provide an overview of methods intended for the sterilisation of salmonids for aquaculture, assess the potential for genetic impact of those methods, and assess other positive or negative effects on biological diversity by the same methods.

VKM appointed a project group with expertise in ecology, fish health, and molecular biology to write a report in response to the terms of reference. An interdisciplinary approval group with similar expertise was tasked to review the report. Two external reviewers also read and commented on the report before it was assessed by the approval group.

Methods

Literature searches were performed following discussions by the project group, VKM staff, and a senior librarian at the Norwegian Institute of Public Health (NIPH). The librarian performed the searches in January 2024, which resulted in 1328 references. These were screened by the project group, reducing the number to 485 relevant articles and book chapters. Additional manual searches were also performed, including the screening of articles cited in the most recent literature.

Sterile fish in aquaculture can be obtained using a variety of strategies. They include triploidisation, hormone-, drug- and vaccine treatment, and techniques that temporarily or permanently affect expression of the genes involved in fish reproduction through silencing, knockdown, or knockout of one or more genes. While triploidisation leads to the imbalanced segregation of chromosomes in meiosis (halving of three), resulting in functional sterility, other methods typically target biological processes important for reproduction, such as the hypothalamic–pituitary–gonadal (HPG) axis, primordial germ cell (PGC) development or gametogenesis.

Results

Each of the methods used in salmonid fishes, or other relevant fish species, were categorized by mechanism of sterility and assessed with respect to the level of development, robustness, reproducibility, proportion of sterility obtained, use in salmonids or other cultured fishes, comparative performance versus non-modified fish, health, welfare, and mortality, and environmental risk categorisation in cases where sterile individuals where accidentally of intentionally released into the environment.

Fish farming is a big industry in Norway, where each year 450 million farmed salmonids are released into net pen cages in the sea after being artificially reproduced and reared to the smolt (seawater-adapted) stage in freshwater facilities. For each method, VKM therefore assessed its potential for large-scale use in Norwegian fish farming, while noting that many methods now currently are being tested in the laboratory. Hence, these will need a period of further innovation and testing to reach a commercial scale.

Methods that produce transient genetic changes leading to sterility by introducing antisense oligonucleotides that knockdown genes necessary for the development of gonads are interesting for two reasons. First, methods that involve immersion of eggs in solutions containing the antisense oligonucleotides may meet the requirements of inducing sterility in large numbers of individuals simultaneously. Secondly, administration of these antisense oligonucleotides to eggs does not result in permanent genetic changes yet is functional for a critical time for germ cell or gonad development.

Methods that result in permanent genetic changes leading to sterility include CRISPR (or similar techniques for targeted gene change) to knockout genes that are crucial for

reproduction. Among these genes are those involved in the HPG axis, PGC development, and gametogenesis.

Necessary steps may include breeding large numbers of fertile individuals that are heterozygote for the knockout genotype, and genotyping tens of thousands of microinjected, fertility-rescued individuals to select targeted knockout homozygotes from a large variety of microinjected CRISPR varieties. These varieties may (or may not) include fertile heterozygotes. This suggests that until a well-described sterile genotype is established, several generations may need to be produced in strict containment.

Vaccination is a promising method since it is a well-developed technique to protect against disease agents in hundreds of millions of smolts in Norwegian aquaculture. However, vaccination aimed at inhibiting gonadal development (immunosterilisation) has not reached a stage where it ensures permanent sterility in fish.

Interspecific hybridisation is used with some merits in stocking sterile fish for recreational fishing but is not shown to be a valuable strategy in commercial aquaculture. If used, triploid interspecific hybrids seem necessary to avoid breaking down borders between species.

All-female lines are not a sterilisation method but may reduce the unwanted effects of triploidy (like male gonadal development and spawning activity) and could be a viable strategy if used in a species where escaped individuals cannot meet a male of the same species in nature.

Triploidy is so far the only method for sterilisation that can be used in commercial-scale aquaculture. VKM's assessment of health and welfare for triploid salmon (VKM 2023:22) has identified problems related to stress response, environmental tolerance, and welfare in triploid farmed salmon. More research is needed before these problems can be alleviated. All-female lines for triploidy may be better than mixed-sex lines, partly because females seem more robust than males and partly because they do not engage in spawning attempts like triploid males do.

Uncertainty

Several approaches to introduce sterility have been proven successful in zebrafish in the laboratory, however lower or more variable success has been achieved when applying these methods in salmonids.

Limited or variable efficiency has been observed for methods like immunosterilisation, including lower efficiency both in the target proteins and the vaccination strategy.

Sterilisation using Morpholino or Gapmer oligonucleotides and transient knock down by immersion is a technique with a high potential for large-scale use, but the success rate is still uncertain for salmonids.

Conclusion

Escaped farmed Atlantic salmon are found in many rivers in Norway and genetic introgression from farmed to wild salmon has been documented in two thirds of wild salmon populations in Norway. This introgression leads to changes in smoltification and maturation, growth rate, timing of migration, straying rate, and ultimately, the fitness and viability of wild salmon populations.

Triploidisation is an effective method of sterilisation in fish with efficiency near 100% by hydrostatic pressure shock treatment of fertilised eggs to induced triploidy. 70-90% of the treated eggs survive until hatching relative to diploid fertilisation (90-95% survival).

Triploid males and females are sterile, but males may enter rivers and participate in spawning. All-female triploid farmed salmon would prevent spawning interaction by triploid males and also increase triploid robustness. The proportion of spontaneous triploid salmon in fish farms (2%) is roughly ten times as high as their proportion among escaped farmed salmon in rivers (0.2%), suggesting that triploid escapes have lower survival than diploid escapes.

Fish welfare issues in aquaculture have been reported for triploid Atlantic salmon, including higher sensitivity to environmental stressors and higher susceptibility to some disease agents. This may increase the transmission of disease agents from farmed to wild fish.

If using all-female triploid Atlantic salmon in aquaculture effects on biodiversity in rivers are expected to be reduced because significantly fewer escaped female salmon would enter rivers. Hence, all-female triploids might also reduce disease transmission in rivers.

Breeding programs for improved health and welfare of triploid Atlantic salmon may solve challenges with triploid aquaculture. VKM is however not aware of any existing breeding programs for improved health of triploid Atlantic salmon. Improved performance of traits like length and weight in diploid salmon may also improve the same traits in triploid salmon. However, genetic parameters for health- and welfare traits in triploid salmon are currently not known.

One method to improve the production of triploid fish includes producing triploid offspring by fertilisation of diploid eggs by tetraploid sperm. This method has been used on an experimental scale for 30 years but has seen limited success in Atlantic salmon.

In the short term, better welfare for triploid fish in aquaculture is more likely to be achieved by improving the farm conditions.

Other sterilisation methods, like temporal gene knockdown, are less effective in producing total sterility than triploidisation. However, escapes from such a population will still reduce the risk of genetic impact on wild salmonids compared to traditional farming, due to few fertile escapees.

Common for most new methods for sterile salmonid production is that they still are in the experimental stage. So far, these methods show variable success with respect to reaching sterility. Methods that are based on a temporal knockdown of germ cell formation by immersion of eggs in a solution with antisense oligonucleotides appear most promising with respect to efficiency and up-scaling for use in aquaculture. This is because egg immersion may

be used on thousands of eggs in each batch, and the induced changes are not genetically permanent and cannot be inherited by wild fish populations.

Methods involving permanent genetical changes that rely on the use of a high number of actual or potential heterozygote fertile individuals to produce sterile homozygotes are considered to be a higher threat to wild salmon genetic integrity if fertile fish with one sterile allele escape. Development and production of sterile salmon in land-based facilities could reduce the genetic risk to wild salmon populations.

The Effects on behaviour, welfare and on biological diversity have not been studied using any of the alternative methods for sterilisation of salmonids and can therefore not be risk assessed.

Data gaps

Alternative methods to triploidisation for generation of sterile salmonids are still at an early stage of development. Data on performance traits and pathogen susceptibility, health and welfare under controlled closed containment and realistic large-scale aquaculture conditions is lacking.

The limited understanding of the health and welfare traits in triploids and the lack of estimates on genetic parameters for the same traits limit the possibility for genetic improvement by a breeding program.

Sammendrag på norsk

Bakgrunn

Miljødirektoratet har bedt VKM om å vurdere både de positive og negative effektene på biologisk mangfold som følge av potensiell bruk av sterile laksefisk i akvakultur i Norge. Bruk av steril laks i oppdrettsnæringen vil ha positive effekter på biologisk mangfold ved at det forhindrer genetisk innblanding av oppdrettslaks, dersom disse rømmer, til ville laksebestander. Sterilitet kan oppnås gjennom triploidisering og andre metoder. VKM har nylig vurdert effekter ved triploidisering med hensyn til helse og velferd i produksjon av oppdrettet atlantisk laks i Norge (VKM 2023:22). Miljødirektoratet har også bedt om at den nåværende vurderingen inkluderer andre metoder for sterilisering av oppdrettsfisk.

Store mengder rømt oppdrettslaks har blitt oppdaget i norske elver siden slutten av 1980-tallet, og rømt oppdrettslaks er registrert i alle lakseelver i Norge. Den rømte oppdrettslaksen kan krysse seg med villaks, og deres avkom konkurrerer med ville ungfisker om plass og mat. Denne kryssingen resulterer i genetiske endringer og redusert tilpasningsevne hos villaks, noe som reduserer produktiviteten og levedyktigheten til de opprinnelige laksebestandene. I Norge er to tredjedeler av villaksbestandene påvirket av genetisk innblanding fra rømt oppdrettslaks.

Flere tiltak for å begrense de negative effektene av rømt oppdrettslaks ble skissert i St. prp. nr. 32 (2006-2007) Om vern av villaksen og ferdigstilling av nasjonale laksevassdrag og laksefjorder, inkludert bruk av steril laks.

Mandatet fra Miljødirektoratet ba en oppsummering av kunnskap og en vurdering av de positive og negative konsekvensene knyttet til:

Biologisk mangfold ved bruk av laksefisk sterilisert ved triploidisering.

- Innenfor denne kategorien ble VKM bedt om å beskrive effektiviteten av triploidisering, forekomsten og påvirkningen av oppdrettet og triploid oppdrettsfisk i miljøet, samt andre positive eller negative effekter av triploid laksefisk sammenlignet med tradisjonelt oppdrett.

Biologisk mangfold ved bruk av alternative metoder for produksjon av steril oppdrettsfisk.

- Innenfor denne kategorien ble VKM bedt om å vurdere om det er mulig å forbedre velferden til triploid fisk i oppdrett gjennom avl eller andre metoder, gi en oversikt over metoder beregnet på sterilisering av laksefisk for akvakultur, vurdere potensialet for genetisk påvirkning av disse metodene, og vurdere andre positive eller negative effekter på biologisk mangfold ved de samme metodene.

VKM oppnevnte en prosjektgruppe med ekspertise innen økologi, fiskehelse og molekylærbiologi for å skrive en rapport som svar på mandatet. En tverrfaglig godkjenningsgruppe med lignende ekspertise ble bedt om å gjennomgå rapporten. To eksterne fagfeller leste og kommenterte også rapporten før den ble vurdert av godkjenningsgruppen.

Metoder

Litteratursøk ble utført etter diskusjoner i prosjektgruppen, VKM-ansatte og en seniorbibliotekar ved Folkehelseinstituttet (FHI). Bibliotekaren utførte søkene i januar 2024, noe som resulterte i 1328 referanser. Disse ble gjennomgått av prosjektgruppen, og antallet ble redusert til 485 relevante artikler og bokkapitler. Ytterligere manuelle søk ble også utført, inkludert gjennomgang av artikler sitert i den nyeste litteraturen.

Steril fisk i akvakultur kan oppnås ved hjelp av ulike strategier. Disse inkluderer triploidisering, hormon-, medikament- og vaksinebehandling, og teknikker som midlertidig eller permanent påvirker uttrykket av genene involvert i fiskens reproduksjon gjennom silencing, knockdown eller knockout av ett eller flere gener. Mens triploidisering fører til ubalansert segregering av kromosomer i meiose (halvering av tre), noe som resulterer i funksjonell sterilitet, retter andre metoder seg typisk mot biologiske prosesser viktige for reproduksjon, som hypotalamus-hypofyse-gonade (HPG)-aksen, primordiale kimceller (PGC)-utvikling eller gametogenese.

Resultater

Hver av metodene brukt på laksefisk eller andre relevante fiskearter ble kategorisert etter steriliseringsmekanisme og vurdert med hensyn til utviklingsnivå, robusthet, reproduserbarhet, andel oppnådd sterilitet, bruk i laksefisk eller andre oppdrettsfisk, sammenlignende ytelse mot ikke-modifisert fisk, helse, velferd og dødelighet, og miljømessig risikokategorisering i tilfeller hvor sterile individer ved et uhell eller med vilje ble sluppet ut i miljøet.

Fiskeoppdrett er en stor industri i Norge, hvor hvert år 450 millioner oppdrettslaks slippes ut i merder i sjøen etter å ha blitt kunstig reprodusert og oppdrettet til smoltstadiet (sjøvannstilpasset) i ferskvannsanlegg. For hver metode vurderte VKM derfor potensialet for storskala bruk i norsk fiskeoppdrett, samtidig som de bemerket at mange metoder nå testes i laboratoriet. Derfor vil disse trenge en periode med videre innovasjon og testing for å nå kommersiell skala.

Metoder som produserer forbigående genetiske endringer som fører til sterilitet ved å introdusere antisense oligonukleotider som knockdown gener nødvendige for utvikling av gonader, er interessante av to grunner. For det første kan metoder som involverer immersjon av egg i løsninger som inneholder antisense oligonukleotider oppfylle kravene til å indusere sterilitet i et stort antall individer samtidig. For det andre resulterer administrasjon av disse antisense oligonukleotidene til egg ikke i permanente genetiske endringer, men er funksjonelle i en kritisk periode for kimcelle- eller gonadeutvikling.

Metoder som resulterer i permanente genetiske endringer som fører til sterilitet inkluderer CRISPR (eller lignende teknikker for målrettet genendring) for å knockout gener som er avgjørende for reproduksjon. Blant disse genene er de som er involvert i HPG-aksen, PGC-utvikling og gametogenese. Nødvendige trinn kan inkludere avl av et stort antall fertile individer som er heterozygote for knockout-genotypen, og genotyping av titusenvis av mikroinjiserte, fertilitetsreddede individer for å velge målrettede knockout-homozygoter fra et stort utvalg av mikroinjiserte CRISPR-varianter. Noen av disse variantene kan også være fertile

heterozygoter. Dette antyder at inntil en velbeskrevet steril genotype er etablert, kan flere generasjoner trenge å bli produsert i streng inneslutning.

Vaksinasjon er en lovende metode siden det er en velutviklet teknikk for å beskytte mot sykdomsagens i hundrevis av millioner smolt i norsk akvakultur. Imidlertid har vaksinasjon rettet mot å hemme gonadeutvikling (immunosterilisering) ikke nådd et stadium hvor det sikrer permanent sterilitet hos fisk.

Interspesifikk hybridisering brukes med noen fordeler i utsetting av steril fisk for rekreasjonsfiske, men er ikke vist å være en verdifull strategi i kommersiell akvakultur. Hvis brukt, synes triploide interspesifikke hybrider nødvendige for å unngå å bryte ned grenser mellom arter.

Populasjoner bestående av kun hunnlinjer er ikke en steriliseringsteknikk, men kan redusere de uønskede effektene av triploidi (som hannlig gonadeutvikling og gyteaktivitet) og kan være en levedyktig strategi hvis brukt i en art hvor rømt individer ikke kan møte en hann av samme art i naturen.

Triploidi er så langt den eneste metoden for sterilisering som kan brukes i kommersiell skala akvakultur. VKMs vurdering av helse og velferd for triploid laks (VKM 2023:22) har identifisert problemer knyttet til stressrespons, miljøtoleranse og velferd hos triploid oppdrettslaks. Mer forskning er nødvendig før disse problemene kan avhjelpes. Alle-hunnlinjer for triploidi kan være bedre enn blandede kjønnslinjer, delvis fordi hunner synes mer robuste enn hanner og delvis fordi de ikke deltar i gyteforsøk som triploide hanner gjør.

Usikkerhet

Flere tilnærminger for å introdusere sterilitet har vist seg vellykkede i sebrafisk i laboratoriet, men lavere eller mer variabel suksess har blitt oppnådd når disse metodene anvendes på laksefisk. Begrenset eller variabel effektivitet har blitt observert for metoder som immunosterilisering, inkludert lavere effektivitet både i målproteinene og vaksinasjonsstrategien. Sterilisering ved bruk av Morpholino eller Gapmer oligonukleotider og forbigående knockdown ved immersjon er en teknikk med høyt potensial for storskala bruk, men suksessraten er fortsatt usikker for laksefisk.

Konklusjon

Rømt oppdrettslaks finnes i mange elver i Norge, og genetisk innblanding fra oppdrettslaks til villaks har blitt dokumentert i to tredjedeler av villaksbestandene i Norge. Denne innblandingen fører til endringer i smoltifisering og modning, veksthastighet, tidspunkt for migrasjon, vandringsrate og til slutt, tilpasningsevne og levedyktighet hos villaksbestandene.

Triploidisering er en effektiv metode for sterilisering av fisk med en effektivitet nær 100% ved hydrostatisk trykksjokkbehandling av befruktede egg for å indusere triploidi. 70-90% av de behandlede eggene overlever til klekking sammenlignet med diploid befruktning (90-95% overlevelse). Triploide hanner og hunner er sterile, men hanner kan gå opp i elver og delta i gyting. Ren hunnlinje triploid oppdrettslaks vil forhindre gyteinteraksjon fra triploide hanner og også øke triploid robusthet. Andelen spontane triploide laks i fiskeoppdrett (2%) er omtrent

ti ganger så høy som deres andel blant rømt oppdrettslaks i elver (0,2%), noe som tyder på at triploide rømlinger har lavere overlevelse enn diploide rømlinger.

Fiskevelferdsproblemer i akvakultur har blitt rapportert for triploid atlantisk laks, inkludert høyere følsomhet for miljøstressorer og høyere mottakelighet for noen sykdomsagens. Dette kan øke overføringen av sykdomsagens fra oppdrettsfisk til villfisk. Ved bruk av ren hunnlinje triploid atlantisk laks i akvakultur forventes effektene på biologisk mangfold i elver å bli redusert fordi betydelig færre rømt hunnlaks vil gå opp i elver. Dermed kan bruk av ren hunnlinje triploider også redusere sykdomsoverføring i elver.

Avlsprogrammer for forbedret helse og velferd for triploid atlantisk laks kan løse utfordringer med triploid akvakultur. VKM er imidlertid ikke kjent med noen eksisterende avlsprogrammer for forbedret helse for triploid atlantisk laks. Forbedret ytelse av egenskaper som lengde og vekt i diploid laks kan også forbedre de samme egenskapene i triploid laks. Imidlertid er genetiske parametere for helse- og velferdsegenskaper i triploid laks for øyeblikket ikke kjent.

En metode for å forbedre produksjonen av triploid fisk inkluderer produksjon av triploid avkom ved befruktning av diploide egg med tetraploid sperm. Denne metoden har blitt brukt på eksperimentell skala i 30 år, men har hatt begrenset suksess i atlantisk laks. På kort sikt er bedre velferd for triploid fisk i akvakultur mer sannsynlig å oppnås ved å forbedre oppdrettsbetingelsene.

Andre steriliseringsteknikker, som forbigående gen-knockdown, er mindre effektive i å produsere total sterilitet enn triploidisering. Imidlertid vil rømlinger fra en slik populasjon fortsatt redusere risikoen for genetisk påvirkning på ville laksefisk sammenlignet med tradisjonelt oppdrett, på grunn av få fertile rømlinger. Felles for de fleste nye metoder for produksjon av sterile laksefisk er at de fortsatt er i eksperimentstadiet. Så langt viser disse metodene variabel suksess med hensyn til å oppnå sterilitet. Metoder som er basert på forbigående knockdown av kimcelleformasjon ved immersjon av egg i en løsning med antisense oligonukleotider synes mest lovende med hensyn til effektivitet og oppskalering for bruk i akvakultur. Dette er fordi eggimmersjon kan brukes på tusenvis av egg i hver batch, og de induserte endringene er ikke genetisk permanente og kan derfor ikke arves av ville fiskepopulasjoner.

Metoder som involverer permanente genetiske endringer som er avhengige av bruk av et høyt antall faktiske eller potensielle heterozygote fertile individer for å produsere sterile homozygoter, anses å være en høyere trussel mot villaksens genetiske integritet hvis fertile fisk med ett sterilt allel rømmer. Utvikling og produksjon av steril laks i landbaserte anlegg vil redusere den genetiske risikoen for villaksbestander.

Effektene på atferd, velferd og biologisk mangfold har ikke blitt studert ved bruk av noen av de alternative metodene for sterilisering av laksefisk og kan derfor ikke risikovurderes.

Kunnskapshull

Alternative metoder til triploidisering for generering av sterile laksefisk er fortsatt på et tidlig utviklingsstadium. Data om ytelsestrekk og patogenmottakelighet, helse og velferd under kontrollerte lukkede inneslutninger og realistiske storskala akvakulturforhold mangler. Den begrensede forståelsen av helse- og velferdsegenskaper i triploider og mangelen på estimater på genetiske parametere for de samme egenskapene begrenser muligheten for genetisk forbedring gjennom et avlsprogram.

Abbreviations and glossary

Abbreviations

ASOs	Antisense oligonucleotides
CRISPR	Clustered regulatory interspaced short palindromic repeats
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ES	Embryonic stem cells
FAO	Food and Agriculture Organization of the United Nations
FDA	U.S. food & drug administration
FSH	Follicle-stimulating hormone
GM	Genetically modified
GMO	Genetically modified organism
GnRH	Gonadotropin-releasing hormone
Gsdf	Gonadal soma-derived factor
HPG	Hypothalamic–pituitary–gonadal axis
ICES	International Council for Exploration of the Seas
IPR	Intellectual Property Rights
ISA	Infectious salmon anaemia
KLH	Keyhole limpet hemocyanin
LH	Luteinising hormone
MO	Morpholino oligonucleotide
MDHT	17α-methyldihydrotestosterone
miRNA	MicroRNA
mRNA	Messenger RNA
NASCO	North Atlantic Salmon Conservation Organization
NIPH	Norwegian Institute of Public Health
OHA	17 β-hydroxyandrostenedione
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PZP	Porcine zona pellucida
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

RNAi	RNA interference
SGR	Specific growth rate
siRNA	small-interfering RNA, also known as short interfering RNA or silencing RNA
shRNA	short (or small) hairpin RNA
TALEN	Transcription activator-like effector nuclease
TGFβ	Transforming Growth Factor Beta
UTR	Untranslated region (genetics, gene expression)
VKM	Vitenskapskomiteen for mat og miljø (Norwegian Scientific Committee for Food and Environment)
VRL	Vitenskapelig råd for lakseforvaltning (Norwegian Scientific Advisory Committee for Atlantic Salmon)
WISH	Whole mount *in situ* hybridiSation
ZFN	Zinc finger nuclease

Glossary

Allele	An allele is one of two or more versions of DNA sequence (gene) at a given genomic location. An individual inherits two alleles, one from each parent. If the two alleles are the same, the individual is homozygous for that allele. If the alleles are different, the individual is heterozygous. Naturally occurring functional alleles are called wild-type alleles (WT), while abnormal and often non-functional alleles are called mutants.
Androgen	A natural or synthetic steroid hormone that regulates the development and maintenance of male characteristics in vertebrates.
Anovulation	The failure of mature eggs (oocytes) to be released from the ovaries during the folliculogenesis or spawning process.
Antibody	Y-shaped protein belonging to the immunoglobulin superfamily which is used by the immune system to identify and neutralize antigens such as bacteria and viruses.
Antigen	In immunology, a molecule, moiety, foreign particulate matter, or an allergen, such as pollen, that can be bound by a specific antibody. The presence of antigens in the body may trigger an immune response.
Antisense	In molecular biology and genetics, 'antisense' of a DNA or RNA -strand, refers to the complementary sequence of the 'sense'-sequence.
Apoptosis	A regulated and controlled cell death that occurs in multicellular organisms.
Adjuvant	In pharmacology, a drug or other substance, or a combination of substances, that is used to increase the efficacy or potency of certain drugs, e.g. vaccines.
Aquaculture	Breeding, rearing, feeding, and harvesting of fish, shellfish, algae, and other organisms in aquatic environments.
AquAdvantage	A commercialised genetically modified salmon.
Atlantic salmon - *Salmo salar*	A fish species belonging to the salmon (Salmonidae) family, native to the North Atlantic region. They spawn in rivers, spend the juvenile phase in rivers or lakes, and most individuals perform long-distance feeding migrations in the ocean before returning to their native river for spawning. In Norway, wild populations are found in more than 450 rivers, and the species is also extensively used as farmed fish in aquaculture.

Autoimmunity	The system of immune responses of an organism against its own healthy cells, tissues, and other natural body constituents. (Autoimmunity is often a pathological response)
Base-pairing	Two complementary DNA nucleotide bases that pair together.
Biodiversity	Variety of life on Earth at all its levels, from genes to ecosystems, and can encompass the evolutionary, ecological, and cultural processes that sustain life.
Blastocyst	A blastocyst is a cluster of dividing cells made by a fertilised egg. It is the early stage of an embryo.
Broodstock	Broodstock or brood fish are the parent fish from which fry and fingerlings are produced. The success of stocking programs for wild fish, fish farming and aquaculture industries depends upon a reliable supply of healthy fry/fingerlings that have a sound genetic base.
Cas	A CRISPR-associated (Cas) endonuclease, or enzyme, that acts as molecular scissors to cut DNA at a location specified by a guide RNA (gRNA).
Cataract	Permanent or reversible alteration of lens opacity/loss of transparency due to disturbances in osmotic homeostasis of the lens, intraocular infections and parasites, environmental, hereditary, toxicological, and nutritional factors, for instance insufficient levels of the amino acid histidine.
Chromosome	Threadlike structures made of protein and a single molecule of DNA that serve to carry genomic information in the nucleus of the cells.
Coho salmon - *Oncorhynchus kisutch*	A fish species belonging to the salmon (Salmonidae) family, native to the North Pacific region.
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats. Molecular machinery enabling gene/genome edits by precisely cutting DNA and then letting natural DNA repair processes join the DNA cuts. The system consists of the Cas enzyme and a guide RNA (gRNA). CRISPR-Cas is used as a molecular tool to change single nucleotides, introduce short insertions/deletions (indels), or insertion of new genes.
Cytotoxic	Harmful to living cells, i.e., a substance or process that can damage or kill cells.
Electroporation	The process of using an electric pulse to introduce DNA into cells by creating temporary pores in the cell membrane.

Embryo	The initial stage of development for a multicellular organism.
Embryogenesis	The developmental process by which an embryo is formed and developed.
Endonucleases	Enzymes that cleave the phosphodiester bond within a polynucleotide chain (DNA or RNA).
ERA	Environmental Risk Assessment
Erythrocytes	The red blood cells (RBC), the functional components of blood responsible for transporting gases and nutrients.
Eukaryote	Any cell or organism that possesses a clearly defined nucleus.
Fish health	Absence of disease or normal functioning and behaviour of the fish.
Fish welfare	Experiences, perceptions, and quality of life as perceived by the fish itself.
Flow cytometry	A laser-based lab test that can detect chemical and physical characteristics of cells or particles.
Folliculogenesis	The process by which the female germ cell develops within the ovary and matures into a fertilisable egg.
Fry	Juvenile fish at the stage of development when the yolk sac has almost disappeared, and the swim bladder is functional to the point where the fish can move around and perform limited foraging to nourish itself.
Gamete	A reproductive cell of an animal or plant. In animals, female gametes are called ova or egg cells, and male gametes are called sperm.
Gametogenesis	A process by which diploid or haploid precursor cells undergo cell division and differentiation to form mature haploid gametes.
Gapmer	A short single-stranded DNA antisense oligonucleotide (ASO) with a central portion of DNAs and modified RNA-like nucleotides on on both sides of the DNA sequence. Gapmer ASOs bind to their target mRNA to form DNA-RNA hybrids that recruit RNase H for the cleavage of these mRNAs.
Gene knockdown	Refers to techniques by which the expression of one or more of an organism's genes is reduced.
Gene knockout	Inactivation of a gene as a result of genetic changes (insertion/deletion/substitution).
Gene silencing	Interruption or suppression of the expression of a gene at transcriptional or translational levels.

Genetic modification	The process of inserting novel DNA/genes from the same or foreign species or deleting genes. Common to all is the use of recombinant DNA technology.
Genetic ploidy	Refers to the number of sets of chromosomes in a cell. Most animal cells are diploid, containing two chromosome sets.
Genome editing	The process of editing DNA with techniques such as CRISPR to target genetic changes to a specific location in a genome. Most often with the aim to change single nucleotides or produce short DNA insertions/deletions (indels).
Genotype	The product of all genes/allelic variants in an organism or may alternatively be used to indicate allelic combinations of a single gene.
Germ cells	Embryonic cells with the potential of developing into gametes.
Gonads	Reproductive gland, a mixed gland that produces the gametes and sex hormones of an organism.
Gonadotropin	Gonadotropins are glycoprotein hormones secreted by gonadotropic cells of the anterior pituitary of vertebrates.
gRNA/Guide RNA	Short RNA sequence recognized by Cas proteins, and an essential component of CRISPR engineering tools. Part of the sequence is complementary to the region to be edited, while other parts of the sequence bind the Cas protein.
Granulosa cells	Somatic supporting cells of the ovary that play critical roles in follicle and germ cell development.
Heterozygous	As related to genetics, refers to having inherited different versions (alleles) of a genomic marker (e.g., a gene) from each biological parent.
Histidine	One of the 20 amino acids used to make proteins. It is an essential amino acid, meaning that fish are not able to produce it in the body and must obtain it through their diet.
Histology	Method to study the microscopic structure of cells, tissues, and organs.
Homologous recombination	A type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA.
Homozygous	As related to genetics, refers to having inherited the same versions (alleles) of a genomic marker (e.g., a gene), from each biological parent.

Hybridisation	The process of combining two different varieties of organisms to create a hybrid offspring that is genetically related to both parents.
Hybrid swarm	A population of hybrids that has survived beyond the initial hybrid generation, with interbreeding between hybrid individuals and backcrossing with its parent types.
Immunocontraception	Inducing sterility by activation of the immune system, often via vaccination with proteins essential for germ cell development that trigger an autoimmune response and apoptosis at the target tissue expressing these proteins or receptors.
Immunohistochemistry	Technique to visualize tissue composition and presence of specific proteins in thin tissue sections by using target antibodies as detection systems.
Immunosterilisation	Sterilisation by triggering the immune response to target a specific protein essential for germ cell development by autoimmunity and induced apoptosis.
In situ	*"On site"* investigation of tissue appearance and presence of specific proteins. *In situ* is also used to signify the natural environment of the organism.
Intergeneric hybridisation	Crossing of two species of different genera, same genus.
Interspecific hybridisation	Crossing of two species from the same genus.
Intra-peritoneal	Injection in peritoneum/the bowel, most often used injection site for vaccination of fish.
Introgression	Movement of genes from one species or population into the gene pool of another.
Karyotype	The general appearance of the complete set of chromosomes in the cells of a species or in an individual organism.
Locus	A genetic locus (loci in plural) refers to a specific physical region of a chromosome that defines an area harbouring a gene or genes.
Luciferase assay	Technique used to investigate a variety of cellular processes using bioluminescent light output as a sensitive measure of biological activity.
Meiosis	Cell division resulting in two germ cells each possessing half the number of chromosomes of the original cell.
Microinjection	Injection into a single cell (often a recently fertilised embryo).
Microsatellites	In genetics refers to repetitive segments of DNA scattered throughout the genome in noncoding regions between genes or within genes.

Morpholino oligonucleotides (MOs)	Chemically modified synthetic antisense oligonucleotides, that consist of ~ 25 nucleotides with morpholino rings as backbone structure and bind to targeted RNA sequences by base-pairing.
Morphology	The form/shape and structure of organisms (including fish).
Mutagenesis	Refers to the process of inducing changes in the genetic material of an organism, e.g. through exposure to certain chemicals or radiation.
Mutant	A mutant is a genetically changed allele, or an organism that carries a mutation.
Mutation	A change in the nucleotide sequence of DNA that happens during replication prior to cell division. In this document, we also include in the term changes to DNA sequences that are made by genome editing tools like CRISPR-Cas.
Neomales	Sexually reproductive males but genetically female fish. Females with sperm cells only containing the X-chromosome, thereby only capable of producing female offspring. Neomales are produced by androgen treatment.
Non-canonical	A feature that deviates from the common or established rule sets (the canonical model).
Nucleases	Any enzyme that cleaves nucleic acids by breaking phosphodiester bonds between nucleotide molecules.
Nucleic acids	Naturally occurring chemical compounds that serve as the main information-carrying molecule of the cell and that directs the process of protein synthesis, thereby determining the inherited characteristics of every living thing.
Off-target effects	Unintended effects when DNA is altered at sites in the genome not intentionally targeted.
Oligonucleotide	Short DNA or RNA molecules, oligomers, that have a wide range of applications in genetic testing, research, and forensics.
Oocytes	Female germ cells, immature eggs.
Oophoritis	Inflammation in one or both ovaries
Orchitis	Inflammation of the testes
Pacific salmon	Salmonid fishes of several species belonging to the genus *Oncorhynchus*. Native to the North Pacific region.
Parr	Juvenile salmon between the fry and smolt stage. They are named for the vertical marks on their sides called “parr” marks.

Peptide	Short string of 2 to 50 amino acids, formed by a condensation reaction, joining together through a covalent ester bond. Sequential covalent bonds with additional amino acids yield a peptide chain and the building block of proteins.
Phage	Phages (short for bacteriophage) are virus that infects bacteria. Phages are used as tools in biotechnology, genetic engineering and therapeutics.
Phenology	The study of natural phenomena that recur periodically in plants and animals and of the relationship of these phenomena to seasonal changes and climate.
Phenotype	The composite of an organism's observable characteristics or traits. An organism's phenotype is dictated by the expression of an organism's genetic code, its genotype, the influence of environmental factors, and the interactions between these.
Polar body	A small byproduct of the meiotic division of an oocyte, which remains within the zona pellucida after undergoing apoptosis.
Primordazine	A small molecule compound identified by chemical screening in zebrafish embryos. It functions as a selective inhibitor of primordial germ cell (PGC) development by targeting poly(A)-tail-independent noncanonical translation.
Primordial germ cell (PGC)	Germline stem cells that give rise to gametes in vertebrates and are separated from somatic cells in early development.
Proteomics	The study of the interactions, function, composition, and structures of proteins and their cellular activities.
Ribonuclease	A type of nuclease that catalyses the degradation of RNA into smaller components.
RNA interference	RNAi, RNA-mediated gene silencing pathway found widely in eukaryotes, including mammals.
Salmonids	Fishes belonging to the family Salmonidae, which includes more than 200 species of char, grayling, lenok, Atlantic and Pacific salmon, taimen, trout and whitefish.
Saprolegniosis	Disease caused by oomycete pathogens (fungus-like organisms), that can cause serious losses to fish both in commercial hatcheries and fish farms and can affect wild stocks of salmonids when they return to their spawning grounds.

Secondary sexual traits	Characteristics that distinguish the two sexes of a species, but that are not directly part of the reproductive system.
Sertoli cells	Supporting cells of the testes that are essential for spermatogenesis.
Smolt	Juvenile life stage of salmon, when they migrate down the river, prepared to enter the sea for the ocean feeding migration. The term is also used in aquaculture, at the stage when salmon are ready to be transferred from freshwater hatcheries to net pens in the sea.
Smoltification	The process of physiological and morphological changes when a salmon juvenile is adapting to saltwater and becomes ready to move from freshwater to saltwater.
Somatic cells	Any biological cell forming the body of a multicellular organism other than germ cells.
Spermatids	Haploid cells that are formed by the second division in meiosis of a spermatocyte and that differentiate into spermatozoa.
Spermatocyte	A cell that is derived by mitosis from a spermatogonium (undifferentiated male germ cell) and ultimately gives rise by meiosis to four haploid spermatids.
Spermatozoa	(Singular: spermatozoon) also known as sperm, refers to the mature male sex cells or gametes.
Splicing (RNA)	The process by which introns are cut out of the primary mRNA transcript, and the exons are joined together to make mature mRNA.
Transcription	The process of making an RNA copy of a gene's DNA sequence. One of the various types of RNAs, called messenger RNA (mRNA), carries the gene's protein (or other product) information encoded in DNA.
Transgenesis	Refers to the process of introducing an exogenous or modified gene into an organism, resulting in the incorporation of the (trans)gene into the host's genome.
Translation	The process by which a cell makes proteins using the genetic information carried in messenger RNA (mRNA).
Triploid salmon	Sterile fish with three complete chromosome sets.
Vitellogenic oocytes	Vitellogenesis is the process through which maturing oocytes in the ovary accumulate yolk. Vitellogenic oocytes refer to oocytes within this maturing process.

Western blot	A widely used analytical technique in molecular biology and immunogenetics to detect specific proteins in a sample.
Wild type	The phenotype of the typical form of a species (or a given trait or genetic marker) as it occurs in nature.

Background as provided by the Norwegian Environment Agency

The Norwegian Environment Agency refer to the collaboration agreements with the Norwegian Scientific Committee for Food and Environment (VKM), and hereby asks VKM to carry out an assessment of positive and negative effects on biological diversity when using sterile salmonid fish in aquaculture in Norway. Sterility is currently achieved through triploidisation, mainly by pressure treatment. The assessment shall also include other methods for sterilizing farmed fish.

The Norwegian Environment Agency's Need for the Assignment

Escaped farmed salmon and sea lice are the biggest threats to Norwegian salmon. The interbreeding of escaped farmed salmon with wild stocks leads to changes in the salmon's life history, making it less adapted to life in nature. This has resulted in reduced production in the rivers. Despite many measures, the proportion of escaped farmed salmon in many spawning stocks is above a sustainable level. A sterile farmed salmon could be an important tool to reduce this impact.

Background

Ever since salmon farming began in Norway, farmed fish have escaped and many have made their way into rivers and spawning with wild fish. As early as the 1980s, large numbers of escaped farmed salmon were detected in rivers in Western Norway. Similar situations arose in Central and Northern Norway during the 1990s and escaped farmed salmon have been recorded in all salmon rivers in Norway. For rainbow trout, an introduced species in Norway, it is also known that many individuals escape from farms and some of these migrate into rivers to spawn. It is a national goal to preserve and rebuild wild salmon stocks of a size and composition that ensures the diversity within the species and utilizes its production potential, cf. St. prp. no. 32 (2006-2007). On the protection of wild salmon and the completion of national salmon rivers and salmon fjords. The responsibility for achieving this goal is distributed across several sectors. In St. prp. no. 32, several measures were pointed out to limit the negative effects of escaped farmed salmon. One of these was the use of sterile salmon. Triploid salmon production has been tested on a commercial scale in Norway since 2013. The Norwegian Food Safety Authority has sent a separate order to VKM to assess the consequences of triploidisation for the health and welfare of farmed fish. Other methods for sterilizing fish than triploidisation, including the use of genome editing techniques, are currently being tested.

International Commitments

Norway ratified the Convention for the Conservation of Salmon in the North Atlantic Ocean (NASCO) in 1982. The organization aims to contribute to the conservation, restoration, enhancement, and rational management of salmon stocks in the North Atlantic through consultation and cooperation. In 2003, the member countries adopted the Williamsburg Resolution, which aims, among other things, to limit the impacts of aquaculture (such as sea lice and genetic influence from escaped farmed salmon) on wild salmon stocks.

Relevant Norwegian Legislation

The objective of the Act relating to Salmonids and Inland Freshwater Fish etc. is: "to ensure that natural stocks of anadromous salmonids, freshwater fish, and their habitats, as well as other freshwater organisms, are managed in such a way as to maintain natural diversity and productivity in accordance with the Nature Diversity Act and in such a way that the diversity and productivity of nature are preserved. Within these frameworks, the Act shall provide a basis for the improvement of stocks with a view to raising yields, for the benefit of holders of fishing rights and sports fishermen." Biological diversity is defined as "the diversity of ecosystem and species variability and intraspecies genetic variability, and the ecological relationships between ecosystem components" cf. the Nature Diversity Act § 3 letter c. The management objective for species in the Nature Diversity Act § 5 is that species and their genetic diversity are maintained in the long term and that species occur in viable populations in their natural ranges. In the Nature Diversity Act § 13, it is stated that the King may establish recommended quality norms for biological diversity. The quality norm for wild stocks of Atlantic salmon (Salmo salar) shall contribute to the conservation and restoration of wild stocks of Atlantic salmon to a size and composition that ensures diversity within the species and utilizes the salmon's production and harvesting potential. The quality norm consists of two sub-norms. The sub-norm for genetic integrity consists of the elements of species hybridisation, the degree of genetic introgression from escaped farmed salmon, and selection.

Aquaculture Act § 10 Environmental Standard:

"The Ministry may prescribe, by administrative decisions or regulations, detailed provisions to ensure environmentally responsible aquaculture, including requirements for preventive measures, requirements for the tagging of aquatic organisms, the use of aquatic organisms that cannot reproduce, and the use of alien organisms."

Protection of Biological Diversity

It has been documented that the interbreeding of escaped farmed salmon with wild stocks has led to changes in the life history strategies of wild salmon (Bolstad et al. 2017). The mandatory broodstock control for 2021 (NINA report 2133) shows that 14.6% of the broodstock was discarded after genetic analyses because they likely originated from escaped farmed salmon. Among the rivers, the proportion of discarded broodstock varied from 0% to 56%. Escaped farmed salmon is considered one of the biggest threats to wild salmon. In the Norwegian Scientific Advisory Committee for Atlantic Salmon's report "Status of Norwegian Salmon Stocks 2021," it states: "Escaped farmed salmon, sea lice, and infections related to salmon farming are the largest threats to wild salmon. Insufficient measures are being taken to stabilize or reduce these threats." Genetic analyses of wild salmon in 228 salmon rivers show that two-thirds of these are affected by genetic introgression from escaped farmed salmon. The Institute of Marine Research writes in its "Risk Report Norwegian Fish Farming 2022 – Risk Assessment" that there is a high risk of further genetic changes in wild salmon populations due to escaped farmed salmon in 7 out of 13 production areas and a moderate risk of further genetic changes in wild salmon due to escaped farmed salmon in 3 of the production areas. Salmon was listed for the first time on the Norwegian Red List for Species in 2021 as near threatened. Sea lice and escaped farmed salmon are considered non-stabilized stock threats. The Norwegian Scientific Advisory Committee for Salmon (VRL) has also assessed other threats

to wild salmon in its reports from 2021 and 2022, including the escape of rainbow trout from fish farms.

Spontaneous Triploidisation

Studies show that spontaneous triploidisation (2%) in farmed salmon is normal and has been observed in all farming regions in Norway (Glover et al. 2015). The proportion was significantly higher in some cages (10-28%). Spontaneous triploidisation was observed in escaped farmed salmon that were recaptured both in the sea and in freshwater. Studies (Jørgensen et al. 2018) show that naturally occurring triploidisation in wild salmon is many times lower than in farmed salmon. We do not know if spontaneous triploidisation presents the same challenges in salmon aquaculture as triploidisation through pressure treatment, or if knowledge of the mechanisms behind spontaneous triploidisation can be used to develop methods for producing triploid salmon in a different way that poses fewer health and welfare challenges.

Terms of reference as provided by the Norwegian Environment Agency

The Norwegian Environment Agency request VKM to provide a summary of knowledge and an assessment of the positive and negative consequences related to:

1. Biological diversity with the use of salmonids sterilized by triploidisation.
    A. Describe the effectiveness of the triploidisation process.
    B. Describe the extent to which fertile and triploid farmed fish are found in the environment and assess the effects this has on wild salmon stocks.
    C. Assess whether there are other positive or negative effects on biological diversity related to the use of triploid salmonids, compared to traditional farming.
2. Biological diversity with the use of alternative methods for the production of sterile farmed fish.
    A. Describe the status if it has previously been investigated whether it is possible to further develop methods for triploid salmon production through breeding or other measures, so that triploid farmed salmon achieve the same or better welfare as diploid salmon.
    B. Provide an overview of other methods intended for the sterilisation of salmonids for aquaculture, as well as the current state of knowledge for these methods.
    C. Assess the potential for genetic impact on wild fish stocks from the use of salmonids farmed using the methods identified under point 2B.

Assess whether there are other positive or negative effects on biological diversity related to the farming of salmonids using the methods identified under point 2B.

1 Introduction

Aquaculture production has seen a tremendous growth in the last decades, and the production now equals the catch in traditional fisheries (FAO 2024). Atlantic salmon (*Salmo salar*) is among the fish species that represents the largest aquaculture production by mass in marine aquaculture (FAO 2024). Norway produces more than half of all farmed Atlantic salmon produced globally. In 2023, more than 457 million farmed Atlantic salmon were kept in net pens along the Norwegian coast[1]. This amount was 1100 times the number of wild Atlantic salmon that returned from the ocean towards the Norwegian coast to spawn in rivers the same year ('pre-fishery abundance', VRL 2024).

The Norwegian wild Atlantic salmon populations are now at a historically low level. The number of salmon returning from the ocean to the Norwegian coast was the second lowest ever in 2023, estimated to only 400,000 wild salmon returning (VRL 2024). The greatest declines in the wild salmon populations have occurred in western and central Norway. The two biggest threats to Norwegian wild Atlantic salmon are salmon farming and climate change (VRL 2024). The biggest threats from salmon farming are salmon lice released into the environment, escaped farmed salmon and transmission of other infections (virus, bacteria etc.).

Farmed Atlantic salmon are typically produced in open net pens, in close interaction with the environment. The net pens use mesh sizes that contain the fish while sea water flows through together with disease agents, feed remains and excrements. Net pens may be 50 meters or more in diameter, 20-50 meters deep and hold up to 200,000 farmed salmon. Various unwanted events may result in escape of farmed fish from the net pen into the natural environment. Bad weather, or accidents with boats or boat propellers may rip the net and generate wholes[2]. Escape events may also occur due to accidents during handling and transport of fish and during treatment against salmon lice (Jensen et al. 2010).

The number of escaped farmed salmon reported by the fish farming companies to the Norwegian Directorate of Fisheries has been reduced the last 15 years compared to previous years (VRL 2024). The annual number of reported escaped farmed salmon was on average 105 000 salmon since 2014[2]. The reported number of escaped farmed salmon are underestimates of the actual number, because the numbers of farmed salmon reported to have escaped during each event are often uncertain estimates by the fish farmer, and not all escape events are reported[3].

Escaped farmed salmon may enter rivers and participate in the spawning together with wild salmon. Wild salmon populations differ genetically between watersheds, and even between sections of watersheds likely due to adaptations to the local environmental conditions (Garcia de Leaniz et al. 2007). Farmed salmon were established from a limited number of wild source populations that later have been exposed to more than 15 generations of selective breeding and domestication since the early 1970s (Glover et al. 2017). Consequently, farmed salmon differ genetically from all wild salmon populations, and farmed and wild salmon individuals are

[1] https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/Matfiskproduksjon

[2] https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Roemmingsstatistikk

[3] https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Roemmingsstatistikk/om-dataene-rommingsstatistikk

typically different in many traits including growth, morphology, life history, behaviour, and physiology (Glover et al. 2017). Interbreeding between wild and escaped farmed salmon causes changes in important characteristics in wild salmon populations (Glover et al. 2017, Bolstad et al. 2021).

Genetic changes due to farmed salmon introgression have been documented or indicated in 168 Norwegian wild salmon populations (67% of 250 analysed wild salmon populations, with the analysed populations representing 95% of the total wild salmon resource in Norway (Diserud et al. 2023). One third of the wild salmon populations have more than 10% introgression of genetic material from farmed salmon compared to their original wild salmon populations (Diserud et al. 2023). About one third of the 250 populations analysed show no signs of genetic introgression from escaped farmed salmon.

Experiments in controlled rivers show that interbreeding between farmed and wild salmon is associated with loss of wild salmon production and viability (McGinnity et al. 1997, 2003, Fleming et al. 2000, Skaala et al. 2012, 2019). Genetic introgression erodes the natural genetic variation, which reduces fitness of native wild salmon populations (Tufto 2017). Such loss of genetic variation is associated with several ecological and life-history changes in wild populations including growth rate in fresh and sea water, age at smoltification and maturation, size at maturation, straying rate, and phenology of migration (Bolstad et al. 2017, 2021, Glover et al. 2017, 2020, Skaala et al. 2019).

A risk assessment of Norwegian aquaculture production suggests moderate-to-high risk of further introgression of farmed escapes (Glover et al. 2020). This may increase the widespread introgression that is already documented. Glover et al. (2020) therefore concluded that as long as aquaculture production continues at its present level and form, there is continued risk of further introgression of farmed salmon in many native populations.

Farmed salmonids in open pens are exposed to an infection pressure from diseased fish in surrounding farming facilities and from wild fish. Multiple infections occurring simultaneously within a farming facility are therefore common, with transmission of infections within and between net pens (Wiik-Nielsen et al. 2016). Wild salmon are potentially even more susceptible to infections transmitted from farmed salmon because wild salmon are also hosts for a variety of infections and parasites that are absent in farmed salmon. Studies have shown that compared to wild salmon, escaped farmed fish have higher odds of carrying infections commonly observed in the aquaculture industry, often with multiple infections simultaneously (Garseth et al. 2013, Madhun et al. 2015, 2024). When infected and diseased escapees enter rivers, they act as a source of infection for wild salmon populations both within and outside aquaculture regions (Håstein and Lindstad, 1991).

How can the described problems with current aquaculture practice and escaped farmed salmon be alleviated? One measure to avoid transmission of infections is to reduce the likelihood of escape from aquaculture facilities. To reduce genetic introgression in wild salmon populations, a possible measure is to reduce the likelihood of successful reproduction if the farmed fish escape. VKM has been requested by the Norwegian Environment Agency to summarize and assess knowledge related to using sterile fish in aquaculture as a means of reducing their capability of reproduction with wild fish. The aims of this report are specifically to (1) assess negative and positive consequences for biodiversity by using salmonids sterilised by triploidy, and (2) assess negative and positive consequences for biodiversity by using alternative methods for sterilisation.

VKM has recently issued three other reports that are relevant for this assessment. The first report is a review of CRISPR and other genome-editing technologies and implications for risk assessment when these techniques are used (VKM 2021:18). The second report is a risk assessment of a release experiment with sterile CRISPR-edited salmon in net pens (VKM 2023:20). The third report is a review related to health and welfare of triploid farmed salmon in commercial aquaculture (VKM 2023:22).

1.1 Sterile salmon in aquaculture to reduce environmental risks

The negative consequences that aquaculture in open net pens impose on wild populations have been internationally recognized for more than three decades. Escaped farmed salmon has been noted as a challenge for the genetic integrity of wild salmon populations since the 1980s (Maitland 1986, Gausen and Moen 1991). In the North Atlantic Salmon Conservation Organization (NASCO), which assembles all the nations holding wild salmon populations, a special resolution (Williamsburg) describes how aquaculture needs to be performed to minimize environmental impacts. The International Council for Exploration of the Seas (ICES) has together with NASCO organized several meetings to discuss aquaculture-wild interactions (1990, 1997, 2005). ICES assembled two of their advisory groups to report on genetic introgression and salmon lice in 2016 (ICES 2016). Relevant reports produced in Norway are Official Norwegian Reports (NOU 1999:9)[4], White Papers to Parliament on wild salmon (St.mld. 2006-2007), and Strategy for environmentally sustainable aquaculture (FKD 2009), all of which discuss escaped farmed salmon and interbreeding with wild salmon as a central theme. A more recent regulation emanating from the 2009 Nature Diversity Act (*Kvalitetsnormen for ville bestander av atlantisk laks* (*Salmo salar*)[5]) lists genetic integrity as one criterion for classification of the status of wild salmon populations.

Sterile fish may be favoured over fertile individuals in aquaculture for several reasons not related to biodiversity. Methods for inducing sterility in fish has for instance been used to safeguard Intellectual Property Rights (IPR) by breeding companies. Moreover, when the fish matures sexually, several physiological processes take place that make maturing fish less valuable as food (or for the food market). These alterations include changes in external morphology as well as in flesh colour and fat content, which make sterile fish better suited for marketing.

Because of gender differences in the timing of sexual maturation (males often earlier than females), the possibility to produce all-female groups has also been considered favourable for fish producers. The possibility to produce all-female groups is relevant to this risk assessment only if the farmed fish species is reared outside its natural range and escaped individuals cannot meet wild males of the same species. Moreover, selective breeding is used to reduce early sexual maturation so that market size is reached before the onset of maturation.

Triploidy to secure sterile fish for aquaculture production or stocking (mainly sport fishing) has been applied since the 1980s. The motivation for imposing sterility has been to avoid

[4]https://www.regjeringen.no/contentassets/5ee11a55cf5c4f7bb79016dc25f6e8bd/no/pdfa/nou199919990009000dddpdfa.pdf

[5]https://lovdata.no/dokument/SF/forskrift/2013-09-20-1109

reproduction in the wild (of both intentionally and accidentally released fish) and to secure that a high proportion of reared fish reaches market size (or body size at catch) that do not imply sexually mature individuals. Moreover, triploidy has been used as one of several barriers to hinder environmental problems caused by the production of genetically modified Atlantic salmon (termed “AquAdvantage salmon”).

The knowledge about triploid sterile salmonids in the wild is based on experimental releases (Cotter et al. 2000) and on the molecular identification of triploid individuals in fish farms and among escaped farmed salmon (Glover et al. 2016). Apart from salmonids, triploidy has been induced in several other fish species used in stocking and aquaculture. Thus, the knowledge base for triploidy as a sterilisation method is extensive, although – as pointed out in VKM Report 2023:22 – the number of experiments on early life stages and for alleviating some of the problems experienced when using triploid farmed salmon on a large scale, is still limited.

The other methods for sterilising fish that VKM described in this report are still at an early stage of development or were abandoned a long time ago for market reasons. The comparison between methods with respect to negative and positive consequences for biodiversity in this VKM report is therefore limited and uncertain.

2 Methodology and data

2.1 Literature search and selection

2.1.1 Search strategy

Three separate literature searches (two main searches + one supplementary search) were performed to address the questions in the terms of reference. The project group discussed and agreed on the search terms and databases to be used together with a senior librarian at the Norwegian Institute of Public Health (NIPH), who performed the searches. The searches were divided into the following categories:

1) *Methods for production of sterile fish*

2*) Environmental effects of farmed sterile fish*

3) *Sterile salmonids* (supplementary)

All searches were performed in CAB Abstract, ISI Web of Science and Scopus.

The literature searches were performed on January 10, 2024.

The search strategies are included in Appendix I-III.

2.1.2 Primary screening of articles

The combined results from all three literature searches, 1328 potentially relevant articles, were divided into three equally distributed libraries of ~440 articles uploaded into the collaborative review tool Rayyan, https://www.rayyan.ai/. In the primary screening, articles in each of the three libraries were reviewed and labelled in accordance with relevance to the terms of reference by two project members, who did this independent of each other, and without knowing each other's screening results. Afterwards, contradicting results (inclusion/exclusion) were solved by further scrutiny of the articles in question in collaboration between the two project members. In situations where it was unclear if the publication was of relevance, it was retained rather than discarded. Each project member participated in reviewing one of the three libraries, so a total of six project members (excluding two VKM staff) were involved in the primary screening.

The primary screening resulted in 485 articles and book chapters, of which 84 were included in the opinion.

To strengthen the scientific data of the opinion, and because the systematic literature search with the chosen databases returned fewer relevant hits than anticipated, additional manual searches for papers and relevant grey literature were also performed. Manual searches included snowballing, i.e. screening of articles that were referred to in papers found in the main literature, and performing searches e.g., via Google, Google Scholar, and various databases and journals. The manual searches resulted in 38 relevant papers and documents included in the opinion (Figure 1.)

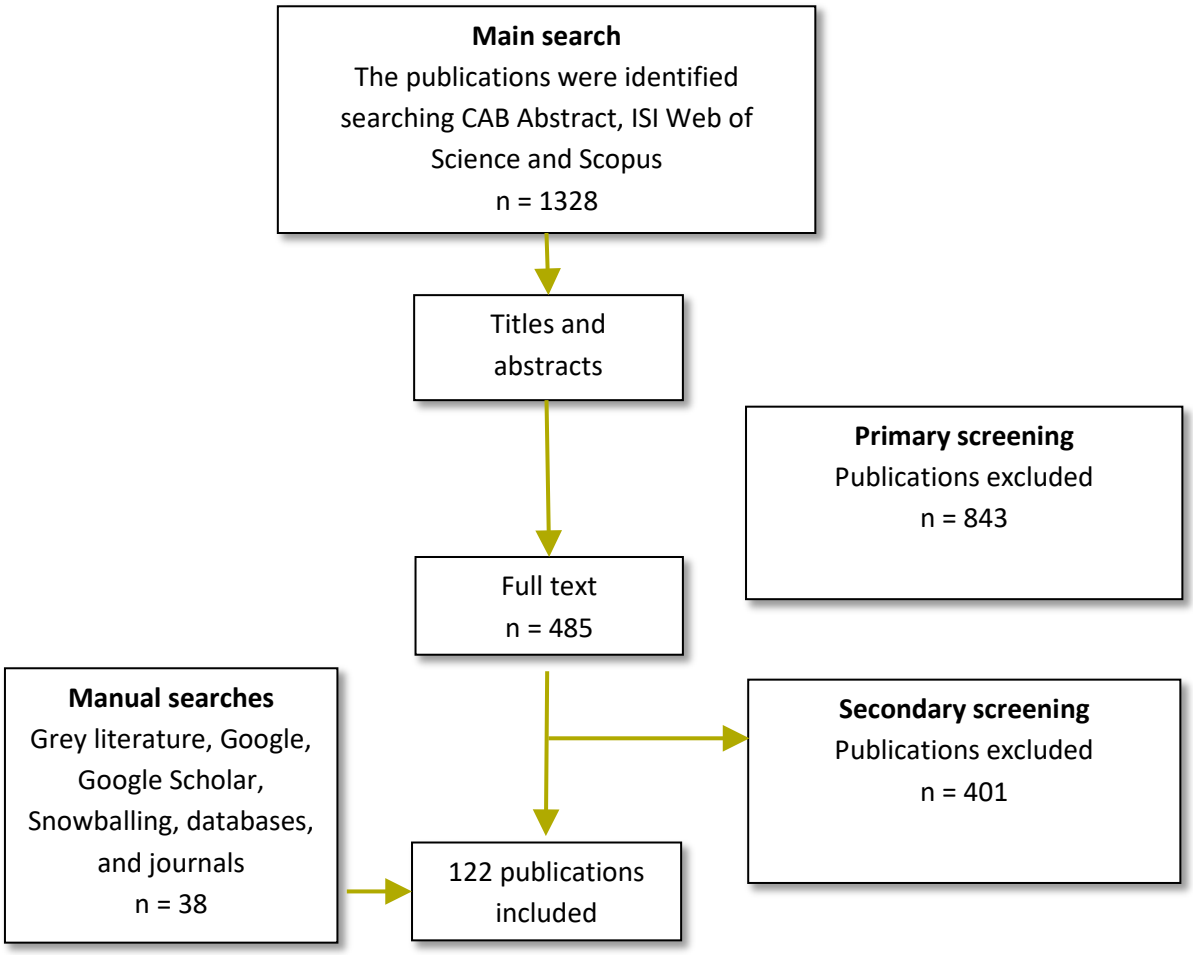

Figure 1: Flowchart for the literature search and the subsequent selection of publications included in the report.

3 Methods for production of sterile fish in aquaculture

Various strategies can be used to achieve sterile fish in aquaculture. An overview of strategies that have been used in aquaculture as well as methods that have been used in other fish species, including model organisms for research, are summarised in Tables 1, 2 and 3, and described in more detail below. Available strategies include triploidisation, hormone-, drug- and vaccine treatment, and techniques that temporarily or permanently affect expression of specific reproductive genes through gene silencing, knockdown, or knockout. Triploidisation leads to imbalanced segregation of chromosomes in meiosis (halving of three is not possible) resulting in sterility or offspring with aberrant chromosomes and no survival after the "eyed egg" stage. The other methods typically target biological processes important for reproduction, such as the hypothalamic–pituitary–gonadal (HPG) axis, primordial germ cell (PGC) development or gametogenesis (Xu et al. 2022).

Tables 1, 2 and 3 (pp. 58-60) summarise the different methods with respect to their mechanisms for generating sterile individuals, the reproducibility and stage of development, the proportion of sterile individuals generated, the method(s) used to verify sterility, its use in salmonids and other fish species, comparative information about performance in aquaculture, and (potential) effects on biodiversity including wild conspecifics.

Table 1 describes methods that generate permanent alterations of the genome resulting in sterile individuals. Among these methods are CRISPR-generated sterility achieved by knocking out genes that are crucial to formation of germ cells or to gonadal development, transgenesis, and mutagenesis by irradiation or other methods. These methods rely on generation of a diploid individual where both alleles confer sterility and need to be 100% accurate in the selection of offspring to ensure that sterility alleles in a heterozygous state cannot go unnoticed.

Table 2 describes methods that strongly reduce the expression of the same vital genes as mentioned above (formation of germ cells or gonadal development) by knocking down transcription or translation of these genes. With these methods, no permanent genetic alterations occur, and inhibition of transcription and/or translation is temporal and affects production, stability or readability of a selected mRNA. Silencing of genes by vaccination, as well as sterility achieved by hormonal treatment, are other methods for non-genetic sterility. Some of these methods are used in combination, e.g., for production of all-female triploid lines.

Table 3 describes other methods for achieving life-long sterility including triploidisation. Triploidisation is the only method used on a large scale today. An example of other and less applied methods included here is interspecific hybridisation.

3.1 Methods that result in permanent changes to the genome sequence

3.1.1 Gene knockout

Genes that are crucial for reproduction are typically involved in the hypothalamic–pituitary–gonadal (HPG) axis, primordial germ cell (PGC) development or gametogenesis (Xu et al. 2022).

Individuals that are heterozygous for a mutation in a gene important for reproduction will often be fertile, while individuals that are homozygous will be infertile. Hence, controlled breeding of individuals that are heterozygote carriers of permanent deletions/mutations of genes affecting fertility can be used to generate sterile offspring. The robustness of this strategy will be highly dependent on the gene(s) targeted and less dependent on the methodology used to generate the gene knockout (KO) by deletion, insertion or substitution. Different technologies that may be used to permanently alter genes will be briefly presented below.

3.1.1.1 Homologous recombination in embryonic stem cells

The first methodology developed for targeting of genes in mammals in the late 1980s involved many steps: genetic manipulation in embryonic stem (ES) cells, fusion of such genetically altered ES cells with an embryo at the blastocyst stage, and implantation of the hybrid blastocyst into a surrogate mother (described in detail in VKM 2021:18). The resulting chimeric offspring from the growing blastocyst will consist of a mixture of normal cells (from the host blastocyst) and cells harbouring the genetic mutation (from the injected ES cells). If the injected ES cells are parts of the gonads and used to generate germ cells, the intended genetic mutation can be inherited to the next generation. Several of these methodological steps are, however, less transferable to species with external fertilisation, including fish. While the strategy in theory can be adjusted to achieve genetic manipulation in fish, it has hardly been used.

3.1.1.2 Double strand DNA endonucleases

In the beginning of 2000, a new generation of genetic engineering tools and technologies was developed based on enzymes generating site-specific double stranded DNA (dsDNA) breaks. These tools trick the cell to introduce mutations when it repairs the dsDNA break using its own DNA repair system(s). Many engineered endonucleases have been developed, with variation in their efficiency, design complexity and specificity. A detailed description of these various tools can be found in a recent VKM report (VKM 2021: 18).

The most widely used endonuclease tools are the zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeats (CRISPR). ZFN and TALEN tools are large protein-complexes containing two essential segments: a large modular-based repetitive region that binds to a specific DNA sequence (determines the DNA-binding specificity), and a Fok-I restriction endonuclease that cleaves DNA. While a ZFNs module binds to a DNA triplet, a TALEN module binds to a single nucleotide. Otherwise, ZFN and TALEN work essentially the same way. Both ZFN and TALEN must be used in pairs to generate a dsDNA break. The repetitive nature of the ZFN and TALEN repeated regions make them difficult to design and clone/synthesise. In addition, some nucleotide combinations cannot be specifically recognised by a unique protein motif, resulting in a small percentage of genomic regions that cannot be efficiently targeted with these tools.

CRISPR is somewhat different from the ZFN and TALEN tools; consisting of a short RNA sequence called guide RNA (gRNA) and an enzyme generating a dsDNA break (often called Cas). The gRNA contains two domains; one domain binds specifically to a complementary DNA region in the host genome, while the second domain binds to the Cas enzyme that insert a dsDNA break. The CRISPR technology is superior to the others with its optimal combination of simple and inexpensive design, high efficiency, and high specificity. Out of the three, the CRISPR technology is likely to be the dominating gene editing tool used in the future.

The ZFN, TALEN and CRISPR tools differ in their success rate for generating a dsDNA break and inserting the desired genetic modification (VKM 2021: 18), but the outcome in terms of the genetic modification will be the same. Except for some variation in the risk for off-target effects (introduction of unwanted genetic modifications), the outcome will not be dependent on which of the techniques being used to insert the genetic modification. A considerably more critical factor in gene targeting is selection of the gene(s) being targeted. Genes involved in the hypothalamic–pituitary–gonadal (HPG) axis, primordial germ cell (PGC) development or gametogenesis have different importance in various species, affecting their ability to efficiently cause sterility when disrupted or mutated.

Loss of function studies for genes involved in the HPG axis have focused on model fish species. A long list of genes involved in the HPG axis has been targeted, with variable effects on sterility (reviewed in Xu et al. 2022). TALEN has been used to disrupt the *gnrh1*, *gnrh2*, *gnrh3*, *fshb*, *fshr*, *lhb*, *kiss1*, *kiss2*, and *lhcgr* genes in medaka (*Oryzias latipes*) or zebrafish (*Danio rerio*), while ZFN has been used to target *lhb* in channel catfish (*Ictalurus punctatus*). Generation of sterile males is inefficient using single disruption of genes involved in the HPG axis, however more efficient by a combined deletion of either *fshr/lhcgr* or *fshb/lhb* (which also generates only male offspring). Results are more promising in females, where single disruption of some of the genes has resulted in anovulation or arrested folliculogenesis. However, the studies in multiple model fishes have revealed inconsistent and species-specific effects on sterility when genes in the HPG axis have been disrupted. Based on current evidence, disruption of genes in the HPG axis seems to be unpredictable (since it is species and gender dependent) and is therefore unreliable as a universal strategy to achieve sterility in fish.

PGCs are precursors for gametes that are separated from the somatic cells during early development. These cells will later give rise to gonads and production of sperm in males and oocytes/eggs in females. Successful disruption of PGC formation may potentially affect sterility in both sexes. Genes such as *nanos*, *dnd*, *sdf1*, and *cxcr4* have been identified with functional roles in PGC formation (reviewed in Xu et al. 2022). Among these genes, the *dnd* gene is the most studied and seems to have a conserved and indispensable role in PGC formation in multiple species. Based on current available data, the *dnd* gene is the most optimal gene to target to achieve sterility in aquaculture. CRISPR has been used to disrupt the *dnd* gene in Atlantic salmon, demonstrating that Atlantic salmon homozygote for the *dnd* gene are germ cell free (Kleppe et al. 2017). Disruption of *nanos* genes seems more gender dependent. Use of CRISPR to achieve genetic ablation of *nanos2* in Nile tilapia (*Oreochromis niloticus*) gives partial sterility in males, while ablation of *nanos3* gives partial sterility in females (Li et al.2014). Disruption of *foxh1* in Nile tilapia results in arrested oogenesis and sterility in females (Tao et al.2020), while disruption of eEF1A1b leads to abnormal spermatogenesis and sterility in males (Chen et al.2017). Based on these findings, effects obtained by genetic disruption of the *dnd*

gene seem most robust and will be used further when discussing the use of a knockout strategy to achieve sterile individuals in aquaculture.

Provided that the gene being targeted is essential for fertility, the robustness of a knockout approach is high (Table 1). The ZFN, TALEN or CRISPR tools that can be used to insert the desired genetic mutation are all robust, although the simplicity of the CRISPR system will strongly favour the use of this genetic engineering tool in the future. Specificity is high and side effects (unwanted genetic mutations) are low with all methods, provided that the binding site(s) in the genome has been carefully selected and represent unique sequence(s) in the genome. But, as with all gene ablations, the phenotypic effect obtained will rely on the functional importance of the gene being targeted. Permanent knockout of the *dnd* gene resulted in sterility in Atlantic salmon (Wargelius et al. 2016). Sterility has also been achieved with temporal knockdown of the *dnd* gene in species such as loach (*Misgurnus anguillicaudatus*) (Fujimoto et al. 2010), goldfish (*Carassius auratus*) (Goto et al. 2012), sterlet (*Acipenser ruthenus*) (Linhartová et al. 2016) and zebrafish (Wong and Zohar 2015). These results predict that sterility will be achieved also with a knockout of the *dnd* gene in the same species. Disruption of the *dnd* gene seems to result in a predictable and highly efficient male and female sterility in multiple species, suggesting it may be a universal target to achieve sterility in aquaculture.

Breeding of individuals with a genetic mutation affecting fertility poses some challenges. Sterile offspring can be achieved with natural breeding, but not without producing a high number of individuals with an undesirable genotype (and phenotype). Since both males and females are sterile when homozygote for the mutation, the only natural breeding strategy that can be used is crosses of heterozygote carriers of the mutation, resulting in 25% individuals that are homozygously normal, 50% that are heterozygote for the mutation, and 25% that are homozygous for the mutation. The need for exclusion of 75% of all produced offspring will be economically costly.

Another critical component for the use of genetically modified fish in commercial fish farming will be genotypic identification of offspring produced by heterozygous mating. Many methods can be used to detect the presence of a disrupted *dnd* gene, whereof the most common strategy will be qPCR analysis. This method is robust when used in a research laboratory. However, the method will not be reliable (error free) in a production setting, where millions of individuals must be genotyped. For all practical aspects, no method currently exists that can accurately determine differences in genetic alleles in millions of individuals with extreme reliability and at a low cost. In addition, there will be a need to segregate the millions of genotyped individuals and only keep those individuals with homozygous mutations in the *dnd* gene. When selecting animals, there will be a risk of unintendedly mixing up a low percentage of individuals resulting in inclusion of animals with incorrect genotype. Due to these practical aspects, natural mating of heterozygous carriers with deletion in the *dnd* gene to achieve sterile offspring for aquaculture will be costly and environmentally unsafe.

The obstacles described with the use of a knockout strategy and heterozygote breeding in commercial aquaculture can be overcome if combined with other strategies. The sterility achieved with permanently ablated *dnd* gene alleles can e.g. be overcome with co-injection of stabilised mRNA encoding the wild type *dnd* gene at (or soon after fertilisation) (Güralp et al. 2020). This rescue strategy produces offspring with spermatogonia and primary oocytes up to

at least three years of age, although these fishes are genetically sterile due to the lack of a functional *dnd* gene. Since these are genetically sterile, any offspring produced from these rescued salmon will inherit a non-functional *dnd* gene and be genetically sterile. It is, however, still uncertain if such rescued fish with a non-functional *dnd* gene develop fully functional gonads capable of repeatedly producing fertilisable gametes. In zebrafish, *dnd* is also a maternal gene expressed in the gonads of adult fish, and it may have functions in later germ-cell development in zebrafish (Weidinger et al. 2003). Prior to widespread use of *dnd* mRNA rescue to circumvent sterility in *dnd*-mutated parents, a complete rescue of the gonads and normal sexual maturation throughout the whole lifespan needs to be confirmed for every species if more than one reproduction is favoured.

Comparable performance analyses to determine virility and fitness of sterile *dnd* mutated salmon in the natural environment have not been performed. During contained experimental studies, salmon homozygote for mutated *dnd* did not undergo puberty (Kleppe et al. 2017) and performed at par with control fish in terms of deformities, smoltification, fillet quality, growth and occurrence of deformities (Kleppe et al. 2022). The studies were not designed to detect differences in susceptibility to infectious agents (VKM Report 2023:20). Sterile *dnd* mutated salmon that do not undergo puberty, also fail to initiate gonadal sex hormone production. This may be considered beneficial in cultured fish from a health, filet quality and animal welfare perspective. However, any permanent genetic modification poses a risk to the wild salmon populations if individuals carrying such genetically engineered mutations are fertile and escape. If escapees carrying *dnd* gene mutations are mixed with wild populations, the allelic mutation is likely to be permanently introduced in the wild population and nearly impossible to eliminate (VKM 2023: 20). Even if present at low levels, it may affect breeding success of such wild salmon populations.

In a risk assessment of potentially sterile salmon (VKM Report 2023:20), VKM found that there was a higher risk associated with using first-generation offspring of microinjected CRISPR-edited parents, whose fertility was rescued by microinjecting mRNA for *dnd*. A major reason for this assessment was uncertainties regarding whether all *dnd* allelic mutations had been characterized and if all of them were (fully) sterile. The VKM Report stated that “a more conservative alternative approach to reduce the number of unique mutations would be to generate individuals based on strategies frequently used to breed research animals. I.e., starting with identification of male and female fish that are heterozygote for the same mutation in the *dnd* gene (e.g., the exact same 8-der) to collect oocytes and sperm. Oocytes are then fertilized and co-injected with the rescue RNA. Such a cross can be expected to produce around 25% fish that are homozygous for the ablated *dnd* gene but fertile due to the rescue. Detailed molecular characterization can then be performed on individuals to identify the 25% individuals with this unique modification. Risks caused by mosaicism in the F0 founders, involving screening for multiple alternative alleles, will then be removed. A reliable method can also be developed to detect the single unique genetic modification” (VKM 2023:20, p. 78).

Having reached a stage where only one well characterized *dnd*-knockout (*dnd*-KO) allele is used in the aquaculture population, homozygous sterile individuals can be generated from rescued parents with temporal expression of *dnd* mRNA. However, production of these must be numerous (in the tens of thousands) if aiming at production of a large number of *dnd-KO*

sterile individuals. In reality, several salmon generations will be needed to reach a high number of *dnd*-KO homozygous individuals, and these generations need to be produced in land-based closed facilities to avoid the risk of partially fertile individuals (e.g. heterozygous for a *dnd* mutation) escaping and transferring genetic sterility to wild salmon populations. Moreover, another problem with the permanent genetic alteration approach is the high risk of inbreeding and reduced genetic variation of the several generations of broodstock required for production of the breeders. High risk for low genetic variability needs to be addressed prior to large scale use of *dnd* mRNA rescuing in genetically infertile breeders (microinjection of *dnd* mRNA to generate fertile, but genetically sterile *dnd* parents).

Other strategies that rely on temporal, and not permanent, deletion of genes affecting fertilisation, will not pose a genetic risk to the wild salmon populations and will be less affected by inbreeding. Use of engineered CRIPSR tools that do not cause permanent genetic modifications but are designed to block translation of the *dnd* gene in a critical time period, could be an alternative for a more environmentally safe strategy. It has, however, not been experimentally verified if CRISPR-mediated repression of *dnd* gene expression efficiently prevents gonad formation and result in sterility.

3.1.2 Radiation (Mutagenesis)

Radiation was one the first approaches used for sterilisation, inducing apoptosis and impaired gametogenesis (Kobayashi and Mogami 1958). In these experiments, fry of rainbow trout (*Oncorhynchus mykiss*) were exposed to X-ray of 1Gy and 5Gy. Irradiation does not seem to totally remove gonadal tissue, and regeneration of gonads may occur. Irradiation seems not to be suitable for implementation in fish farming, since it can be dangerous for both fish and operators and requires expensive equipment for commercial use.

No difference in body weight was reported between irradiated fish (1Gy and 5Gy) and control, only ovary weight differences (Kobayashi and Mogami 1958). Irradiation is however not a relevant method since it can cause negative health and welfare effects in fish. Mutagenesis will generate genetic allelic variants that differ from those found in wild salmon populations. Radiated fish that still are capable of reproducing will be a genetic treat to wild salmon populations.

3.1.3 Transgenesis

The principles behind transgenic sterilisation are similar to those used for gene knockout or gene knockdown. With transgenesis, a gene construct is designed to act on target genes and impose sterility by disrupting gametogenesis or embryogenesis. Typically, the processes that are targeted to induce sterility belong to the HPG (hypothalamic–pituitary–gonadal) axis, PGC (primordial germ cell) development and gametogenesis (Xu et al. 2022). Examples of implementation of this strategy include both model fish (zebrafish), aquaculture fish (rainbow trout, common carp (*Cyprinus carpio*), channel catfish), using various tools such as antisense RNA to target the genes *gnrh* (HPG axis), *dnd* (PGC development) and *nanos* (gametogenesis), as well as short hairpin RNA (shRNA) to target *dnd* and *nanos* genes (Xu et al. 2022).

Transgenesis has been used to produce the only genetically modified salmonid (named "AquAdvantage") that is commercially available today [6]. In this case, the inserted transgene improves growth rate of the Atlantic salmon and is not inserted to induce sterility. The transgene is a recombinant DNA construct consisting of a growth hormone gene from chinook salmon (*Oncorhynchus tshawytscha*) combined with a promoter from ocean pout (*Zoarces americanus*). For the AquAdvantage strain, sterility is secured by applying triploidy and commercial production is so far limited to a physically contained, land-based facility in the US.

Whereas transgenic growth enhancement easily can be inherited in a population of farmed salmon, inheritance of transgenic sterility will be troublesome. Sterility needs to be circumvented for each new generation. One way to restore fertility can be to combine transgenic methodology that induces sterility with another pharmacological method that silence expression of the transgene and thus restore fertility (Su et al. 2015). Another possibility can be to develop two different transgenic lines that are fertile when pure-bred, but sterile when cross-bred (Zhang et al. 2015).

At present, it is difficult to foresee the use of a fertile-transgenic-line-1 X fertile-transgenic-line-2 breeding scheme to produce sterile offspring in large-scale aquaculture. Xu et al. (2022) contend that the regulatory processes for producing transgenic lines for aquaculture production suggest that gene editing techniques for achieving sterility are more likely than transgenesis to be applied in the future. Transgenesis also generates individuals with permanent genetic alterations that can be inherited if fertile individuals escape and mate with wild salmon.

3.2 Methods that do not include any permanent changes to the genome sequence

3.2.1 Knockdown using siRNA

As an alternative to permanent and inheritable gene manipulation, transient gene downregulation can be achieved with the use of antisense oligonucleotides. The use of siRNA to silence gene expression in fish has been studied using small dsRNA and siRNA (Golpour et al. 2016). RNAi (RNA interference) is an RNA-mediated gene silencing pathway found widely in eukaryotes, including mammals. RNAi originally evolved as a defense system against foreign RNA molecules invading eukaryotic organisms. RNAi specificity is based on sequence-specific recognition of a single stranded RNA target sequence through nucleotide base pairing. The short effector interfering RNA can either be delivered as exogenous double-stranded RNA (siRNA, ~22 nucleotides long) or endogenously expressed single stranded RNA (miRNA). Both can initially be expressed in the form of longer hairpin RNA that will be processed and modified by the ribonuclease DICER (or Dicer-like enzyme) in the host cell into functionally active siRNAs. Binding of the siRNA to target RNA forms segments with double-stranded RNA (dsRNA), which may result in inhibition of transcription or translation, or cleavage of the duplex RNA resulting in degradation of the RNA-transcript. When used in cell cultures or living

[6] https://www.fda.gov/animal-veterinary/aquadvantage-salmon/aquadvantage-salmon-fact-sheet

organisms, the synthetic dsRNA introduced will selectively and robustly suppress expression of specific genes of interest (Saurabh et al. 2014). The siRNA induced gene knockdown effects are highly sequence specific, but siRNAs may generate several types of off-target effects, particularly, the so-called microRNA-like mechanisms of gene regulation. Optimized siRNA design and/or nucleotide modifications (e.g. replace the 2'-OH group in ribose with an O-methoxyethyl group), or DNA substitutions in siRNA duplexes can be used to reduce the microRNA-like off-target effects (Jackson et al. 2006; Chiu et al. 2004).

Non-specific effects in fish embryos treated with siRNAs or dsRNAs, due to toxicity or type-1 interferon stimulation, have been reported. Similar non-specific effects were observed when injecting *dnd*-siRNA in zebrafish. RNAi based systems are still in the early experimental phase and research is needed to solve issues related to toxicity and type-1 interferon stimulation (Golpour et al. 2016).

In addition to morphological and histological analysis, siRNA effects can partly be determined by qPCR or RNA sequencing (RNA-seq) to evaluate alterations in gene expression levels. This strategy is only reliable when binding of the siRNA results in efficient cleavage of the duplex RNA. More reliable methods are western blot or proteomics to measure protein levels of the gene being targeted, luciferase assay and immunohistochemistry using antibodies. More research is needed to determine if antisense, dsRNA or siRNA interference techniques offer a reliable approach to achieve farmed fish sterility on a commercial scale.

3.2.2 Knockdown with antisense oligonucleotides

3.2.2.1 Morpholino oligonucleotides (MO)

Morpholinos (MO) are chemically modified synthetic antisense oligonucleotides (~ 25 nucleotides long) that bind to targeted RNA sequences by base-pairing. A morpholino oligomer consists of nucleic acids bound to a backbone of morpholine rings linked by phosphorodiamidate groups. Because of their unnatural backbone structure, MOs are highly resistant to degradation by nucleases (Summerton et al. 1999, Hudziak et al. 1996). MOs are designed to bind sequence-specifically to target nucleic acid molecules and block the translation initiation complex of messenger RNA (mRNA) sequences. Unlike RNAi, they do not cause degradation of their target mRNA but affect protein synthesis of target mRNAs by inhibiting splicing or prevent translation. This blocking of gene function by MOs is a transient process that does not result in any permanent alterations of genome sequences. MOs are used as research tools for reverse genetics to knockdown genes to investigate their function(s) (Subbotina et al. 2016, VKM Report 2021: 18). For efficient intracellular entry, MOs can be delivered by microinjections, electroporation, or association with delivery molecules.

MOs have been employed for studying the role of genes and disrupting their expression during fish embryonic development (Golpour et al. 2016). PGCs are progenitor cells of both male and female germline cells, impaired development of which will lead to individuals devoid of any gametes. Successfully disrupting PGC development can affect fertility in both sexes. Several genes involved in PGC development have been identified, including *nanos*, *dnd*, *sdf* and *cxcr4*. Among these genes, *dnd* is one of the most-studied genes, revealing its important role in PGC development in various fishes (Xu et al.2022). There is no data about the proportion of sterile

farmed fish produced using morpholino, but around 80% or greater knockdown for mRNA or protein is commonly used as the benchmark for efficacy of the morpholino technique.

In addition to morphological and histological analysis, the effects of MOs can be determined by western blot, proteomics to measure protein levels, and immunohistochemistry using antibodies against the protein of interest. The modulation of MOs induced knockdown on the expression of other genes can be evaluated by qPCR or RNA-seq methods.

3.2.2.2 Morpholino (MO) and Gapmer oligonucleotides microinjection

MOs are delivered immediately following fertilisation using a microinjection apparatus. Injection of MOs is carried out using the same basic protocol as microinjection of mRNAs. MOs are typically injected at the 1–8 cell stage. Once inside the cell, a MO specifically binds to their target mRNA and functionally inhibits protein synthesis by blocking mRNA translation or by preventing proper pre-mRNA splicing. MOs are used as antisense knockdown tools in zebrafish embryos.

Ciruna et al. (2002) produced PGC depleted fish using *dnd*-MO, a morpholino oligonucleotide that blocks (PGC) development, by microinjecting zebrafish embryos at the 2–8 cell stage with *dnd*-MO. Skugor et al. (2014) knocked down the *dnd* gene in Atlantic cod (*Gadus morhua*) by microinjecting *dnd*-MO into two-cell embryos. The development and migration of presumptive PGCs in wild type embryos of Atlantic cod were monitored by whole-mount in situ hybridisation (WISH) analysis of *vasa* expression. They observed a decrease in *vasa*, *nanos3*, and *tudor* gene expression levels in fish treated with *dnd*-MO.

Two different types of antisense oligonucleotides have been tested in Atlantic salmon. Tveiten et al. (2023) tested methods for production of sterile Atlantic salmon embryos by targeting the *dnd* gene with two different antisense oligonucleotides; morpholino or Gapmer. Like morpholino, Gapmer is an antisense oligonucleotide (ASO) but consists of a central portion of DNA nucleotides flanked with modified RNA-like nucleotides on both sides of the DNA sequence. To investigate the effect of *dnd* gene depletion on the fish development, more than 2000 eggs were injected with *dnd*-Gapmer oligonucleotides and embryos were raised to juvenile stages and to adults. They observed that randomly sampled fish injected with *dnd*-Gapmer were morphologically undistinguishable from their control siblings. Sterile (*dnd*-Gapmer knockdown fish) and fertile (control fish) female gonads displayed striking difference between a prominent orange structure and a faint translucent string, respectively, while the morphological differences between the testes in sterile and fertile fish were less obvious. Further, WISH staining of the gonads with *vasa* antisense probe demonstrated complete depletion of the PGC population in the *dnd* knockdown fish, in marked contrast to the intact PGC populations in wild type and fertile fish. Successful *dnd* gene knockdown and sterilisation was achieved with Gapmer oligonucleotides, but not with morpholino oligos (MO-injection). Germ cell-depleted embryos developed into morphologically normal male and female salmon with rudimentary gonads devoid of gametes (Tveiten et al. 2023).

Microinjection of MOs had a severe impact on the survival rate during embryogenesis, and low survival is more challenging to tackle and cannot be mitigated by using a large number of fertilised eggs. As MOs are sequence-dependent, unsuccessful *dnd*-MO effects may be due to the specificity of the oligos used, or the *dnd*-Gapmer oligonucleotides in the study are more suitable for salmon than *dnd*-MO.

There is no information available on the use of MO-injection technique in commercial scale sterilisation of farmed fish. More data is needed to determine if MO-injection technique offers a reliable approach to achieve farmed fish sterility on a commercial scale. Manual microinjection methods are laborious and prone to errors in the detection and selection, and therefore an efficient large-scale MO or Gapmer-oligonucleotides delivery system needs to overcome these limitations.

3.2.2.3 Morpholino oligonucleotides (MO) immersion

Wong et al. (2015) developed a technology using bath immersion to induce temporary gene silencing with the molecular transporter, Vivo, conjugated to MO to induce sterility to fish. Vivo-MO is a morpholino conjugated to a transporter delivery moiety, comprised of an octa-guanidine dendrimer, that improves uptake of the morpholino oligonucleotides by cells. Vivo-conjugated MO against dead-end (*dnd*-MO-Vivo), effectively disrupted PGC development, which led to the elimination of germ cells and resulted in the production of reproductively sterile zebrafish. Direct uptake through the chorion is restricted to small molecules because there is a limitation of chorion permeability. The Vivo molecular transporter used in the immersion method penetrates the chorion and promotes uptake of *dnd*-MO by PGCs in zebrafish embryos. The immersion strategy is adaptable, and it can easily be scaled up to simultaneously treat many eggs or embryos, since bath immersion is used to administer MOs instead of microinjection.

When zebrafish embryos were treated with *dnd*-MO-Vivo by bath-immersion, the *dnd*-MO-Vivo penetrated the chorion of embryos and entered target cells, and under optimal conditions, 100% sterility was achieved when zebrafish embryos were treated immediately after fertilisation. The bath-immersion method was first developed and optimized in zebrafish (Wong et al. 2015), and the method has been subsequently adapted to coho salmon (*Oncorhynchus kisutch*), rainbow trout, Atlantic salmon, and sablefish (*Anoplopoma fimbria*).

Xu et al. (2023) performed a proof of principle study of producing sterile coho salmon where unfertilized eggs were bath-immersion treated with *dnd*-MO-Vivo in different immersion media (Xu et al. 2023). Sterile fish lacking germ cells, and those with arrested germ cells/atretic oocytes, were obtained at 14 and 20 months of age, although at a low percentage (2.3% to 10% based on females). Gonadal histology, *vasa or nanos3* gene expression levels were used to compare fertile and sterile gonads, as well as retarded ovaries. Two apparent ovarian bulbs were recognized when coho salmon reached 14-months age, and smaller two lobes of the testes were observed in male fish attached to and spanning the length of the roof of the abdominal cavity. Examination of the gonads revealed that gonad development was absent except for two pieces of thin filament-like tissue in 4%–20% of treated fish, depending on the *dnd*-MO-Vivo concentration. These individuals were defined as sterile because no germ cells were found in their gonads. Further, there was a marked size difference between fertile and sterile gonads when the ovaries, testes and sterile gonads were evaluated.

For sablefish, treated and control fish were reared for two-and-a-half years before the final assessment of gonadal development (Xu et al. 2022). Of the *dnd*-MO-Vivo treated sablefish at one-year old, 12% of fish were found to have reduced germ-cell numbers, and 10% of fish were found to be sterile with no detectable germ cells in the early developing gonads, which were comparatively small. At 2.5 years of age, 11% of treated fish were sterile. Sterile gonads of sablefish were small and filament-like without detectable germ cells and could be easily

distinguished from control ovaries and testes, which were more firm, solid, and notably larger in size (Xu et al. 2022).

No data was found on the use of MO-immersion technique in large-scale sterilisation of farmed fish. However, the technique has a great potential for aquaculture application of fish sterilisation. A patent has been filed for this technique[7] and from the initial results, the technique seems to work in Atlantic salmon. However, more data are needed to assess whether the MO-immersion technique can be used to achieve farmed fish sterility on a large commercial scale.

3.2.3 Vaccination

Immunocontraception and immunosterilisation techniques are designed to block essential steps or elements necessary to complete the reproductive process (Naz 2011). The concept is applied in veterinary medicine and has for instance been used to control reproduction in feral, zoo and free-ranging wild mammals (reviewed by Kirkpatrick et al. 2011, Naz 2011). Immunocontraception and immunosterilisation vaccines for certain mammalian species are commercially available with species-specific approval (by U.S. Environmental Protection Agency) for fertility-control in wildlife and invasive species (Fitzpatric et al. 2011, Naz 2011, 2016, Bechert and Fraker 2018, Eckerström-Liedholm et al. 2024).

Sterility by autoimmunity against gonadal proteins is shown in zebrafish (Presslauer et al. 2014). Targeting germ cell-specific proteins resulted in disturbed functions of somatic supporting cells, likely through disturbed crosstalk signalling. Six-week-old juvenile zebrafish were immunised against three gonadal proteins (conjugated to a carrier protein, keyhole limpet hemocyanin KLH): anti-Gsdf, anti-Gdf9, and anti-Cd205, with a repeated injection 15 days later. The gonadal soma-derived factor (Gsdf) supports somatic cells of ovary and testis, granulosa and Sertoli cells and is a member of TGFβ (transforming growth factor beta) superfamily, while Gdf9 (growth differentiation factor 9) is involved in follicle development. Cd205 is found in spermatocytes, spermatids, spermatozoa, and vitellogenic oocytes and is likely involved in antigen presentation. Anti-Cd205 treatment stimulated a strong immune response in both sexes, resulting in pro-apoptotic signals in somatic cells and oocytes. The anti- Gdf9 treatments promoted apoptotic signals in testes and caused abnormal development of ovaries, while anti-Gsdf treatments only had effects in testes.

Sterility by injecting peptides targeting gonadotropin receptors is shown in rainbow trout (Sambroni et al. 2009). The vaccine treatment was a monthly injection for six months of a peptide construct including filamentous phages displaying decapeptides of the rainbow trout FSH (follicle-stimulating hormone) receptor and of the LH (luteinising hormone) receptor. Significant results on sterility were observed in all-male populations. However, the inhibitory effect on spermatogenesis was reversed 10 weeks after treatment, indicating a transient effect on sterility. The success within and between treatments, developmental stages and sex was variable and transient. There were also signs of disorganised tissues in the peripheral areas, suggesting a possible inflammatory reaction because of the adjuvant used for the vaccination.

[7] https://acdpharma.com/legemiddel/steril-fisk/

There is lack of data pertaining to health and welfare in the two available publications from experiments in fish (Presslauer et al. 2014, Sambroni et al. 2009). In zebrafish, successful vaccination inducing sterility seemed to reduce their weight and length.

Immunocontraceptive and immunosterilisation vaccines may have a potential for use in aquaculture but must be further developed. These vaccines must offer a single dose and multi-year efficacy to be relevant. So far, the magnitude of immunosterilisation in rainbow trout is relatively weak compared to in mammals. Improved effects may be achieved with vaccination before the onset of maturation. Further optimisation of the treatment includes timing (prior to puberty onset), dose, and type of antigen peptide, improved adjuvants for induced immune response, as well as investigating long-term effects of the treatment.

In fish, there seems to be limited research on immunocontraception either because of challenges with optimisation, the method might have been abandoned due to unfavorable results like weaker autoimmune response to gonadal proteins, or other causes. There are obvious differences in reproduction biology and fertilisation between mammals (endothermic with internal fertilisation) and salmonids (ectothermic, external fertilisation) and therefore not straightforward to compare or implement mammal-based technologies in fish. In mammals, the two primary categories of contraceptive vaccines are based on gonadotropin-releasing hormone (GnRH) and porcine zona pellucida (PZP) antigens (Naz 2011, Bechert and Fraker 2018). GnRH based vaccines aim at interfering with the hypothalamus-pituitary-gonad signalling pathways responsible for reproduction by inhibiting the release of hormones that stimulate gonad development. The different targets of the immunisation may be associated with positive and negative specific side effects. For instance, immunisation against GnRH inhibits behaviours linked to sex hormones, as GnRH also controls pituitary and gonadal hormone responses in both males and females. However, targeting GnRH has been associated with stunted growth and other negative side effects since several organs have GnRH receptors (Kirkpatrick et al. 2011). Other targets have also resulted in unacceptable side effects for mammalian models. For instance, immunisation of male guineapigs with the sperm surface protein PH-20 lead to autoimmune orchitis (Tung et al.1997) and targeting sperm receptor proteins ZP3 in zona pellucida by immunizing female mic e resulted in autoimmune oophoritis (Rhim et al. 1992). The choice of antigen thus represents a key challenge. In addition, immunization route has been an obstacle in mammalian animal models including experimental use of host specific parasites, bacterial vectors, recombinant viruses and phages to deliver the antigen.

Vaccines for immunisation against virus and bacteria in fish have traditionally been delivered either by immersion (fry) or by intraperitoneal injection (parr and pre-smolt). Intra-peritoneal injection vaccines have been formulated with adjuvants to stimulate the immune response and increase the durability of protection. Both antigens and adjuvants have been associated with reduced welfare due to severe side effects observed as inflammatory responses with adherences between organs and melaninisation in the abdominal cavity (Thorarinsson et al. 2023). Skeletal deformities and stunted growth have also been associated with vaccines (Aunsmo et al. 2008, Thorarinson et al. 2023). During recent years, the number of antigens (pathogens) included in the vaccines have increased. The prospects of introducing yet another component and purpose into the vaccination regimen may be undesirable and even unfeasible from a health and welfare perspective.

3.2.4 Cytotoxic drugs

Cytotoxic drugs (antimitotic) like busulfan, produce intrastrand or interstrand crosslinks of DNA, blocks DNA replication, cell proliferation, and differentiation and leads to germ cell depletion. The use of busulfan has been successful for sterility induction, e.g. four doses of busulfan (intraperitoneal injections) at 30 mg/kg and 40 mg/kg to females and males respectively, of one year old Patagonian pejerrey (*Odontesthes hatcheri*) kept at 26°C. However, this was not as successful for rainbow trout (Majhi et al. 2009, Billard et al. 1982). In zebrafish, 88% male sterility was achieved after cotreatment with busulfan and high temperature (Nobrega et al. 2010). However, the sterility after short-term treatment was transient and longer treatment (+45 days) at a high temperature (37°C) was necessary to induce permanent sterilisation in Nile tilapia (*Oreochromis niloticus*) and Mozambique tilapia (*O. mossambicus*) (Ryu et al. 2022). Another study in Nile tilapia failed to induce permanent sterility, because germ cells developed again when returning to normal conditions (Jin et al. 2019). Studies with busulfan in olive flounder (*Paralichthys olivaceus*) show a reduction in *vasa* gene expression to 5% of untreated males (Wang et al. 2021). Species, heat and toxicity tolerance seem to influence the effect of busulfan on germ cell ablation.

3.2.5 Primordazine treatment

The primordazine's mechanism of action is mediated through a primordazine response element located in the 3’ untranslated region (UTR) of the *nanos3* gene, which is an essential gene for germ cell maintenance. Primordazine alters the localisation and represses the translation of *nanos3* RNA in germ cells through the primordazine response element. Dissection of primordazine’s mechanism of action revealed that *nanos3*, *dnd* and other early germ cell genes are translated through the poly(A)-tail-independent noncanonical translation, which is disrupted by primordazine treatment. Injection of polyadenylated mRNA of *nanos3*, *dnd1*, or both modestly but significantly attenuated primordazine's effect on PGC loss, suggesting that PGC loss by primordazine is due in part to reduced translation of *nanos3* and *dnd1.* However, the *nanos3* and *dnd1* mRNA levels were not affected by primordazine (Jin et al. 2018). Primordazine has to our knowledge only been used in zebrafish, and only one publication was found.

A few PGCs survive after primordazine treatment, although most of PGCs begin to disappear 10–12 h post fertilisation. Disruption of the PGC developmental process with primordazine treatment alone is incomplete, and some surviving PGCs go on to populate the adult gonad, suggesting that the few residual PGCs are sufficient to maintain fertility. Primordazine treatment induced PGC loss can be determined by *in situ* hybridisation with PGC markers such as *ddx4*, *nanos3*, and *dnd1* and by immunohistochemistry using anti-DDX4 antibody.

More studies on the usefulness of primordazine treatment alone in fish sterilisation or combined with other sterilisation techniques are needed to be able to evaluate the use of the method in aquaculture.

3.3 Other methods used to produce sterile fish

3.3.1 Triploidisation

Triploidy means that the organism has three chromosomes instead of two (diploid). In fishes, the second polar body from the meiosis (cell division that produces the egg) is not expelled until after the egg is fertilized by sperm. During a short time after fertilisation, there is a possibility to keep both haploid chromosome sets of the egg in addition to the chromosome set provided by the sperm. The result is a fertilised egg with three chromosome sets. Triploid individuals are sterile because their cells cannot undergo meiosis and form germ cells, since halving of three chromosomes results in unbalanced chromosome numbers in the generated daughter cells.

Spontaneous triploidy is found in both wild and cultured populations of many species (Piferrer et al. 2009). In Atlantic salmon, triploidy seems to be slightly more common in farmed salmon than in wild salmon (Glover et al. 2015, 2016). The reason for triploidy from natural causes may be manifold. Likely, spontaneous triploidy is induced by some external (environmental disturbance) or internal (egg quality and egg aging) stress during fertilisation and early egg development.

Artificial triploidy is induced by giving the newly fertilised egg a shock, either by heat treatment or by hydrostatic pressure treatment. Heat treatment is the earliest method (Valenti 1975, Chourrout 1980, Benfey and Sutterlin 1984) and was subject to many experiments at Akvaforsk, Norway, in the 1980s to find the best combination of heat-shock timing, temperature and duration for producing a high percentage triploid offspring with little egg mortality. Pressure treatment performs even better on a commercial scale and is currently the preferred method in use (Benfey 2016). Apart from this improvement, there is little that distinguishes between these two methods, and they are treated as one in this report.

Electric shock has been used to produce triploid offspring of an African catfish (*Clarias gariepinus*) (Okomoda et al. 2020) but is not considered further here because of the low proportion of triploidy achieved. Nor is cold-shock treated here, as its main use is to induce triploidy in warm-water fish species.

A different method to produce triploid fish is to make crosses between tetraploid and diploid individuals. Experiments by Chourrout et al. (1986) showed two problems with this method. Making tetraploids by pressure treatment resulted in high mortality and poor growth, and no ovulated females were found after two years. Tetraploid males were crossed with diploid females to produce triploids. Fertilisation ability of tetraploid males was low with 40% fertilisation (0-97% range) relative to control fish. The authors suggested that the reason for low fertilisation may be that diploid spermatozoa, which are wider than haploid, are more frequently blocked in the micropyle canal (Chourrout et al. 1986).

Advances in making tetraploid rainbow trout and using second-generation tetraploids in crosses with diploid rainbow trout, suggest that triploid individuals generated by such inter-crossed triploids (4N x 2N) may perform better than pressure-induced triploids from the same groups (Weber et al. 2014). This improves performance in 4N x 2N crosses with respect to both fewer vertebrate deformities and higher body weight.

3.3.1.1 Robustness and reproducibility

Triploidy is a robust and reproducible method for making sterile individuals of many fish species. The mechanism for sterility is known and near absolute. Triploid salmonid males show secondary sexual traits and may participate in spawning (with no or very low fertilisation success). This problem can be avoided by producing all-female lines for triploidy as triploid salmonid females do not develop functional gonads or participate in spawning. On a global scale, triploid aquacultured fish have been produced in high numbers for several decades.

3.3.1.2 Development scale

Triploidy has been used in large-scale stocking of rainbow trout since the 1980s (Bye and Lincoln 1986) and in commercial-scale aquaculture since the 1990s. In Tasmania, triploid all-female salmon has been used in fish farming since the 1990s to secure a stable market supply of immature farmed salmon (Thorstad et al. 2008).

Triploidy has been used in large-scale experiments from 2007 and in full-scale aquaculture operations in Norway since 2014. In 2007, a research project was initiated at the Institute of Marine Research to understand the biology of triploid Atlantic salmon raised under aquaculture conditions in Norway. A project aimed at clarifying pathogen susceptibility in triploid salmon was initiated a few years later, involving research institutions, breeding companies and fish-farming companies (Sindre et al. 2018). In 2013, two fish farming companies were awarded “green concessions” from the Norwegian fisheries and aquaculture authorities to farm triploid salmon in Troms and Finnmark counties. The Norwegian Food Safety Authority requested that the companies documented the health and welfare of their triploid salmon. A collaboration between fish farming companies, the fish health services, and the Institute of Marine Research was therefore established. The results of the full-scale experiments and commercial production of triploid salmon have been published in several reports (Stien et al. 2019, 2021ab, 2023) and in international scientific journals (Madaro et al. 2022, 2024 and references therein).

In Norway, more than 30 million triploid individuals have been produced for experimental and commercial use. In Newfoundland, the use of Atlantic salmon in aquaculture production must be based on triploid salmon for any farmed strain originating from Europe.

3.3.1.3 Proportion of sterile offspring

Early experiments with landlocked Atlantic salmon in Canada showed that heat treatment of 5 min. at 32°C, and hydrostatic pressure of 3-6 min. at *c.* 10,000 p.s.i. gave 100% triploid offspring with 70-90% egg survival relative to control fish when the treatment was given 20 min. after fertilisation at 10°C (Benfey and Sutterlin 1984). The combinations of fertilisation temperature, timing, duration and hydrostatic pressure have now been improved, resulting in higher egg survival and 100% triploidy (Benfey 2016). Reports from Norway suggest that hydrostatic pressure gives close to 100% triploidy, whereas egg survival is more variable from similar to diploid egg survival from the same cross to less than 70% survival relative to diploid crosses.

3.3.1.4 Verification methods

There are several methods that can be used to document triploidy in salmon. First, triploid individuals have larger erythrocytes, and this feature has been used to sort triploid from diploids after erythrocyte measurements. Secondly, fish erythrocytes are nucleated, and triploids have a higher DNA content (larger cell nucleus) of erythrocytes, which can be quantified by flow cytometry. Thirdly, triploidy can be documented by genetic analyses finding three different alleles in the same locus, for example, in highly variable microsatellites (Jacq 2020). A recent study comparing these three methods showed that they all gave the same results in a group of individuals screened by all three methods (Glover et al. 2020b).

3.3.1.5 Use in salmonids and other fishes

Sterility by triploidy has been applied to salmonid fishes of the genera *Salmo*, *Oncorhynchus* and *Salvelinus*, and to our knowledge, to most species of these genera. Sterility by triploidy has also been applied to northern pike (*Esox lucius*), yellow perch (*Perca flavescens*), walleye (*Sander vitreus*), sunfish (*Lepomis gibbosus, L. cyanellus, L. macrochirus*), largemouth black bass (*Micropterus salmoides*), crappie (*Pomoxis annularis, P. nigromaculatis*), common carp, grass carp (*Ctenopharyngodon idella*), tench (*Tinca tinca*), Nile tilapia, channel catfish, sablefish, pejerrey (*Odonthestes bonariensis*), white sturgeon (*Acipenser montanus*), Atlantic cod, and others.

3.3.1.6 Comparative performance (vs traditional farmed fish)

Triploid salmonids typically show slightly lower growth and survival compared to normal (diploid) salmonids. VKM (2023) concluded that triploids tended to show a higher growth rate in fresh water and a lower growth rate in sea water than traditional farmed salmon. In experiments at the Institute of Marine Research facilities at Matre from November 2009 (fertilisation) to May 2012 (slaughter), triploid salmon were 15% heavier and 7% longer than diploid salmon at the smolt stage (seawater transfer) in May 2011, whereas diploids were 10% heavier and 1% longer than triploids at slaughter 12 months later (Fraser et al. 2013). These experiments were carried out with ca. 20,000 individuals per ploidy (in triplicate groups of ca. 7000) and with eggs and sperm from the AquaGen breeding program. Seawater mortality was slightly higher in triploids (1.8 to 2.2% per group) than in diploids (1.2 to 1.5% per group), and the proportion of fish classified as superior quality at slaughter was lower in triploids than in diploids. The price obtained per fish per cage was lower in triploids (63.5 to 67.4 NOK) than in diploids (62.3 to 81.1 NOK) at a commercial slaughter (Fraser et al. 2013). The conclusion was that triploids could be produced but at a higher cost to the producer.

A commercial-scale experiment was initiated in Norway in 2017. More than 1.2 million eggs were used to compare triploid and diploid offspring of the same AquaGen strain, distributed among four fish farm companies: one in southwestern Norway, two in middle Norway and one in northern Norway (Madaro et al. 2022). The fish were released into sea cages as smolts in April to June 2018 and compared with respect to performance and welfare indicators. The overall results were similar to those reached by Fraser et al. (2013) except that they were dramatically different with respect to mortality in the sea. More than 40% of the smolts released in the sea cages in the southwestern Norway fish farm were lost before slaughter, and the losses were larger in the diploid group (44.8%) than in the triploid group (41.8%). In the three other fish farms, the losses were higher among the triploid groups (11.7-15.5%) than

among the diploid groups (2.5-9.4%) and were primarily related to the periods with sea lice treatment (Madaro et al. 2022).

Triploid salmon were smaller than diploid salmon at the end of the seawater trials, but there was no significant difference in specific growth rate between triploids and diploids in this large-scale experiment (Madaro et al. 2022). In the three companies where diploid and triploid groups were compared, diploid salmon showed the highest percentage superior quality salmon at slaughter. On the other hand, in the groups with higher mortality in southwestern Norway (excluded from test), the percentage superior quality salmon was highest in the triploid group.

One conclusion from this commercial-scale experiment was that from an economic perspective, the higher loss and lower quality in triploids resulted in lower income for the fish farmers (Madaro et al. 2022). Another conclusion was that there is a need for further research into reducing losses of triploid sterile salmon under farming conditions.

3.3.1.7 Health, welfare, mortality

VKM has recently compared the health and welfare of triploid and diploid Atlantic salmon under commercial farming conditions (VKM Report 2023: 22). Under similar farming conditions, triploid salmon have a higher occurrence of skeletal deformities and cataract than diploid salmon and require diets with higher contents of phosphorus and histidine to mitigate these effects. Triploid salmon respond more intensely than diploid salmon when exposed to both acute and chronic stress (Madaro et al. 2024). Triploid salmon are less robust during handling, and cope less well under conditions with low oxygen level and high temperatures. Stien et al. (2023) reported that under field conditions, farmers and fish health personnel viewed triploid salmon as inferior and less robust than diploid salmon and thus made other management and treatment decisions for triploids. Triploid salmon thus requires other management conditions than diploid salmon with regards to nutrition, handling and rearing temperatures. The latter aspect is relevant for future aquaculture production where adjustment to climatic change and increasing sea surface temperatures may be crucial. On the other hand, the susceptibility of triploids to high temperatures could mitigate the biodiversity risks of escaped triploids, as these conditions may reduce their viability.

Studies designed to compare specific susceptibility to infectious agents between triploid and diploid salmon have shown varying results. Under experimental conditions, triploid and diploid salmon have the same susceptibility to the causative agent of pancreas disease (salmonid alphavirus –3, SAV-3) (Moore et al. 2017, Sindre et al. 2018) and infectious pancreatic necrosis (IPN virus) (Sindre et al. 2018). Under field conditions, triploid salmon were more susceptible to winter ulcers than diploid salmon (Stien et al. 2019). In the infection trial with *Tenacibaculum* spp. there was overall low mortality and no difference in mortality between triploid and diploid salmon, but the trial results indicated that triploid salmon died faster than diploid salmon (Sindre et al. 2018). Infection trials with *Moritella viscosa* also gave varying results and were possibly confounded by obstacles during the trials (Sindre et al. 2018). In the first trial there was higher mortality in triploid salmon, but these were fed conventional feed for diploid salmon prior to the infection trial (feeding with a special feed tailored to triploid salmon started one week prior to the trial). In the repeated trial, rapid mortality associated

with an elevated infection dose may have masked potential differences between the groups (Sindre et al. 2018).

Under field conditions, triploid salmon had higher odds for primary infectious salmon anemia (ISA) outbreaks compared to diploid salmon (Aunsmo et al. 2022). Triploid salmon are not different from diploid salmon with respect to sea-lice induced mortality (Frenzl et al. 2023), but are more susceptible to another ectoparasite, *Gyrodactylus salaris*, than their diploid counterparts (Ozerov et al. 2010).

Sindre et al. (2018) investigated regulation of genes involved in immune responses before and after infection with SAV-3 and detected differences in expression of specific immune genes between healthy triploid and diploid Atlantic salmon. Differences related to immune genes were also detected after SAV-3 infection but could not be linked to differences in clinical manifestation of infection in triploid versus diploid salmon.

Further research into susceptibility of triploid sterile salmon to infectious agents is needed. However, the combined burden of disease, intermittent low oxygen levels and high temperatures experienced by fish under farming conditions are difficult to reproduce in closed containment experiments.

3.3.1.8 Environmental risk assessment (ERA)

Triploidy has been known as a simple and inexpensive method to drastically reduce the number of fertile individuals in fish intended for release or for rearing in facilities that are not escape-proof. Triploidisation cannot fully guarantee that treated fish will not reproduce. On the other hand, as the success rate is approaching 100% for pressure-induced triploidy (Benfey 2016), the extended use of triploidy in aquaculture will strongly reduce the proportion of fertile farmed fish.

In Ireland, the migration behaviour of diploid and triploid salmon in the wild was compared based on controlled releases of micro-tagged triploid salmon of both mixed-sex and all-female groups (Cotter et al. 2000). The fish were released from either a smolt-releasing locality, or from coastal sea cages 100 km away at the post-smolt stage. The return of salmon to coastal and freshwater capture sites was monitored in the Irish national coded wire tag recovery program. The highest return rate to freshwater was 2.25% and was found in the mixed-sex diploid group released as smolts. Recaptures in freshwater was 0.9% for all-female diploid fish, 0.6% for the mixed-sex triploids, and 0.2% for the all-female triploids. The return of all-female triploids to freshwater suggests that maturation is not a prerequisite for returning to rivers. Less than 0.1% of the post-smolts released from sea cages were recaptured in fresh water.

Triploid female salmon do not show signs of sexual maturity, whereas triploid males mature sexually, show spawning behaviour like diploid males and produce sperm that may fertilize diploid females. Fjelldal et al. (2014) found that triploid males competed with diploid males and were able to induce digging behaviour and egg deposition in diploid females. Cotter et al. (2000) crossed a triploid male with a diploid female and found that 1.6% of the resulting fry survived until first feeding compared to 92.7% of fry from diploid controls. Murray et al. (2018) stripped ten triploid and ten diploid males and found a survival of less than 1% until the eyed egg stage for the eggs fertilized by triploid males compared to 61% survival among the diploid controls. The sperm of a triploid male is aneuploid (i.e. has chromosome aberrations) and is a

likely reason for the poor survival of offspring sired by triploid males. Aneuploid animals that lack a balanced chromosome complement, typically die early in development (Benfey 2016).

If triploid farmed salmon males escape in large numbers, it is conceivable that they can reduce the spawning success of wild salmon. However, Glover et al. (2016) found that fewer triploid individuals entered rivers as escaped farmed salmon (0.18%) than expected from their proportion among farmed salmon in net pens (2%). Glover et al. (2016) and Cotter et al. (2000) concluded that the use of triploid salmon in aquaculture can reduce the environmental impact of escaped farmed salmon on wild salmon both in terms of their reduced rate of return and inability to interbreed successfully with native wild populations. The production of all-female triploid lines of farmed salmon will effectively stop spawning of triploid individuals. Triploid all-female lines therefore also stop genetic introgression of escaped farmed to wild salmon.

If salmon farming were based on all-female triploid lines, escapes from fish farms should cause minor problems for wild salmon other than the potential transmission of disease agents while in the sea. Ecological problems caused by long-lived immature salmon in the sea are more likely related to predation on smaller fish than to effects on wild salmon. As far as we know, longevity has not been estimated for triploid salmon, but two extra years at sea have been indicated for hormonally sterilised fish (Solar et al. 1986). In the experiment in Ireland, the salmon that had been two years at sea rather than one year were three diploid and four triploid salmon (Cotter et al. 2000). The much lower proportion of escaped triploid farmed salmon found in rivers compared to the proportion of spontaneous triploidisation found in salmon farms suggests that the number of escapes must be very high before ecological problems caused by triploid all-female farmed fish occur.

A study comparing fine-scale movement and survivorship of released triploid and diploid rainbow trout in two Washington State lakes, concluded that triploids had a decreased rate of emigration out of the lake, similar survivorship, and similar diel movements and home range in comparison to diploid rainbow trout (Pease et al. 2023).

The number of farmed salmonids in Norway is high (more than 457 million farmed Atlantic salmon in 2023) and expected to increase. Infectious diseases are widespread and a major cause of mortality and reduced welfare (Sommerset et al. 2024). In this context, even minor changes in pathogen susceptibility in triploid fish used in large scale production can have significant consequences for farmed and possibly also for wild salmon. Research regarding the susceptibility of triploid salmon to specific pathogens is limited to a small number of pathogens, and for several of these, the findings are not conclusive. More laboratory-based research is needed on specific pathogen-host interactions, and there is also a need for more transmission trials under controlled closed containment conditions. Research and surveillance under field conditions strongly suggest that triploid salmon are more fragile and with regards to standard handling operations and environmental conditions (Stien et al. 2019, 2021ab, 2023). Under field conditions, this fragility may shift the tipping point of disease development from infection to disease (Snieszko 1974). A more susceptible, or in this context, fragile fish group constitute a biosecurity risk for surrounding farmed and wild fish by increasing the likelihood of establishment of an infectious agent in a population, and subsequently by increasing the propagation and shedding of infectious agents to the surrounding environment.

To our knowledge, there is little (if any) difference between triploid and diploid salmon in their vulnerability to sea lice (Frenzl et al. 2023). The high mortality of wild salmon caused by sea lice spreading from salmon farms will therefore not be alleviated by triploidisation. Farmers and fish health personnel report that they view triploid salmon as less robust than diploid salmon and therefore make other treatment decisions than for diploid fish (Stien et al. 2023). If this leads to more reluctance to treat triploids against lice, it may result in more sea lice being released to the environment. On the other hand, if triploid salmon are managed and treated as diploid salmon, their fragility may lead to more infectious diseases and shedding of pathogens to the environment.

On the other hand, if all-female triploid lines were used, disease transmission might be reduced in freshwater, as only very few triploid females escape to rivers. Moreover, there is a higher susceptibility to infections in males, due to immunosuppressive effect caused by testosterone (Caballero-Huertas et al. 2024).

3.3.2 Interspecific hybridisation

Fishes are more prone to interspecific hybridisation than most other vertebrates. Among the salmonid fishes, interspecific hybrids are known for all genera including species of *Salmo*, *Oncorhynchus* and *Salvelinus*. Typically, interspecific hybrids occur in small proportions (a few percent or lower) but they may be common in perturbed environments/populations or where one species is introduced outside its native range.

Hybrids between Atlantic salmon and brown trout (*Salmo trutta*) are more common among offspring of female farmed salmon than among offspring of female wild salmon (Youngson et al. 1993). In rivers where the Atlantic salmon population has been strongly reduced because of *Gyrodactylus salaris*, salmon-x-trout hybrids may be as numerous as salmon among juvenile fish (Johnsen et al. 2005). Salmon-x-trout hybrids are highly viable and thought to be functionally sterile due to major karyotypic differences. Back-crosses of F1 hybrids to either parental species show high mortality and those surviving in experiments appear to be gynogenetically diploid or triploid offspring (Galbreath and Thorgaard 1995). A likely triploid back-cross has also been found among juvenile salmonids from River Driva, Norway (pers.comm.).

Intergeneric hybrids are known between brown trout and brook trout (*Salvelinus fontinalis*). In South Norway, where brook trout were released for maintaining a fishery in acidified localities in the 1970s, brook trout established self-sustaining populations in many watercourses. In a few of them, findings of so-called tiger trout (a cross between brown trout and brook trout) have been documented.

As hybrids may grow well but often remain sterile, they may be of interest to aquaculture and releases of non-reproducing individuals. Several experiments were executed at the Sunndalsøra aquaculture station to find the best fish for aquaculture in Norway. Rainbow trout showed the highest growth rate in fresh water, whereas Atlantic salmon showed the best growth in salt water. Arctic charr tolerated the highest densities in fresh water. To our knowledge, no interspecific crosses showed better overall performance than either of the parental species, although – depending on the preferred traits – it may be possible to find crosses that meet them better than the pure species. For example, in North America, the so-

called splake (cross between brook trout and lake trout *Salvelinus namaycush*) is used extensively in stocking because it grows better than lake trout and reaches a higher size than brook trout, and only occasionally produces offspring beyond the F1 generation.

Large-scale rearing or releases of interspecific hybrids may be problematic for wild populations if F1 hybrids reproduce. Hybrid swarms are known for several fishes (cyprinids, ictalurids) although not known for salmonids. Recently, the large-scale aquaculture based on Atlantic salmon in Pacific North America (Washington State and British Columbia) has led to a series of experiments to investigate the potential for crosses with Pacific salmon and rainbow trout. Devlin et al. (2021) produced artificial crosses between Atlantic salmon and seven Pacific salmon and concluded that "Most cross types were found to produce low numbers of hatched embryos, but none survived to sexual maturation. Survivors consisted of diploids and triploids containing both Atlantic and Pacific salmon parental genomes. Thus, introgression of DNA between Pacific and Atlantic salmon may occur to form F1 hybrids, but transmission to subsequent generations is expected to be rare and occur only over evolutionary time scales."

Finally, Devlin et al. (2021) noted that most intergeneric crosses between Atlantic and Pacific salmonids experienced very low viability in early stages of development.

If interspecific hybrids should be used in commercial production, the best way is suggested to make triploid hybrids to added assurance of sterility (Galbreath and Thorgaard 1994, 1995).

3.3.3 Androgen treatment

Sexual maturation can occur before fish reach marketable size, causing energy and resources routed to gonadal development, gametogenesis, development of secondary sexual characteristics and reproductive behaviours, which may result in compromised somatic growth, health, flesh quality and animal welfare (Xu et al. 2022). Monosex/all-female Atlantic salmon populations are preferred by some producers since males are more susceptible to disease and reduced flesh quality if undergoing full maturation, which occurs earlier in males than in females. In Norway, all-female Atlantic salmon are used in recirculating aquaculture system (RAS) because male salmon are more prone to early maturity in these facilities than in open water net-pens.

The most frequent approach to achieve monosex populations involves application of exogenous sex steroid hormones during a developmental window when sex-related gene expression is initiated, and phenotypic sex can be reversed (known as the 'labile period') (Budd et al. 2015). However, administration of a high dosage of synthetic androgen through immersion and/or feeding can effectively ablate germ cells. This method has been used to induce sterilisation in many species (Manzor 1989, Hunter et al. 1982, Billard 1982, Luckenbach et al. 2016) and 100% sterility was achieved in coho salmon (Goetz et al. 1979). High dose treatment of 17α-methyltestosterone in coho salmon in the diet for a period of 10 weeks post swim-up (25 and 50 µg/L) induced alterations in the gonads resembling neither normal males nor normal females (Goetz et al. 1979). These altered gonads were composed largely of connective tissue with only occasional germ cells. Various proportions of male-, female- and intersex gonads were observed in groups that received androgen (Goetz et al. 1979). Regarding comparative characteristics, rainbow trout and Atlantic salmon treated with androgen were more susceptible to adverse conditions during the period of steroid

administration and had a slower growth rate (Johnstone 1978) compared to fish without androgen treatment. However, the body weight after 150 days was not different upon androgen treatment. Restored sterility was shown one year after withdrawal of a high dose of 17α-methyltestosterone in diet, which initially induced sterile-appearing gonads following treatment in sablefish juveniles (Luckenbach et al. 2016). Residues from the androgen treatment may reach the aquatic environment and have an impact on non-target species causing malformations and hatching delays, as seen in exposure studies of zebrafish embryos (Gediel Rivero-Wendt 2016).

Fish directly treated with steroids may face restrictions or prohibitions on sale for human consumption (e.g., by the European Union under Council Directive 96/22/EC), as well as consumer backlash, even though the treatments are typically completed one or more years prior to harvest, and residual steroid levels are undetectable (Xu et al. 2022).

One method to achieve a female monosex population in species with XX/XY sex determination is the indirect feminisation technique. This method uses ova from normal females (XX) that are fertilised with milt from masculinised females (XX), capable of producing spermatozoa but genotypical females and termed neomales (Piferrer, 2001). Such neomales are produced by hormonal treatment with 17α-methyltestosterone (MT) of a mixed sex population in the feed from the first day of feeding and 800 degree-days. An even higher success rate of masculinization after fertilizing normal female eggs with sperm from these neomales was reported after immersing the fry in 17α-methyldihydrotestosterone (MDHT, 400 µg/L) for 2 hours at 14 days post hatch (dph), and again at 21 or 28 dph (Lee et al.2004). The effect of MDHT was improved when the dissolvent dimethyl sulfoxide (DMSO) was applied together with the hormone (Brown et al. 2021). The alterations induced by MDHT treatment was similar to those induced in genotypic males during testicular differentiation. This is characterised by a maintained suppression of *cyp19a1a* expression and upregulation *amh* and *gsdf* expression and gonad-like tissue development.

Other androgens, such as the naturally occurring 17 β-hydroxyandrostenedione (OHA), can also be applied to induction of functional males (Brown et al. 2021).

Major factors that need to be considered for the sex reversal of salmonid species are:

I. The timing of sexual differentiation, prior to puberty onset

II. Methods of steroid administration and their dose

III. Species-specific characteristics important for the efficacy of sex reversal. Preferably, there should also be indistinguishable characteristics for large scale sex determination. Semen from masculinised females can be cryopreserved and efficiently integrated into hatchery practices.

Monosex populations, although fertile, are used in fish farming, including all-female trout, coho salmon and Atlantic salmon, as well as all-male tilapia aquaculture, to enhance economic return (Wong 2018, Lee 2004, Goetz et al. 1979). Monosex populations are often used in addition to sterility by triploidy, since all-females results in a more robust populations with increased flesh quality compared to males. Karayucel et al. (2017) found that all-female triploid rainbow trout were more susceptible than all-female diploid rainbow trout to

suboptimal environmental conditions, especially higher water temperatures. During some months of this study, the all-female triploids had significantly higher mortality than the all-female diploids. High dose androgen treatment for direct sterility is not used for fish intended for food; only treatment of ancestors to obtain neomales or low dose treatment in juvenile fish is allowed for the European food industry.

3.4 Summary of assessed methods currently available for the production of sterile fish in aquaculture

The methods assessed in chapters 3.1 - 3.3 differ in some characteristics, most importantly in whether the sterilisation results in permanent (and thus theoretically heritable) changes to the genome. In the following tables, we summarize the methods. Table 1 lists the methods that result in permanent changes to the genome, while Table 2 lists the methods that do not include any genomic alterations. Table 3 contains the remaining methods such as unspecific large scale genomic alterations and androgenisation, which does not necessarily produce sterile fish but rather a mono-sex population that is functionally sterile if the opposite sex is unavailable.

Table 1: Methods used to produce sterile fish that results in specific permanent changes to the genome sequence.

Method	**Mechanism**	**Robustness and reproducibility (1-3*)**	**Development stage (1-5**)**	**Sterility: proportion of sterile offspring**	**Verification method**	**Used in salmonids?**	**Used which in other species?**	**Comparative performance (vs traditional aquacultured fish)**	**Welfare, Health, Mortality (vs traditional aquacultured fish)**	**ERA*** considerations (in terms of genetic introgression to wild populations)**
Gene removal / Knockout	CRISPR/Cas9	3	2/3	25%-100%	qPCR or other methods	Atlantic salmon	Nile tilapia	Similar growth and filet quality	Not analysed, lack of puberty	Higher threat than diploid aquaculture fish
Gene removal / Knockout	TALEN	3	2/3	25%-100%	qPCR or other methods	No	Medaka, Zebrafish	Not analysed	Not analysed, lack of puberty	Higher threat than diploid aquaculture fish
Gene removal / Knockout	ZFN	3	2/3	25%-100%	qPCR or other methods	No	Channel catfish	Not analysed	Not analysed, lack of puberty	Higher threat than diploid aquaculture fish
Radiation (Mutagenesis)	Inducing apoptosis and impaired gametogenesis	2-3	1-2	No data available	Morphology, histology,	Rainbow trout	Not known	Will always vary between treatments	Will always vary between treatments. Lack of puberty	Higher threat than diploid aquaculture fish
Transgenesis	Insertion of genetic elements from another species	2-3	2-3	12-100%	PCR detection of transgene	Rainbow trout	Zebrafish, channel catfish, carp	Not analysed	Not analysed	Parent transgenic lines may be fertile. Higher threat than diploid aquaculture fish

* **1** = Low robustness / reproducibility, **2** = Room for improvements, but usually gives the desired outcome, **3**= High robustness / reproducibility

** **1**= Only theoretical, **2**= Lab stage only, **3**= Closed experiments, **4**= Large-scale experiments, **5**= Industrial scale aquaculture

*** ERA = Environmental risk assessment

Table 2: Methods used to produce sterile fish that do not include permanent changes to the genome sequence.

Method	Mechanism	Robustness and reproducibility (1-3*)	Development stage (1-5**)	Sterility: proportion of sterile offspring	Verification method	Used in salmonids?	Used which in other species?	Comparative performance (vs traditional aquacultured fish)	Welfare, Health, Mortality (vs traditional aquacultured fish)	ERA*** considerations (in terms of genetic introgression to wild populations)
Gene silencing / Knockdown	siRNA	2	2	Not determined	qPCR or other methods	Rainbow trout	Zebrafish	Not analysed	Not analysed, lack of puberty	Lower threat than diploid aquaculture fish
Gene silencing / Knockdown	Morpholino oligonucleotides (MO) or gapmer microinjection	2	2	Close to 100%	qPCR or other methods	Atlantic salmon	Zebrafish, Atlantic cod	Not analysed	Not analysed, lack of puberty	Lower threat than diploid aquaculture fish
Gene silencing / Knockdown	Morpholino oligonucleotides (MO) immersion	2	2	Close to 100%	qPCR or other methods	Coho salmon	Zebrafish and sablefish	Not analysed	Not analysed, lack of puberty	Lower threat than diploid aquaculture fish
Vaccination	Induced autoimmunity against gonadal proteins blocking development of functional reproductive organ	1	2-3	Variable and transient results	In situ hybridisation with the targeting proteins	Atlantic salmon, Rainbow trout	Zebrafish	Possible growth retardation	Adjuvants may cause inflammatory reactions	Lower (or similar if transient) threat than diploid aquaculture fish
Busulfan (cytotoxic) + heat	Cytotoxicity/ antimitotic drug (i.p injection) Reduction in vasa expression	1-2	2-3	Variable results due to species, heat and cytotox-icity tolerance	Morphology, Vasa expression	Ranibow trout (less successful)	Tilapia, Flounder, Zebrafish	Lack of information	Lack of information	Possible fertile escapee if transient sterilisation. Antimitotic agent may also affect non-target organisms. Lower threat than diploid aquaculture fish
Primordazine	Induced sterility by repressing gene translation	2	2	Not 100%	In situ hybridisation	No	Zebrafish	Not analysed	Not analysed	Lower threat than diploid aquaculture fish

Table 3: Other methods used to produce sterile fish.

Method	Mechanism	Robustness and reproducibility (1-3*)	Development stage (1-5**)	Sterility: proportion of sterile offspring	Verification method	Used in salmonids?	Used which in other species?	Comparative performance (vs traditional aquacultured fish)	Welfare, Health, Mortality (vs traditional aquacultured fish)	ERA*** considerations (in terms of genetic introgression to wild populations)
Triploidisation	Induced extra set of chromosomes, either through thermal-, or pressure shock	3	5	Close to 100%	Erythrocyte measurement, flow cytometry or genetic analysis	Atlantic salmon, brown trout, rainbow trout, (several) Pacific salmon, brook charr	Common carp, catfish, tilapia, European sea bass and several other species	Superior freshwater growth, inferior seawater growth; lower tolerance to high temperatures	Lower standards of health and welfare; higher early mortality and stress-related mortality	All-female triploids are sterile; male triploids develop gonads and participate in spawning. Lower threat than diploid aquaculture fish
Interspecific hybridisation	Crossing different, yet related species	3	5 (tiger trout)	25% (M), 97% (F)	Morphology and genetic analysis	Atlantic salmon, Rainbow-, Brown-, and Brook trout (brown F X brook M = tiger trout)	Cyprinids, Ictalurids	Faster growth	Popular in stocking programs	Tiger trout are intergeneric hybrids and (largely) sterile and may be subject to triploidisation Lower threat than diploid aquaculture fish
Androgen treatment, low dose	Monosex populations	3	5	0%	Morphology, qPCR / genotyping	Atlantic salmon, Coho salmon	Bluegill	Higher flesh production	Less susceptible to diseases	"Functional sterility" dependent on finding no conspecifics of opposite sex. Lower threat if the opposite sex is not available in the wild
Androgen treatment, high dose	High dose for sterility induction	1-2	2-3	Up to 100%, possibly transient	Morphology	Coho salmon, Atlantic salmon	Sable fish	Slower growth rate, same final weight	More susceptible to adverse conditions during treatment	Possible fertile escapee if transient sterilisation, possibility that androgen residues affect non-target organisms in the near environment. Lower threat than diploid aquaculture fish

4 Method comparison and discussion

Genetic introgression of farmed to wild Atlantic salmon is one of the major environmental problems of Norwegian fish farming. Genetic introgression already affects two thirds of 250 investigated salmon populations in Norway and leads to changes in life history, ecology, and phenology of wild populations, and reduces their adaptations and viability. These problems will continue into the foreseeable future (Glover et al. 2020) and are pertinent for other fish species like Atlantic cod, if today's aquaculture technology persists unchanged. The use of sterile fish in aquaculture can alleviate problems related to genetic introgression of the escaped farmed fish to wild populations but will have limited effect on transmission of disease to wild salmon populations.

Sterile fish in aquaculture will reduce the problem of genetic introgression if sterility can be introduced with methodology that allows mass production and does not cause additional problems, like inherited semi-sterility from individuals with partially restored fertility to wild conspecifics or increased spread of diseases. Farming of Atlantic salmon is fundamentally different to farming of domestic mammals in Norway, as farmed salmon coexist with wild salmon populations and interact with them ecologically and genetically when they escape. Moreover, the open net-pen technology in fish farming invites epidemiological interactions between farmed and wild salmon.

Salmon farming is also different to domestic mammals in Norway with respect to scale – each generation consists of 400-450 million individuals. This production involves the spawning of 50 000 to 100 000 farmed salmon parents.

In this report, VKM has assessed several methods to induce sterility in salmonids with respect to mechanisms to achieve sterility, the stage of development for these methods, their use and performance in aquaculture, and their risk to the environment. The risks to the environment depend on i) to what extent the method secures sterility as intended, and ii) other potential risks of the method to biological diversity and the wild salmon population.

As a starting point, we view all methods that produce a high number/proportion of sterile individuals as potentially useful for future use, as long as siblings or parental fish that potentially escape do not pose a higher risk than conventionally farmed fish. Any method that generates permanent alterations of the genomic sequence is expected to pose a genetic risk to the wild salmon populations with the current farming practice (open net pen-based farming). This is because the potential for escapes to the wild and interactions with wild fish cannot be overlooked.

The following methods seem to meet the objectives:

- Temporal knock-down: Injection / Immersion using morpholino or Gapmer oligonucleotides to knockdown genes essential to primordial germ cell development, alternatively in combination with busulfan
- CRISPR-mediated temporal repression of *dnd* gene expression.
- Vaccination against proteins involved in gonad development at a life stage where this is effective and with a vaccination strategy giving stable result.
- Triploidisation by heat or pressure treatment and potentially by crossing diploids with tetraploids. Health and welfare of triploid salmon may be improved by the following measures:
    - Selection for better triploid performance. Currently, we know little of the traits that underlie health and welfare problems of triploids, and absence of genetic parameters hinders breeding for better welfare.

    - Reduced density at life stages with reduced welfare.
    - Optimized temperature regimes (either by choice of location, water treatment or farmed strain).
    - Closed or semi-closed containment to separate sea lice from triploid salmon, as treatment against sea lice (and not the lice themselves) is currently the biggest welfare problem for farmed salmon in sea cages.
- Use of fish species with no or low risk of introgression, like rainbow trout.
    - For rainbow trout, androgen treatment (all-female) could potentially be an alternative to sterilisation of Atlantic salmon. Rainbow trout rarely form feral populations in Norway, and escaped farmed females are unlikely to meet rainbow trout males and pose a risk to the wild population.
    - However, as rainbow trout is a non-native species listed among the World's 100 worst invasives, triploidy should be used in combination with monosex to add a second level of security with respect to sterility.
    - Regarding triploidisation and stress tolerance, the rainbow trout seem to show fewer negative effects on welfare compared to Atlantic salmon.
- Interspecific hybrids may be a potential method, but additional testing is necessary to find the best combination of male and female parent (and those parents would be fertile if meeting conspecifics).
    - Triploid interspecific hybrids (e.g. between Atlantic salmon and brown trout) may provide a better solution to sterility than diploid interspecific hybrids, provided that a better comparative performance than traditional triploid Atlantic salmon can be demonstrated.
- Genetic knockout of an essential gene for the formation of germ cells (e.g. *dnd* gene) leading to genetic sterility (homozygote genotype), combined with a rescue approach at an early stage leading to functional gonads, can be used. However, this approach depends on ensuring that no fish with undesired genetic background is generated at the final stage of production. The parental generations must be kept in closed land-based containment to avoid possible parental escape of heterozygous individuals, potentially transferring genetic sterility to wild Atlantic salmon. The strategy is also vulnerable to inbreeding, which may have undesirable effects on e.g. growth performance and robustness against diseases.

4.1 Stage of development

Fish farming requires large populations and few of the presented sterilisation techniques are suitable for large-scale production at the current stage of development. Injection or immersion of newly fertilised eggs using morpholino or Gapmer oligonucleotides to knockdown genes essential to primordial germ cell development (e.g. by silencing or downregulating *dnd* mRNA) seems to be one method that can eventually be used to induce sterility to farmed fish. Microinjection is at an advanced stage of development with respect to successful knockdown, but the current methodology is time-consuming and limited in the number of eggs that can be handled. Immersion of eggs into a solution that allows introduction of RNA into the fertilised eggs seems to be a better method allowing large numbers of eggs to be treated simultaneously. At present, immersion methods are at an early stage of development where knockdown success is variable and not necessarily reproducible (or transferable from one species to another).

CRISPR-mediated temporal repression of *dnd* gene expression has similar potential as morpholino or Gapmer oligonucleotides but is still at an early stage of development. Introduction of the CRISPR-tools into fertilised eggs may pose challenges for large scale production, and the success-rate for sterility has presently not been clarified.

Immunosterilisation and contraception have been developed and are available for mammals. Only two studies were found using these methods in fish, and the technology is currently at an early stage of development.

Triploidy is at an advanced stage of development and 30-35 million triploid Atlantic salmon have been held in net pens over the last 10-12 years in Norway. VKM (2023: 22) concluded that research is needed to mitigate some of the health and fish welfare issues that have accompanied large-scale production of triploid farmed Atlantic salmon. Studies on rainbow trout on a semi-commercial scale in recirculating systems seem to suggest that this species is more amenable to triploidy than Atlantic salmon with respect to growth and survival (Crouse et al. 2023). Today, rainbow trout farming makes up less than 10% of Norwegian aquaculture.

All-female lines of Atlantic salmon are already used in recirculating aquaculture systems in Norway. All-female lines of farmed Atlantic salmon will still meet conspecific wild males if they escape or meet farmed males from other populations that have escaped from net pens at sea. All-female lines of rainbow trout could be considered functionally sterile if all farmed rainbow trout were female. But as long as we do not know why rainbow trout rarely form self-sustaining populations in Norway (Hindar et al. 1996), this would be a precarious form of sterility as wild fertile males could exist on a small scale. If some of the aquaculture production in Norway was shifted from Atlantic salmon to rainbow trout, an increased propagule pressure from escaped rainbow trout should be expected. This would increase risks of ecological effects.

Interspecific hybrids have been widely used in stocking of salmonid fishes for sport fishing in North America often involving triploidy to secure no genetic introgression to native salmonids. The level of development is limited with respect to performance in commercial aquaculture, as to our knowledge, only freshwater growth and mortality of interspecific hybrids have been studied in Norway.

4.2 Efficacy

Most methods for achieving sterility are at an early stage of development and most of the estimates of successful sterility are based on very low numbers of individuals and studies. The limited available data makes it difficult to conclude on their efficiency and potential side effects.

Gene silencing or knockdown by morpholino or Gapmer oligonucleotide microinjection have a potential to cause nearly 100% sterility. This seems to be the most promising method, since it is functional for the treated individuals, but sterility is not inherited by the next generation. Tveiten et al. (2023) achieved up to 80% sterility in Atlantic salmon eggs microinjected with *dnd*-Gapmer, in an experiment where they followed 2000 microinjected eggs to the smolt and adult stages. Microinjection is prone to errors and occasionally high egg mortality occurs. This method has not yet reached large scale production.

Immersion of eggs into a solution to transfer *dnd*-MO or *dnd*-Gapmer or CRISPR constructs into the egg (or embryo) is likely a more suitable method when scaling up. So far, proof of principle of achievement of gene silencing has been shown in bath-immersion with *dnd*-MO-Vivo of unfertilized coho salmon eggs (Xu et al. 2023), but at a low rate of success both with respect to the amount of surviving eyed eggs (1-64%) and proportion of sterile females at 14-20 months of age (2-10%).

Vaccines for achieving sterile fish is currently at a development stage where sterilisation seems to be less successful in aquaculture compared to in mammals, both in terms of efficiency and the ability to sustain lifelong sterility. The number of other necessary vaccines in fish farming may pose a challenge to the success of this immunosterilisation approach, since frequent vaccination and handling of fish is stressful with negative effects on fish welfare.

Low androgen treatment is a well-developed method for monosex induction. However, sterility is not induced. If used for species with a small likelihood of reproducing in the neighboring ecosystem, this strategy can be used. Aquaculture of all-female rainbow trout is one example. This strategy has very low possibility for introgression, as rainbow trout is a spring spawner and Atlantic salmon an autumn spawner, and when artificially spawned, there is no survival to hatch for Atlantic salmon-by-rainbow trout hybrids (Devlin et al. 2021). However, increased rainbow trout production needs to be analysed with respect to the possibility of establishment of self-reproducing rainbow trout populations (Hindar et al. 1996) and ecological effects of escaped farmed rainbow trout. Interspecific hybrids (and interspecific triploid hybrids) are used in stocking in North America. These were abandoned long ago for aquaculture in Norway because those crosses were not optimal for Norwegian environmental conditions. Efficacy of breeding has not been determined in recent experiments, but hybrids between triploid Atlantic salmon and brown trout may have a faster freshwater growth compared to parental species (Fraser et al. 2021). However, after 293 days of seawater rearing Fraser et al. (2022) concluded that Atlantic salmon x brown trout triploid hybrids had similar growth to triploid Atlantic salmon and also suffer from the same welfare issues.

4.3 Implications for genetic impact on wild salmon populations

The current open net-pen technology that is dominating Norwegian aquaculture is associated with escapes of farmed salmon interbreeding with wild salmon. Methods that lead to fewer reproductively capable farmed escapes will therefore reduce the threat caused by farmed-to-wild genetic introgression.

Sterility methods will not have to be perfect to achieve results. For example, a method achieving 80% sterile individuals would be 80% as good as total sterility. We expect that the remaining 20% are like normal farmed fish and not individuals that could be unknown diploid carriers of a sterility allele. Therefore, large-scale methods not altering the genome but only affecting the early-stage development and with life-long sterility in the farmed fish, could be a useful way of reducing genetic impact on wild salmon population.

4.4 Other positive and negative effects on biodiversity

We know little of the effects on biodiversity if a large number of sterile salmon should escape. For triploid salmon, we know that males develop gonads and participate in spawning and could disrupt diploid salmon's spawning success by not contributing to any offspring. Female triploid

salmon do not develop gonads and do not mature, but a small proportion may enter rivers, as observed for immature triploid salmon. Triploid escaped farmed salmon enter rivers in much lower proportions (0.18%) than estimated from proportion of spontaneous triploid salmon (2.0%) in fish farms (Glover et al. 2016), so their eventual effect in rivers is likely limited and unlikely to exceed the effects of diploid escapees. At present, no experiment has been undertaken to test whether this could be an ecological problem for small fish or other freshwater biodiversity. Escaped farmed triploid salmon that enter rivers may carry disease agents like diploid farmed salmon.

If sterile salmon stay at sea longer than fertile escaped salmon, one might want to know in which marine habitats they stay (open ocean, fjords or other coastal habitats), and how such marine predators could affect marine biodiversity. At present, it is unclear how long sterile farmed salmon may live in sea water (5 years have been indicated, VKM 2023: 20). The current knowledge suggests that their effect in river habitats is limited, based on observation of the behaviour of escaped fertile salmon. If a large-scale accidental escape of sterile salmon occurred, information from controlled experiments could be used for generating informed risk assessments. Likely, predatory effects on smaller fish and other taxa stand out as a potential novelty if large numbers of big and sterile salmon escape. Experiments that could help assess such predatory effects have to our knowledge not been performed.

4.4.1 Can breeding or environmental adjustment improve triploid performance?

Both family and strain differences have been shown in comparative studies of growth performance of triploid Atlantic salmon.

Broodstock of Atlantic salmon in the St. John River, Canada, which had undergone one generation of selection for increased growth, were used to compare growth of diploid and triploid full-sib groups during a 75-week period (Friars et al. 2001). The study showed a higher final body mass of fish in the diploid compared to the triploid groups and a weak correlation between the different ploidies. The authors noted that some families seemed intolerant to triploidy.

Another Canadian study investigated the effects of strain and ploidy on growth performance of salmon post-smolts. They found no effect of ploidy on specific growth rate for the Norwegian farmed Atlantic salmon strain Mowi, which tended to be intermediate between wild and farmed salmon of the St. John River strain during a 12-week trial (Sacobie et al. 2012). The lowest growth rate in the experiment was observed for a triploid salmon of another Canadian farmed salmon strain. The study concluded that there can be significant variation among strains for triploid specific growth rate. Based on several reports, including Taylor et al. (2013) who reported that diploid and triploid Atlantic salmon within the same family showed similar performance in weight, length and other commercially important harvest traits, VKM (2023: 22) concluded that family ranking of performance indicators is generally similar between different ploidies, suggesting current breeding programs for diploids can also be beneficial for triploids. In line with this, a study on rainbow trout suggests that selective breeding for growth performance based on diploid individuals is expected to effectively improve performance in triploids (Leeds and Weber 2019).

We are not aware of breeding programs currently operating with the purpose of producing the best-performing triploid salmon. A breeding program is possible given that both production and health and welfare traits are recorded for triploid sibs of the diploids used in the breeding program (Friars et al. 2001). Currently, the biggest hurdle for such a program is the weak understanding of the mechanisms of the health and welfare problems of triploid salmon and the lack of estimates of genetic parameters of stress tolerance, health and welfare in triploid salmon. This needs to be in place to benefit from developments in establishing genotyping and statistical tools in a breeding program for improved health and welfare of triploids from diploid parents (Grashei et al. 2020, Roche et al. 2024).

A breeding program for triploid salmon will take several 3-year generations to lead to significant progress in performance. Knowledge on genetic parameters and the likely difference in the breeding objective (including relative economic values) for triploids compared to diploids is lacking for Norwegian farmed salmon populations (see also VKM 2023: 22). On a shorter time scale, adjusting diet and rearing conditions is probably an easier option for increased performance, health and welfare of triploid salmon compared to achieving this with a breeding program.

VKM (2023: 22) assessed several mitigating measures and uncertainties with respect to welfare and health issues of triploid farmed salmon. They concluded that many of the differences in health and welfare can be fully or partially mitigated if conditions are optimized for triploids. They also noted that some of the necessary adjustments are not feasible under the present commercial farming conditions (VKM 2023:22). Open net-pen technology implies uncontrolled transmission of disease agents and unpredictable temperature and oxygen conditions that challenge the health and welfare of triploid farmed salmon. Short-term solutions to health and welfare problems in triploid Atlantic salmon are likely a reduction in the density of farmed salmon or a change to all-female or triploid rainbow trout or other farmed species (if low impact on other fish species can be confirmed if escape occurs). In addition, considering the use of closed farming systems, as an extra safety level would reduce escapes.

4.4.2 Fish health and welfare

Infectious diseases and infection pressure from salmon farming is a non-stabilised expanding population threat to wild salmon (VRL 2024). Changes in the infection status, health and welfare in the farmed population is therefore highly relevant for biodiversity by its impact on the health of wild fish.

There is lack of data concerning impact on pathogen susceptibility, fish health and welfare for most or all of the methods presented in this report. Suggestions of differences in growth, survival or performance observed from the field or in small laboratory-based research including few fish with low or skewed representativeness cannot lay the ground for an appropriate risk assessment on the impact of sterility on fish health and welfare. Sterility due to triploidisation is the best studied method with respect to aquaculture. Sterile fish generated with other methods need to be scrutinised both in laboratory-based research and in large-scale research, under relevant farming conditions, to examine pros and cons with regards to fish health and fish welfare.

Development of a method to produce robust sterile salmon that maintain good health and welfare during the prevailing standard farming conditions is crucial. The Norwegian aquaculture industry reported that 62.8 million salmon and 2.5 million rainbow trout died during the sea phase in aquaculture in 2023 (Sommerset et al. 2024). Infectious diseases, along with ulcerations and wounds of the skin and gills as a result of frequent and stressful non-medicinal delousing operations, are the two most important causes of loss (Sommerset et al. 2024). The standard open-net-pen farming conditions that render farmed fish in close interaction with water borne pathogens from neighbouring farms and the environment are, in general, not able to safeguard the health and welfare of farmed fish. Closed containment systems that prevent introduction of salmon lice and eliminate delousing operations could improve health, welfare and survival of both sterile and fertile salmon.

The indirect impact of the method used to achieve sterility on welfare, health and mortality must also be considered. Onset of puberty and sexual maturation can for instance affect the immune system and overall health due to the interaction between reproductive hormones and immune responses (Harris and Bird 2000). Induction of sterility by methods that simultaneously hinder the onset of sexual maturation or expression of gender-associated traits, may thus have a positive impact on health and welfare of farmed fish.

To be effective with regards to hindering genetic introgression, a large proportion of the farmed Atlantic salmon have to be sterile. Currently, the number of farmed salmonids living in open net-pens along the coast is approximately 450 million individuals. All of these are exposed to pathogens before they enter the sea and during the whole period they are reared in the sea. Even a small systematic change in susceptibility or resistance to infectious diseases due to intrinsic or extrinsic factors will thus have a significant impact on overall health and survival of a large number of individuals in the aquaculture industry.

5 Uncertainties

- Several approaches to introduce sterility have been proven successful in zebrafish in the laboratory, however lower success rate has been achieved when applying these methods in salmonids.
- Limited and variable efficiency has been observed for methods like immunosterilisation, including low efficiency both by the target proteins to induce autoimmunity and the delivery strategy. There is currently no vaccination strategy for sufficient antigen delivery at optimal life stages for best immunological effect.
- Sterilisation using Morpholino or Gapmer oligonucleotides and transient knockdown by immersion may become a powerful strategy in the future but has so far shown limited success rate in salmonid eggs. The potential efficiency of several available techniques is therefore uncertain for salmonids.
- The welfare of salmonids in large scale production using techniques is only applied at research level. Even rare anomalies will occur in a high number of individuals when several hundred million salmon are produced.
- The longevity of the effect using transient sterilisation methods is uncertain regarding life-long results.
- Whether different methods of achieving sterility may result in fish with varying behaviours, with some techniques potentially introducing more alterations than just the loss of gametes.

6 Conclusions (with answers to the terms of reference)

Wild Atlantic salmon populations in Norway have been under continuous pressure for decades by genetic influence and other negative consequences relating to escaped salmon from aquaculture. Ongoing climate change suggests there will be a deteriorating situation for wild Atlantic salmon, including increases in the pressure from sea-lice and other infectious disease agents. It is anticipated that the changing climate will drive introduction and establishment of new pathogens and improve the living conditions for several existing pathogens. This is expected to have a negative impact on both farmed and wild salmonids and increase the vulnerability of wild populations to any unwanted effects caused by escaped farmed salmon.

VKM's assessment with respect to **the Terms of Reference** is given in italics below:

6.1 ToR 1: Biological diversity with the use of salmonids sterilised by triploidisation

A) Describe the effectiveness of the triploidisation process.

Triploidisation is effective in preventing reproduction in the wild. Triploidy can be induced by heat shock of eggs, or more commonly, hydrostatic pressure shock shortly after fertilisation. The treatment commonly results in near 100% triploidy, with 70-90% survival relative to diploid fertilisation until hatching.

B) Describe the extent to which fertile and triploid farmed fish are found in the environment and assess the effects this has on wild salmon stocks.

Escaped farmed Atlantic salmon are common in many rivers in Norway and other salmon farming countries. This is an international environmental concern. Introgression of farmed to wild salmon has been documented in many rivers in Norway, where investigations have documented genetic changes in two thirds of wild salmon populations due to interbreeding between escaped farmed salmon and wild salmon. Experiments and field studies show that genetic introgression from farmed to wild salmon leads to changes in traits in wild salmon such as age at smoltification and maturation, growth rate, timing of migration, straying rate, and ultimately, fitness and wild population productivity and viability.

Triploid farmed salmon have been found among farmed escapes in rivers. Studies based on identifying spontaneous triploid salmon unknowingly used in aquaculture show that their proportion in fish farms (2%) is roughly ten times as high as their proportion among escaped farmed salmon in rivers (0.2%). This suggests, together with experience from releases of triploid salmon, that fewer triploid than diploid farmed salmon end up in rivers, if they escape. Triploid males develop gonads and secondary sexual characteristics and may enter rivers and participate in spawning (with no reproductive success). The use of all-female triploid farmed salmon in aquaculture would prevent spawning behaviour of triploids and prevent genetic effects on wild salmon.

C) Assess whether there are other positive or negative effects on biological diversity related to the use of triploid salmonids, compared to traditional farming.

Triploid Atlantic salmon have welfare issues in aquaculture including lower environmental tolerance and higher susceptibility to some disease agents. This may increase epidemiological problems related to the potentially higher transmission of viruses and bacteria between fish farms and from farmed to wild fish.

Effects on biodiversity in rivers are expected to be reduced by use of all-female triploid Atlantic salmon in salmon farming, because fewer triploid than diploid salmon escapees enter rivers. The lower proportion may be because female triploid salmon rarely enter rivers and that the mortality of triploid salmon in nature is expected to be higher than that of diploid salmon.

6.2 ToR 2: Biological Diversity with the Use of Alternative Methods for the Production of Sterile Farmed Fish.

A) Describe the status if it has previously been investigated whether it is possible to further develop methods for triploid salmon production through breeding or other measures, so that triploid farmed salmon achieve the same or better welfare as diploid salmon.

We are not aware of any existing breeding program on triploid Atlantic salmon. It has been generally established that improved performance in diploid salmon may also imply an improvement of the same performance traits (length and weight) in triploid salmon. However, there is limited knowledge on the traits that cause reduced health and welfare for triploid farmed salmon, and the necessary genetic parameters for health- and welfare traits in triploid salmon are not currently known. A breeding program for improved welfare of triploid farmed fish would need several generations to yield results and would also need incentives to be realised.

One method to improve the production of triploid fish includes producing triploid offspring by fertilisation of diploid eggs by tetraploid sperm. In theory, the process should reduce the mortality and stress imposed by the pressure shock method. However, this method has been used on an experimental scale for 30 years with limited success for Atlantic salmon.

In the short perspective, better welfare for triploid fish in aquaculture is more likely to be achieved by improving farming conditions.

B) Provide an overview of other methods intended for the sterilisation of salmonids for aquaculture, as well as the current state of knowledge for these methods.

Several alternative methods exist that can produce sterile farmed fish. Common to most of these methods is that they are still at the experimental stage, and they show variable success with respect to reaching sterility. In this report, we have evaluated methods that are based on permanent genomic changes for achieving sterility, methods that reduce germ cell production without genomic changes, and other methods including vaccination and hormonal treatment. Methods that are based on a temporal knock down of germ cell formation by immersion of eggs in a solution containing antisense RNA seem to be the most promising with respect to efficiency and up-scaling for large-scale use in aquaculture. This is because egg immersion may be

used on thousands of eggs in each batch, and the induced changes are not genetically permanent, and can therefore not be inherited by wild fish populations.

C) Assess the potential for genetic impact on wild fish stocks from the use of salmonids farmed using the methods identified under point 2B. *Some of the evaluated methods, like temporal gene knockdown, are less effective in producing total sterility compared to triploidisation methods. Escapes from such a population will however still pose a reduced risk of genetic impact on wild salmonids compared to farming of 100% fertile fish*

Methods that rely on the use of a high number of actual or potential heterozygote sterile/fertile individuals to produce sterile/sterile homozygotes are considered to result in a higher threat to wild salmon genetic integrity, should they escape. This is based on the demand for a highly reliable method to confirm correct genotyping and selection of each individual for large-scale production. Genetically homozygous sterile parents with restored fertility (physiologically fertile due to rescue) will pose a large genetic impact if escaping by potentially transferring sterility allele(s) to wild populations. If the production of the sterile offspring takes place at land-based contained facilities, this would pose a lower genetic risk to wild salmon.

D) Assess whether there are other positive or negative effects on biological diversity related to the farming of salmonids using the methods identified under point 2B. *At the present state of knowledge for most methods to produce sterile salmon, effects on behavioural (competition, predation, mating behaviour, longevity etc.) and on biological diversity have not been demonstrated and can therefore not be risk assessed. As information on pathogen susceptibility or general welfare under aquaculture conditions is lacking, these issues are not possible to risk assess for methods still at the experimental level. Regarding Atlantic salmon, the use of all-female populations may increase welfare, since female Atlantic salmon are shown to be less susceptible to infections compared to males. A combination of triploidisation and all females would result in fewer individuals entering rivers in case of escape and likely in a lower risk of pathogen transmission in the river than if keeping a mixed-sex triploid population. All females and sterility using other methods could potentially give the same results of fewer individuals entering rivers, but there is no available information regarding this at present.*

7 Data gaps

VKM has identified the following data gaps in relation to this assessment:

- There is a lack of data from experiments on early life stages of sterile fish generated by pressure induced triploidisation.
- Knowledge on traits that lead to reduced health and welfare of triploid salmon is lacking and there is no information on genetic parameters for breeding better triploid salmon with respect to health and welfare.
- Alternative methods for generation of sterile Atlantic salmon presented in this VKM report are still at an early stage of development. Data on pathogen susceptibility, health and welfare under controlled closed containment conditions and realistic large-scale aquaculture conditions is lacking.

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Appendix I

Litteratursøk fra Bibliotek for helseforvaltningen

Dokumentasjon av søkestrategi

Hvilke metoder finnes for å sterilisere oppdrettsfisk?

Kontaktperson: Ville Erling Sipinen og Martin Malmstrøm
Søk: Trude Anine Muggerud
Fagfelle: Marita Heintz
Kommentar: 2 av 2 søk, dette med fokus på metoder for sterilisering.
Dublettsjekk i EndNote: Før dublettkontroll: 873
Etter dublettkontroll: 541

Hvilket spørsmål skal litteratursøket besvare?			
Hvilke metoder brukes/kan brukes til masseproduksjon av steril oppdrettsfisk fortrinnsvis laksefisk (Salmonidae), inkludert atlantisk laks (Salmo salar)? Er det en av metodene brukt til sterilisering av oppdrettslaks som utmerker seg med tanke på effektivitet (sterilisering, masseproduksjon, kvalitet og fiskevelferd).			
Spørsmålet i PICO-format			
Population (pasient)	**Intervention (tiltak)**	**Comparison (sammenligning)**	**Outcome (utfall)**
Salmon, salmonids, farmed fish, aquaculture, Salmo salar, Salmonidae	sterilisation, sterile fish production	CRISPR/CAS9, CRISPR, TALEN, Morpholino, Knock-out, Zink finger, Triploidisation, mutation, microinjection, siRNA, dead-end, dnd, vasa, antisense oligos, ASO, Vivo, MO immersion, transgenic	
Kjente relevante studier			
[Reproductive sterility in aquaculture: A review of induction methods and an emerging approach with application to Pacific Northwest finfish. Lan Xu, Mingli Zhao, Jun Hyung Ryu, Edward S. Hayman, William T. Fairgrieve, Yonathan Zohar, J. Adam Luckenbach, Ten-Tsao Wong. 20 July 2022. https://doi.org/10.1111/raq.12712]			

Database: **CAB Abstract**
Dato: 10.01.24
Antall treff: 354

1	Salmon/ or exp Salmonidae/ or Aquaculture/ or Fish culture/ or Salmon culture/ or (salmon or "salmo salar" or "farmed fish" or (fish adj (farming or ranching)) or pisciculture or "pisci culture" or Salmonidae or salmonoidea or salmon?ids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus or aquaculture or aquafarming or (aqua adj (farming or culture))).ti,ab,id.	140350
2	Sterilization/ or (Sterili#ation or sterili#es or sterili#ing or sterile or sterility).ti,ab,id.	81321
3	CRIPR-Cas9/ or Mutations/ or Small interfering RNA/ or Transgenics/ or (CRISPR or "clustered regularly interspaced short palindromic repeats" or Cas9 or Talen or "Transcription activator like effector nuclease" or Morpholino or "knock out" or knockout or ((zink or zinc) adj finger) or zinkfinger or zincfinger or mutation? or microinjection? or "micro injection?" or siRNA or (("small interfering" or "short interfering" or silencing) adj RNA) or "dead end" or deadend or Dnd or vasa or "antisense	479440

FHI

Litteratursøk fra Bibliotek for helseforvaltningen

Dokumentasjon av søkestrategi

	oligos" or ASO? or vivo or "mo immersion" or transgenic? or aquaculture or aquafarming or (aqua adj (farming or culture))).ti,ab,id.	
4	1 and 2 and 3	354

Database: **Web of Science Core Collection: Science Citation Index Expanded (SCI-EXPANDED) --1987-present, Social Sciences Citation Index (SSCI) --1987-present, Arts & Humanities Citation Index (A&HCI) --1987-present, Emerging Sources Citation Index (ESCI) --2015-present**
Dato: 10.01.24
Antall treff: 418

1	TS=("salmon" OR "salmo salar" OR "farmed fish" OR ("fish" NEAR/0 ("farming" OR "ranching")) OR "pisciculture" OR "pisci culture" OR "Salmonidae" OR "salmonoidea" OR "salmon$ids" OR "Coregonus" OR "Hucho" OR "Oncorhynchus" OR "salmo trutta" OR "trout" OR "Salvelinus" OR "thymallus" OR "aquaculture" OR "aquafarming" OR ("aqua" NEAR/0 ("farming" OR "culture")))	165396
2	TS=("Sterili?ation" or "sterili?es" or "sterili?ing" or "sterile" or "sterility")	97508
3	TS=("CRISPR" OR "clustered regularly interspaced short palindromic repeats" OR "Cas9" OR "Talen" OR "Transcription activator like effector nuclease" OR "Morpholino" OR "knock out" OR "knockout" OR (("zink" OR "zinc") NEAR/0 "finger") OR "zinkfinger" OR "zincfinger" OR "mutation$" OR "microinjection$" OR "micro injection$" OR "siRNA" OR (("small interfering" OR "short interfering" OR "silencing") NEAR/0 "RNA") OR "dead end" OR "deadend" OR "Dnd" OR "vasa" OR "antisense oligos" OR "ASO$" OR "vivo" OR "mo immersion" OR "transgenic$" OR "aquaculture" OR "aquafarming" OR ("aqua" NEAR/0 ("farming" OR "culture")))	2705926
4	#3 AND #2 AND #1	418

Database: **Scopus**
Dato: 10.01.24
Antall treff: 101

#4	#1 and #2 and #3	101
#3	TITLE-ABS((CRISPR or "clustered regularly interspaced short palindromic repeats" or Cas9 or Talen or "Transcription activator like effector nuclease" or Morpholino or "knock out" or knockout or ((zink or zinc) PRE/0 finger) or zinkfinger or zincfinger or mutation or mutations or microinjection or microinjections or "micro injection" or "micro injections" or siRNA or (("small interfering" or "short interfering" or silencing) PRE/0 RNA) or "dead end" or deadend or Dnd or vasa or "antisense oligos" or ASO or ASOs or vivo or "mo immersion" or transgenic or transgenics)) OR AUTHKEY((CRISPR or "clustered regularly interspaced short palindromic repeats" or Cas9 or Talen or	2,634,717

FHI

	"Transcription activator like effector nuclease" or Morpholino or "knock out" or knockout or ((zink or zinc) PRE/0 finger) or zinkfinger or zincfinger or mutation or mutations or microinjection or microinjections or "micro injection" or "micro injections" or siRNA or (("small interfering" or "short interfering" or silencing) PRE/0 RNA) or "dead end" or deadend or Dnd or vasa or "antisense oligos" or ASO or ASOs or vivo or "mo immersion" or transgenic or transgenics))	
#2	TITLE-ABS((Sterilisation or sterilization or sterilises or sterilizes or sterilising or sterilizing or sterile or sterility)) OR AUTHKEY ((Sterilisation or sterilization or sterilises or sterilizes or sterilising or sterilizing or sterile or sterility))	160,047
#1	TITLE-ABS((salmon or "salmo salar" or "farmed fish" or (fish PRE/0 (farming or ranching)) or pisciculture or "pisci culture" or Salmonidae or salmonoidea or salmonids or salmonoids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus or aquaculture or aquafarming or (aqua PRE/0 (farming or culture)))) OR AUTHKEY ((salmon or "salmo salar" or "farmed fish" or (fish PRE/0 (farming or ranching)) or pisciculture or Salmonidae or salmonoidea or "pisci culture" or salmonids or salmonoids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus or aquaculture or aquafarming or (aqua PRE/0 (farming or culture))))	158,747

Appendix II

Litteratursøk fra Bibliotek for helseforvaltningen

Dokumentasjon av søkestrategi

Hvilke miljøeffekter kan sterilisering av oppdrettsfisk ha?

Kontaktperson:	Ville Erling Sipinen og Martin Malmstrøm
Søk:	Trude Anine Muggerud
Fagfelle:	Marita Heintz
Kommentar:	1 av 2 søk, dette med fokus på miljøeffekter.
Dublettsjekk i EndNote:	Før dublettkontroll: 650
	Etter dublettkontroll: 201

Hvilket spørsmål skal litteratursøket besvare?			
Hvilke positive og negative effekter kan (masse)produksjon av sterile oppdrettsfisk (Salmonidae) ha for biologisk mangfold og villaksstammer (v/rømming fra anlegg, smitteoverføring, predasjon på yngel m.m.)?			
Spørsmålet i PICO-format			
Population (pasient)	**Intervention (tiltak)**	**Comparison (sammenligning)**	**Outcome (utfall)**
Sterile salmonids, sterile salmon, sterile farmed fish	Aquaculture, fish production		Environmental effects, biodiversity, introgression, genetic effects, ecological impact, wild salmon, competition, predation, disease transmission, fitness, health, immunology, welfare
Kjente relevante studier			
[Reproductive sterility in aquaculture: A review of induction methods and an emerging approach with application to Pacific Northwest finfish. Lan Xu, Mingli Zhao, Jun Hyung Ryu, Edward S. Hayman, William T. Fairgrieve, Yonathan Zohar, J. Adam Luckenbach, Ten-Tsao Wong. 20 July 2022. https://doi.org/10.1111/raq.12712]			

Database: **CAB Abstract**
Dato: 10.01.24
Antall treff: 215

1	Salmon/ or exp Salmonidae/ or Aquaculture/ or Fish culture/ or Salmon culture/ or (salmon or "salmo salar" or "farmed fish" or (fish adj (farming or ranching)) or pisciculture or "pisci culture" or Salmonidae or salmonoidea or salmon?ids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus or aquaculture or aquafarming or (aqua adj (farming or culture))).ti,ab,id.	140350
2	Sterilization/ or (Steril#ation or steril#es or steril#ing or sterile or sterility).ti,ab,id.	81321
3	exp environmental impact/ or Biodiversity/ or Introgression/ or Genetic effects/ or Biological competition/ or Interspecific competition/ or Intraspecific competition/ or exp predation/ or exp disease transmission/ or Fitness/ or Health/ or Animal welfare/ or Immunology/	648423
4	(((environmental or ecological) adj (impact? or effect? or consequence? or outcome? or reaction? or footprint?)) or (("life cycle" or lifecycle) adj (assessment? or analys#s)) or "cradle to grave analys#s" or "carbon footprint" or Biodiversity or "bio diversity" or (biological adj (diversity or variability)) or "diversity	1741541

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Litteratursøk fra Bibliotek for helseforvaltningen

Dokumentasjon av søkestrategi

	of life" or introgression or (genetic adj (effect? or impact? or consequence? or outcome? or reaction?)) or ((biological or intraspecific or interspecific) adj competition) or "wild salmon" or predation or "disease transmission" or infection? or fitness or health or welfare or wellbeing or "well being" or immunology or triploidisation).ti,ab,id.	
5	3 or 4	1964352
6	1 and 2 and 5	215

Database: **Web of Science Core Collection: Science Citation Index Expanded (SCI-EXPANDED) --1987-present, Social Sciences Citation Index (SSCI) --1987-present, Arts & Humanities Citation Index (A&HCI) --1987-present, Emerging Sources Citation Index (ESCI) --2015-present**
Dato: 10.01.24
Antall treff: 217

1	TS=("salmon" OR "salmo salar" OR "farmed fish" OR ("fish" NEAR/0 ("farming" OR "ranching")) OR "pisciculture" OR "pisci culture" OR "Salmonidae" OR "salmonoidea" OR "salmon$ids" OR "Coregonus" OR "Hucho" OR "Oncorhynchus" OR "salmo trutta" OR "trout" OR "Salvelinus" OR "thymallus" OR "aquaculture" OR "aquafarming" OR ("aqua" NEAR/0 ("farming" OR "culture")))	165,396
2	TS=("Sterili?ation" or "sterili?es" or "sterili?ing" or "sterile" or "sterility")	97,508
3	TS=((("environmental" OR "ecological") NEAR/0 ("impact$" OR "effect$" OR "consequence$" OR "outcome$" OR "reaction$" OR "footprint$")) OR (("life cycle" OR "lifecycle") NEAR/0 ("assessment$" OR "analys?s")) OR "cradle to grave analys?s" OR "carbon footprint" OR "Biodiversity" OR "bio diversity" OR ("biological" NEAR/0 ("diversity" OR "variability")) OR "diversity of life" OR "introgression" OR ("genetic" NEAR/0 ("effect$" OR "impact$" OR "consequence$" OR "outcome$" OR "reaction$")) OR (("biological" OR "intraspecific" OR "interspecific") NEAR/0 "competition") OR "wild salmon" OR "predation" OR "disease transmission" OR "infection$" OR "fitness" OR "health" OR "welfare" OR "wellbeing" OR "well being" OR "immunology" OR "triploidisation")	5,515,670
4	#1 AND #2 AND #3	217

Database: **Scopus**
Dato: 10.01.24
Antall treff: 218

#4	#1 and #2 and #3	218
#3	TITLE-ABS((((environmental or ecological) PRE/0 (impact or impacts or effect or effects or consequence or consequences or outcome or outcomes or reaction or reactions or footprint or footprints)) or (("life cycle" or lifecycle) PRE/0 (assessment or assessments or analysis or analyses)) or "cradle to grave analysis" or "cradle to grave analyses" or "carbon footprint" or Biodiversity or "bio diversity" or (biological PRE/0	6,967,835

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	(diversity or variability)) or "diversity of life" or introgression or (genetic PRE/0 (effect or effects or impact or impacts or consequence or consequences or outcome or outcomes or reaction or reactions)) or ((biological or intraspecific or interspecific) PRE/0 competition) or "wild salmon" or predation or "disease transmission" or infection or infections or fitness or health or welfare or wellbeing or "well being" or immunology or triploidisation)) OR AUTHKEY((((environmental or ecological) PRE/0 (impact or impacts or effect or effects or consequence or consequences or outcome or outcomes or reaction or reactions or footprint or footprints)) or (("life cycle" or lifecycle) PRE/0 (assessment or assessments or analysis or analyses)) or "cradle to grave analysis" or "cradle to grave analyses" or "carbon footprint" or Biodiversity or "bio diversity" or (biological PRE/0 (diversity or variability)) or "diversity of life" or introgression or (genetic PRE/0 (effect or effects or impact or impacts or consequence or consequences or outcome or outcomes or reaction or reactions)) or ((biological or intraspecific or interspecific) PRE/0 competition) or "wild salmon" or predation or "disease transmission" or infection or infections or fitness or health or welfare or wellbeing or "well being" or immunology or triploidisation))	
#2	TITLE-ABS((Sterilisation or sterilization or sterilises or sterilizes or sterilising or sterilizing or sterile or sterility)) OR AUTHKEY ((Sterilisation or sterilization or sterilises or sterilizes or sterilising or sterilizing or sterile or sterility))	160,047
#1	TITLE-ABS((salmon or "salmo salar" or "farmed fish" or (fish PRE/0 (farming or ranching)) or pisciculture or "pisci culture" or Salmonidae or salmonoidea or salmonids or salmonoids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus or aquaculture or aquafarming or (aqua PRE/0 (farming or culture)))) OR AUTHKEY ((salmon or "salmo salar" or "farmed fish" or (fish PRE/0 (farming or ranching)) or pisciculture or Salmonidae or salmonoidea or "pisci culture" or salmonids or salmonoids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus or aquaculture or aquafarming or (aqua PRE/0 (farming or culture))))	158,747

Appendix III

Litteratursøk fra Bibliotek for helseforvaltningen

Dokumentasjon av søkestrategi

Sterilisering av fisk

Kontaktperson: Ville Erling Sipinen
Søk: Trude Anine Muggerud
Kommentar: Dokumentasjon av et «ekstrasøk» gjort i forbindelse med et søk om sterilisering av fisk gjort i 2024.
Dublettsjekk i EndNote: Før dublettkontroll: 1617
Etter dublettkontroll: 739

Database: **CAB Abstracts <1973 to 2025 Week 03>**
Dato: 21.01.25
Antall treff: 657

1	Salmon/ or exp Salmonidae/ or Salmon culture/ or (salmon or "salmo salar" or Salmonidae or salmonoidea or salmon?ids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus).ti,ab,id.	104314
2	Sterilization/ or (Sterili#ation or sterili#es or sterili#ing or sterile).ti,ab,id.	72401
3	1 and 2	657

Database: **Web of Science Core Collection: Science Citation Index Expanded (SCI-EXPANDED)--1987-present, Social Sciences Citation Index (SSCI)--1987-present, Arts & Humanities Citation Index. (AHCI)--1987-present, Emerging Sources Citation Index (ESCI)--2019-present**
Dato: 21.01.25
Antall treff: 533

1	TS=(salmon or "salmo salar" or Salmonidae or salmonoidea or salmon$ids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus)	126523
2	TS=(Sterilisation or sterilization or sterilises or sterilizes or sterilising or sterilizing or sterile)	91898
3	#1 AND #2	533

Database: **Scopus**
Dato: 21.01.25
Antall treff: 427

1	TITLE-ABS(salmon or "salmo salar" or Salmonidae or salmonoidea or salmonids or salmonoids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus) OR AUTHKEY(salmon or "salmo salar" or Salmonidae or salmonoidea or salmonids or salmonoids or Coregonus or Hucho or Oncorhynchus or "salmo trutta" or trout or Salvelinus or thymallus)	98,406
2	TITLE-ABS(Sterilisation or sterilization or sterilises or sterilizes or sterilising or sterilizing or sterile) OR AUTHKEY(Sterilisation or sterilization or sterilises or sterilizes or sterilising or sterilizing or sterile)	144,190

3	1 and 2	427