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REVIEW

REVIEWS IN Aquaculture

Reproductive sterility in aquaculture: A review of induction methods and an emerging approach with application to Pacific Northwest finfish species

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Abstract

Aquaculture is the fastest-growing food-production sector and is striving to become a long-term sustainable approach to meet the rising global demand for seafood. During the expansion and advancement of aquaculture, minimizing ecological impacts should occur concomitantly with maximizing production. Farmed fish, often genetically distinct from their natural conspecifics, may pose significant risks of genetic contamination and ecological imbalance to wild populations if they escape from aquaculture confinement. Growing reproductively sterile fish is the most effective way to genetically contain farmed fish. Atlantic salmon (*Salmo salar*) escape events in the 'Pacific Northwest' region of the United States and Canada have raised alarms over potential ecological impacts and led to legislation in Washington State phasing out the culture of non-native finfish species. Farming sterile native species such as coho salmon (*Oncorhynchus kisutch*) and sablefish (*Anoplopoma fimbria*) in the Pacific Northwest would ease public concerns and promote environmentally and economically sustainable aquaculture. Sterile fish also can mitigate the challenge of precocious maturation, a prominent issue associated with culture of salmonids and many other species, to improve somatic growth, flesh quality and fish health and welfare. Here, we review methods having potential applications for producing sterile fish and introduce our novel immersion-based technology that temporarily silences the *dead end* (*dnd*) gene using Morpholino oligonucleotides to produce sterile coho salmon and sablefish for the first time. The successful induction of sterility in these two iconic Pacific Northwest species without introducing genetic modifications would promote the use of this immersion-based sterilization technology for more aquaculture finfish worldwide.

KEYWORDS
genetic containment, germ cell development, Morpholino-Vivo, precocious maturation, reproductively sterile fish, sustainable aquaculture

1 | INTRODUCTION

With global demand for seafood ever-increasing, wild fish stocks are under the growing pressure of overfishing, which accelerates biodiversity loss and compromises the long-term sustainability of fisheries resources.[1,2] Aquaculture is the fastest-growing food-production sector and is striving to become one of the long-term sustainable ways to meet rising seafood and nutrition demands.[3] Global aquaculture production of fish was estimated to have reached 82 million tonnes, valued at USD 250 billion, exceeding capture fisheries utilized for human consumption in 2018, and is expected to surpass total capture fisheries production in the next few years.[4] Farming selectively-bred, non-native and sometimes genetically-engineered fish with superior performance traits such as fast growth and disease resistance can increase aquaculture yields, making them preferable to industry. Nevertheless, expansion and optimization of aquaculture should focus not only on maximizing production, but also on minimizing ecological impacts, thereby achieving long-term environmental sustainability of our seafood supplies.

Farmed fish, often genetically distinct from their natural conspecifics, pose potential maladaptive changes to genetic diversity and ecological balance in wild stocks if they escape and reproduce.[5,6] Growing reproductively sterile fish is the most effective way to genetically contain aquaculture fish since the sterile escapees will neither proliferate nor interbreed with wild populations. Sterile fish also carry the additional commercial advantage of preventing precocious maturation, a major challenge in many farmed fish species, especially in males (reviewed by Taranger et al.[7]). That is, when sexual maturation occurs before fish reach marketable size, energy and resources are routed to gonadal development, gametogenesis, development of secondary sexual characteristics and reproductive behaviours, potentially resulting in compromised somatic growth, health, flesh quality and animal welfare.[7–9] Deterioration of fish growth and health associated with precocious maturation can be precluded by reproductive sterilization through minimization of energy dedicated to gonad development.[10,11] Moreover, the release of sterile male fish can be used to control invasive fish species in the natural environment.[12,13] In addition, sterility protects breeders' intellectual property rights by making specific proprietary strains of fish infertile to safeguard against unauthorized breeding.

Attempts to induce sterility in fish using gonadectomy and radiation[14,15] have a history dating back to the 1950s.[14,15] Nowadays, the most common and practical approach for producing sterile fish on a commercial scale is through triploidization. Triploidy is generally induced by applying a 'shock' during the second meiotic division, shortly after fertilization, to retain one extra set of maternal chromosomes, which in turn impairs meiosis during gametogenesis and renders the fish sterile[16] (see Section 3.1.1). However, limitations exist with triploid applications in aquaculture and generally include difficulty obtaining 100% triploidy induction and occurrence of some gonadal development in triploid male fish, though most resulting sperm would be aneuploid and incapable of producing viable offspring.[16] Furthermore, triploids are generally more sensitive than their diploid counterparts to suboptimal rearing conditions (e.g., elevated temperature, high rearing densities, poor water quality). They may also exhibit lower survival, especially in early development, and higher rates of jaw and/or skeletal deformities, which have been linked to nutritional deficiencies in some species.[16,17] With global climate change expected to further alter environmental conditions and create suboptimal conditions in many cases, the use of triploids in aquaculture may be hindered in the future.

The recent two decades have witnessed considerable advancements in molecular and genetic tools for application in aquaculture, and many emerging strategies take advantage of transgenesis and genome editing (i.e., genetic engineering) to induce sterility.[18,19] However, there are three major challenges and limitations for these evolving technologies when applied for producing sterile fish. The first challenge is how to solve the paradox of producing genetically sterile fish for multiple generations (discussed in Section 3.2.1 below). The second limitation is that the function of the targeted genes can be plastic in different fish species, thus compromising the universality of the method. The third relates to regulatory complexity and consumer acceptance of genetically engineered fish. For example, the US Food and Drug Administration's approval of the first transgenic animal for food, the growth hormone transgenic 'AquAdvantage' Atlantic salmon (*Salmo salar*), first engineered in 1989, was delayed until 2015.[20,21]

In light of the limitations associated with triploids, and potential regulatory and consumer resistance hurdles associated with genetically engineered fish, alternative technologies that are highly effective and practical without introducing genetic modifications are needed. To this end, we have developed an immersion-based technology that temporarily silences the *dead end* (*dnd*) gene via Morpholino oligonucleotides (MO) to achieve 100% sterility in zebrafish (*Danio rerio*).[22] *dnd* is a germplasm component that encodes an RNA-binding protein responsible for primordial germ cell (PGC) development.[23,24] Disruption of *dnd* by MO in many fish leads to sterility.[25–29] The highly conserved role of the *dnd* gene within the PGC and germline developmental mechanism among different fish[30] makes it feasible to apply this gene silencing-based sterilization method to a variety of aquaculture species.

Salmon species are important components of our freshwater and ocean ecosystems, as they are often viewed as crucial indicators of ecosystem health.[31] Salmonids are the most popular finfish consumed in North America and are on the rise globally. Among several species of salmon, farm-raised Atlantic salmon has dominated the marketplace, partly due to successful breeding programs culminating in traits particularly suitable for aquaculture production, such as consistent growth performance, increased disease resistance and high consumer demand. Given the increase in US salmon consumption and lack of alternatives, the import of Atlantic salmon grew from 19.2 thousand tonnes in 1990 to 326.0 thousand tonnes (USD 3.43 billion) in 2018.[32] As demand increased, global production of Atlantic salmon reached 2.6 million metric tons in 2019.[4]

Market domination of Atlantic salmon, their ability to grow well in cold marine waters, and the availability of domesticated strains fostered its development as the preferred species for aquaculture production in Norway, Chile, Scotland and New Zealand, as well as in the coastal waters of Washington State (United States) and British

Columbia (Canada). However, major escape events, such as ~250,000 fish in Washington State in 2017[33] and ~20,000 fish in British Columbia in 2019,[34] renewed ecological concerns about culturing this non-native salmon species in the 'Pacific Northwest' (Washington, Oregon, Alaska and British Columbia) since they could potentially interact with native Pacific salmon or become invasive. As a result, new legislation in Washington State phases out aquaculture of non-native finfish species in 2022 and bans any new Atlantic salmon net-pen operations. Consequently, farming sterile native species has become the most promising approach to improve the sustainability of net-pen finfish aquaculture in this region.

Several Pacific salmon and trout species in the genus *Oncorhynchus* are native to the Pacific Northwest, including coho salmon (*Oncorhynchus kisutch*), Chinook salmon (*Oncorhynchus tshawytscha*), and steelhead/rainbow trout (*Oncorhynchus mykiss*). They support large commercial, recreational and subsistence fisheries. Aquaculture for many *Oncorhynchus* species has been well established in the United States and elsewhere in the world, so these species have great potential for native fish farming in the Pacific Northwest. For example, rainbow trout is the most widely introduced and cultured *Oncorhynchus* species worldwide.[35] For coho salmon, the early success of sea cage culture initiated in Washington State around 1970 was soon followed by the development of coho salmon farming in Chile, now the primary producer of coho salmon in the world.[36] Although it has not reached the level of Atlantic salmon production, coho salmon is well suited for commercial aquaculture because of its short life cycle and high value.[37] Thus, it was selected as a representative Pacific salmonid for this review.

Sablefish or black cod (*Anoplopoma fimbria*) also has a wide distribution in the northeastern Pacific Ocean from Alaska to northern Mexico and is a commercially important species in the Pacific Northwest. They are among the highest-valued finfish per unit weight in Alaska and the United States west coast commercial fisheries due to their high omega-3 fatty acid content and superior quality of white, firm flesh.[38,39] The high economic value and market demand for sablefish coupled with decreases in wild harvests over the last several decades have bolstered commercial aquaculture development for this species.[38] Recently, techniques for establishing all-female monosex stocks,[40] capitalizing on the greater growth potential of females over males, have demonstrated increased economic benefits for commercial aquaculture of sablefish.[41]

Coho salmon, as a representative Pacific salmonid species, and sablefish, as an emerging marine aquaculture species, are potential alternatives to Atlantic salmon for Pacific Northwest aquaculture. Because commercial grow-out for both species may take place in open-water net-pens, there is an urgent need to mitigate threats of genetic contamination of wild populations that could interbreed with aquaculture escapees. Alternatively, there has been increasing interest in developing closed-containment recirculating aquaculture systems (RAS), which can be ascribed in part to their higher bio-security, better control of rearing conditions and ability to dispose of waste properly, for instance, for coho salmon.[37,42] However, the high levels of precocious maturation experienced in RAS for some salmonids (e.g., up to 80% of males[43] and in a rare case, 67% of females[44] by harvest time for Atlantic salmon) can severely compromise their economic profitability. Farming sterile fish would eliminate the possibility of sexual maturation prior to harvest and allow the fish to reach a larger size by extending the potential rearing period. Therefore, the production and use of sterile coho salmon and sablefish in net-pens and RAS would be one of the most effective approaches to address these concerns and promote cost-effective and ecologically responsible aquaculture in the Pacific Northwest. The future success of sterilizing these two species by the novel immersion-based gene-silencing technology would also have implications for the general applicability of sterility induction in other finfish species cultured worldwide, since the essential role of the *dnd* gene in PGC development is highly conserved among fish. Farming sterile native species will help the aquaculture industry mitigate the undesired maturation and reproduction challenges, thereby supporting local and global progress toward sustainable aquaculture.

In this review, we discuss: (1) the main problems associated with undesired maturation and reproduction in aquaculture scenarios, (2) methods for producing sterile fish for aquaculture and their limitations, (3) the novel immersion-based gene-silencing approach for sterilization, with potential application in two representative aquaculture species, coho salmon and sablefish, and (4) the refinement and further applicability of this novel sterilization technology to other aquaculture species.

2 | CHALLENGES ASSOCIATED WITH UNDESIRED MATURATION AND REPRODUCTION IN AQUACULTURE

Successful reproduction is crucial for continuously breeding fish in aquaculture. However, reproductive competency can be detrimental in escape and precocious-maturation scenarios, where reproduction and maturation are undesired. Additionally, it is unfavourable for producers to combat unauthorized reproduction of their proprietary (often selectively-bred) strains that result from long-term development with intensive investment.

2.1 | Escape and introgression

While farming fish species outside their native range has the risk of them becoming invasive should escape occur, farming native species within their natural range significantly increases the risk of interbreeding with wild conspecifics, both of which can be ecologically and adaptively harmful.[45,46] That is, domesticated fish in aquaculture often differ genetically from wild fish through intentional and unintentional selection, such as genetic improvement of commercial traits, domestication selection and loss of genetic diversity due to the limited number of founders and subsequent genetic drift.[6,47] Consequently, farmed and wild fish usually differ in multiple traits, including morphology, behaviour, physiology and life history.[47,48]

For example, compared with wild populations, domesticated Atlantic salmon generally exhibit lower fitness, spawning success and lower relative survival in the wild.[49,50] These differences in fitness-related traits in domesticated fish, should they mate with wild counterparts, can alter the genetic structure of wild populations and reduce overall population fitness, including changing their life-history traits, reducing population productivity and decreasing adaptation to local environmental conditions.[6,48,51–53]

Introgression from farmed Atlantic salmon has been detected throughout its aquaculture range, including Norway,[54] Canada,[55] the United Kingdom[56] and Ireland.[57] For example, a recent and extensive investigation found significant genetic introgression of farmed Atlantic salmon in nearly half of the 109 rivers surveyed in Norway.[6] The continuous interbreeding and introgression from farmed escapees can impart heritable and population-level reductions in genetic variation, lifetime fitness and viability to wild fish.[53]

Transgenic fish are receiving increased attention because of their potential to improve production efficiency and other desirable traits in aquaculture.[58] However, transgenic escapees from aquaculture containment, if fully fertile, could spread transgenes to wild stocks. In this regard, growth-hormone-transgenic male Atlantic salmon have been demonstrated as able to contribute to subsequent generations in competitive spawning trials with nontransgenics, although they displayed delays in early development and maturation and reduced breeding performance.[59–61] A 'Trojan gene' hypothesis, where the escaped transgenic fish with enhanced mating advantage and reduced viability could cause the extinction of local wild populations, was proposed by Muir and Howard.[5] One scenario was demonstrated empirically using a transgenic male medaka (*Oryzias latipes*) possessing a significant mating advantage but survival disadvantage compared with a wild-type male medaka.[62] When these fitness components were included in their model, the transgene was predicted to spread (due to mating advantage) if transgenic individuals escaped to the wild. It was posited that this could ultimately lead to wild population extinction, in this case, due to the viability disadvantage.[62] In addition to the potential risks of transgene introgression into wild conspecifics, Oke et al.[63] demonstrated transmission of a transgene via hybridization from genetically modified Atlantic salmon to wild brown trout (*Salmo trutta*) through experimental crosses. It remains unclear whether the transgene could fully invade the brown trout genome through backcrossing between hybrids and wild brown trout, although the authors suggested that backcrossing is unlikely.

Inadvertent release or escape of farmed fish that are either nonnative, genetically engineered, or genetically divergent from native populations can have deleterious environmental impacts ranging from competition for food and habitats to interbreeding with native fish populations. Accordingly, interbreeding can cause introgression and lead to genetic diversity and integrity losses among wild populations. Therefore, preventing escapes should be incorporated into redundant containment strategies, including physical and reproductive containments. The latter, that is, farming sterile fish, is the best safeguard against introgression[18] because they cannot breed with each other or wild stocks even after escape from confinement.

2.2 | Precocious maturation

Puberty is the period when immature juveniles advance to mature adulthood, characterized by the onset of gametogenesis in both sexes, functional competence of the hypothalamic–pituitary–gonadal (HPG) axis, followed by the capability to reproduce sexually.[7] The onset of puberty is a flexible process influenced by numerous environmental and biological factors, including photoperiod, water temperature, diet, growth rate, body size, fat deposition and stock genetics, as reported for Atlantic salmon.[43,64] While it may take some fish species many years to attain puberty (e.g., tunas, groupers and sturgeons), many farmed fish, including salmonid, sea bream, sea bass, cod and halibut species,[65] exhibit precocious puberty or maturation before reaching marketable size. Precocious maturation adversely affects fish growth, feed utilization, filet quality, health and animal welfare.[7] Particularly, precociousness is more prominent in male than female fish in many salmonids[66–68] and European sea bass (*Dicentrarchus labrax*).[65] An example of precocious puberty in a 26-g male Atlantic salmon is shown in Figure 1.

With a heavy investment of resources and energy to sexual maturation, including gonadal development, gametogenesis, secondary sexual characteristics and reproductive behaviour, somatic growth typically slows and feed intake is markedly reduced or stops entirely. Consequently, muscle/flesh growth stagnates or decreases before and during the spawning period.[69–71] Maturation also severely deteriorates salmon flesh quality as reduced fat and protein content, increased water content, dramatic change of smell and taste, and loss of flesh pigmentation,[7,8] make them less desirable to consumers.[72] The onset of puberty also can negatively impact the immune system and health status, partially due to the interaction between reproductive hormones and the immune system.[73] These impacts on health can be further exacerbated by sexual dimorphism of pathogenic influences[74] and aggressive/agonistic behaviours that accompany maturation and reproduction, typically seen in salmonids, which can result in skin damage and increased sensitivity to opportunistic infections.[75,76] Sexual maturation also represents a welfare issue in anadromous fish

FIGURE 1 Precocious male maturation in an Atlantic salmon cultured in a recirculating aquaculture system. An outstanding example of precocious male maturation in Atlantic salmon reared in our Aquaculture Research Center at 14-months-old. Note the two conspicuous testes with white colour and solid structure (indicated by arrows). The total body length of this fish was 14.2 cm, and body weight was 25.8 g, whereas the gonads weighed 0.9 g, for a gonadosomatic index of 3.5 in such a small fish. Scale bar = 1 cm.

species, such as many salmonids, farmed in sea cages during grow-out to harvest size. During the maturation process, they gradually lose tolerance to seawater due to compromised hypo-osmoregulatory ability,[9,77] which can cause dehydration, stress, immune vulnerability and ultimately mortality in seawater.[73]

These adverse consequences, coupled with maturation and reproduction, would result in a long delay in reaching harvest size for iteroparous fish species that can spawn multiple times (e.g., Atlantic cod). In semelparous salmonids (that spawn just once, e.g., coho and Chinook salmon), sexual maturation and spawning events exhaust their body reserves completely. The energy cost of reproduction in Atlantic salmon, for instance, was estimated to be about 59% as expended energy reserves.[78] In addition, they suffer high or total mortality post-spawning, so that the loss would be most or all of the edible parts of the fish. In contrast, sterile coho salmon (induced by androgen treatment) continued to grow without flesh deterioration during the period when fertile fish sexually matured.[79–81] In fact, they lived and grew for at least two more years beyond the time of normal maturation and death of their fertile counterparts. The growth advantage of sterile fish was also shown in Nile tilapia (*Oreochromis niloticus*) induced by high temperature[82] and in common carp (*Cyprinus carpio*) induced by dietary androgen administration.[10]

Various strategies have been adopted by industry to reduce precocious maturation incidences, including photoperiod control,[83] feeding control[84] and selective breeding for late maturation,[85] but with mixed success overall. These strategies can delay maturation at best, as fish often still initiate puberty before harvest, jeopardizing marketability. In contrast, germ cell-free Atlantic salmon did not enter puberty, which was correlated with the immature status of steroidogenesis.[86] Furthermore, the absence of sex steroid production associated with puberty in sterile salmon also alleviates the compromised health problem modulated by sex hormone production. In summary, the development and application of reproductively sterile fish can effectively mitigate these undesired maturation and reproduction challenges.

3 | METHODS FOR INDUCING STERILITY WITH APPLICATION POTENTIAL IN AQUACULTURE

3.1 | Traditional approaches

Researchers have explored sterilization methods in fish for many decades, with recorded studies dating back to the 1950s.[14,15] A summary of different strategies to induce fish sterility is provided in Table 1. Gonadectomy and high-energy irradiation were the first of many approaches used for sterilization, although they were not feasible for fish-farming purposes. Surgery is invasive for fish and too labour-intensive, while high-energy irradiation is burdensome, dangerous for both fish and operators and requires expensive equipment for commercial use. Furthermore, it is challenging to entirely remove gonadal tissues by gonadectomy or irradiation, and the probable regeneration of the gonads can nullify the effectiveness of both methods.[15,87]

Alternatively, 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 sterilization in many species,[10,80,81,88,89] and 100% sterility was achieved in coho salmon.[90] In contrast, sablefish juveniles fed a diet containing high levels of 17α-methyltestosterone exhibited sterile-appearing gonads following treatment, although recovery was observed 1 year after the withdrawal of androgen treatment.[89] In addition, sterilization by androgen administration requires lengthy treatment and proper safety evaluation and measures to protect farm employees and the environment from exposure.[91] Moreover, fish directly treated with steroids may face restrictions or prohibitions on their sale for human consumption (e.g., by the European Union under Council Directive 96/22/EC[92]), 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.[91]

Another approach using heat treatment, inspired by hyperthermia-induced male sterility in mammals,[93] has been applied to fish to generate sterile hosts for germ-cell transplantation.[94] Rearing pejerrey (*Odontesthes bonariensis*) larvae and juveniles under prolonged periods of high temperature induced germ-cell depletion for both males and females.[95] Success of heat-induced germ-cell degeneration has mainly been reported for warm-water fishes such as Argentinian silverside (*Patagonina hatcheri*)[95] and Nile tilapia[82] and may not be effective for cold-water species, for example, sablefish.[96] This method was expanded later by a combination of busulfan injection[97] and applied to other fish such as common carp.[98] The combination of heat and busulfan treatment has been shown to be more effective in eradicating germ cells.[97,98] However, busulfan seemed to have a limited effect on spermatogenesis when injected into rainbow trout.[88] As an antimitotic agent, the safety of busulfan to humans and the environment has not been thoroughly evaluated.

Among all the classical methods, the most common and practical one to induce sterility in aquaculture is through chromosome set manipulation, including interspecies hybridization, de novo triploidization or crossing of diploid and tetraploid individuals.

3.1.1 | Triploidy

Triploidization began to be widely used for sterility induction in the 1970s–1980s and is still recognized as the most effective and practical approach for producing sterile fish for large-scale commercial aquaculture. For example, farming of triploid salmonids is still common in the Pacific Northwest. The principle of triploidy induction involves applying a shock, which can be hydrostatic pressure, temperature, electrical or chemical, with precise timing after fertilization to prevent second polar body extrusion during the second meiotic division of embryonic development.[99] An alternative to shock-induced triploids, mating tetraploids with diploids to generate interploid triploids, is expected to yield an all-triploid population, but actually does not in all cases (e.g., rainbow trout[100]). Further, the low viability, high frequency of abnormality and mosaicism and impaired fertility, especially in males, make maintaining tetraploid broodstock less practical for triploid production[16] (and references therein). Thus, triploids are generally

TABLE 1 Summary of methods to induce sterility in fishes with the potential application in aquaculture and their respective sterility results in different species

Strategies	Principle/tools	Target process	Target genes	Species	Sterility[a]		References
					Male	Female	
X-irradiation	Apoptosis/antimitotic	Gametogenesis	—[b]	Rainbow trout (*Oncorhynchus mykiss*)	—	N.A.[c]	[15]
Gonadectomy	Surgery	—	—	Gobiid Fish (*Bathygobius soporator*)	N.A.	—	[14]
				Coho salmon (*Oncorhynchus kisutch*)	N.A.	N.A.	[190]
Androgen treatment	17α-methyltestosterone	Gonadogenesis/ gametogenesis	—	Coho salmon	94% ($n = 48$)[80]; 100% ($n = 22$)[90]		[80,90]
				Common carp (*Cyprinus carpio*)	98% ($n = 57$)		[10]
Heat treatment	Apoptosis	Gametogenesis	—	Pejerrey (*Odontesthes bonariensis*)	100% ($n = 14$)	66.6% ($n = 12$)	[95]
				Sablefish (*Anoplopoma fimbria*)	—	0% ($n = 35$)	[96]
				Nile tilapia (*Oreochromis niloticus*)	—	100% ($n = 60$)	[82]
Heat and busulfan	Apoptosis/antimitotic	Gametogenesis	—	Common carp	100% ($n = 5$)	100% ($n = 5$)	[98]
				Patagonian pejerrey (*Odontesthes hatcheri*)	100% ($n = 5$)	40% ($n = 5$)	[97]
Interspecific hybridization	Gametogenesis impaired	Meiosis	—	Tiger trout hybrid	25% ($n = 110$)	97% ($n = 66$)	[130]
Triploidization	Thermal shock (heat)	Meiosis	—	Atlantic salmon (*Salmo salar*)	Well-developed testis without spermiation	Rare presence of oocytes	[106]
	Thermal shock (cold)	Meiosis	—	European sea bass (*Dicentrarchus labrax*)	No spermatozoa	Rudimentary ovary	[104]
	Pressure shock	Meiosis	—	Coho salmon	Same size of testis to diploids	No oocytes	[191]
	Tetraploid male × diploid female	Meiosis	—	Rainbow trout	N.A.	N.A.	[192]
KD[d]	MO microinjection	PGC development	*dnd*	Loach (*Misgurnus anguillicaudatus*)	100% ($n = 13$)		[25]
				Goldfish (*Carassius auratus*)	100% ($n = 14$)		[26]
				Sterlet (*Acipenser ruthenus*)	100% ($n = 30$)[e]		[27]
	MO immersion	PGC development	*dnd*	Zebrafish (*Danio rerio*)	100% ($n = 37$)		[22]

TABLE 1 (Continued)

Strategies	Principle/tools	Target process	Target genes	Species	Sterility[a]		References
					Male	Female	
KO[f]	CRISPR/Cas9	PGC development	*dnd*	Atlantic salmon	57% ($n = 14$)[g]; 100% ($n = 14$)[h]		[144]
		Oogenesis	*nanos3*	Nile tilapia	N.A.	40% ($n = 10$)	[159]
			foxh1		N.A.	Arrested oogenesis	[160]
		Spermatogenesis	*nanos2*	Nile tilapia	18% ($n = 16$)	N.A.	[159]
			eEF1A1b		Abnormal spermiogenesis	Fertile	[158]
	TALEN	HPG axis	*gnrh1*	Medaka	Fertile	Anovulation	[146]
			fshb	(*Oryzias latipes*)	Fertile	Arrested folliculogenesis	[146]
			lhb		Fertile	Anovulation	[146]
			gnrh3	Zebrafish	Fertile	Fertile	[147]
			gnrh3/ gnrh2		Fertile	Fertile	[148]
			gnrh3/ Kiss1/ Kiss2		Fertile	Fertile	[149]
			fshb		Fertile	Fertile	[152,153]
			lhb		Fertile	Anovulation	[152,154]
			fshr		Fertile	Arrested folliculogenesis[i]	[153,155]
			lhcgr		Fertile	Fertile	[154,155]
			fshr/lhcgr		Majority sterile[j]	All-male	[155]
			fshb/lhb		Majority sterile[j]	All-male	[152,153]
	ZFN	HPG axis	*lhb*	Channel catfish (*Ictalurus punctatus*)	100% ($n = 22$)[k]		[140]
Mutagenesis	Mutagenesis and surrogate propagation	HPG axis	*fshr*	Medaka	—	Retarded oogenesis (n = 20)	[157]
Transgenesis	Nitroreductase/prodrug	Gametogenesis	—	Zebrafish	—	100% ($n = 20$)	[135]
					100% ($n = 38$)	0% ($n = 40$)	[136]
	Antisense RNA	HPG axis	*gnrh*	Rainbow trout	Fertile	Fertile	[150]
				Common carp	12% ($n = 102$)		[151]
		PGC development	*dnd*	Zebrafish	100% ($n = 10$)	100% ($n = 10$)	[138]
		Gametogenesis	*nanos*	Channel catfish	67% ($n = 3$)[l]	100% ($n = 1$)[l]	[145]
	shRNA	PGC development	*dnd*	Channel catfish	63% ($n = 13$)[l]	80% ($n = 10$)[l]	[145]
		Gametogenesis	*nanos*		57% ($n = 7$)[l]	38% ($n = 21$)[l]	[145]

(Continues)

TABLE 1 (Continued)

Strategies	Principle/tools	Target process	Target genes	Species	Sterility[a]		References
					Male	Female	
	Overexpression	PGC development	*sdf1a*	Zebrafish	100% ($n = 64$)	All-male	[137]
	Maternal deposit pro-apoptotic factor	PGC development	—	Zebrafish	N.A.	N.A.	[139]
Vaccination	Autoimmunity against gonadal proteins	Early gonad development	Gsdf, Gdf9 and Cd205	Zebrafish	Retardation, apoptosis and atresia in germ cells and supporting somatic cells, although variable		[177]
	Anti-gonadotropin receptors	Gametogenesis	Fshr, Lhcgr	Rainbow trout	Transient and reversible effects on vitellogenesis and spermatogenesis		[156]
Primordazine	Translation inhibition	PGC development	*nanos3*, *dnd*, *vasa*, etc.	Zebrafish	PGCs ablated, but one or more surviving PGCs often fully populate the adult gonads		[178]

[a]Sterility column denotes either representative results or the best results if multiple conditions were used in some studies; *n*-value inside parentheses indicates the total number of fish examined.
[b]—, not applicable.
[c]N.A., data not available.
[d]KD, knockdown.
[e]Indicated by no PGC positive signal at 4 and 21 days post-fertilization.
[f]KO, knockout.
[g]*dnd*-mutated fish.
[h]*dnd*/*alb* double knockout fish.
[i]All reverse to fertile males after arrested folliculogenesis.
[j]Majority sterile, expect some early spermatogenesis.
[k]Based on no eggs obtained when mating mutant males with mutant females.
[l]Immaturity based on no secondary sexual characteristics observed.

obtained by direct shock induction in most fish. Pressure shocks seem to be more easily controlled, less harmful and preferred over chemical and thermal treatments.[101] The retention of the second polar body induced by the shock results in a triploid embryo containing an additional set of maternal chromosomes. The extra chromosome set renders the triploid individuals functionally sterile due to the inability to pair three sets of homologues in meiosis during gametogenesis, resulting in aneuploid gametes.[102]

The degree of functional sterility in triploids varies by fish species and sex, with gonadal development in females being disrupted more severely than in males. Complete sterility, without any functional egg production, has been achieved in many triploid females, like tench (*Tinca tinca*),[103] European sea bass,[104] turbot (*Scophthalmus maximus*)[105] and Atlantic salmon,[106] although occasional production of mature oocytes was documented depending on the age of the fish and culture conditions.[102,107,108] In contrast, triploid males can develop testes to a similar size as their diploid siblings with the onset of puberty,[109] partially because early spermatogenesis proceeds via mitotic division that is not impaired in triploid males. As a result, triploid and diploid males may have a similar dress-out percentage (i.e., carcass yield at slaughter) due to a similar energy diversion to testis development. With a considerable population of fully functional steroidogenic cells in triploid males, the concomitant hormonal changes and adverse effects during sexual maturation are expected,[16] potentially impacting their health, growth and welfare. The challenge of maturation in triploid males can be resolved by culturing all-female triploid populations through genetic sex control,[110] especially in species where females are preferred due to their superior growth relative to males.[111] Nevertheless, intensive research indicates that triploid fish are potentially less robust than diploids.

The low survival of triploids during the early stages of development can be ascribed mainly to the shock procedure applied to the fish embryos shortly after fertilization, whereas the depressed survival later on, particularly when reared together with diploids, is likely attributed to the triploidy status *per se*.[16] For example, triploids were repeatedly reported to show lower survival than diploid counterparts in coho salmon during the common garden grow-out phase.[112,113] The literature contains mixed results regarding growth, but generally speaking, triploids grow more slowly than or equal to diploids depending on the species and environment prior to maturation, while in the post-maturation phase, they grow 10%–30% faster than their diploid counterparts on account of their partial or complete sterility,[99,114] which can potentially compensate for their early growth depression. In salmonids, triploidy also can result in reduced disease resistance[112,115,116] and increased frequencies of deformities, for example, in the gill, eye, bone and spine relative to diploids.[101,117–119] Moreover, triploids are more sensitive to environmental fluctuations such as temperature and hypoxia.[120,121] This intolerance of environmental extremes is unfavourable to many fish farming operations, particularly when the grow-out stage occurs in open water with daily and seasonal variation, a situation that could be exacerbated in the future by global climate change. The poorer performance of triploids than diploids in suboptimal rearing conditions, such as elevated temperature, high stocking density and communal culture, constrains the further application of the technique.

With regard to Pacific Northwest finfish aquaculture species, triploidy induction has been achieved in several Pacific salmon, including coho salmon, Chinook salmon and steelhead/rainbow trout.[112,122–124] Triploidy has also recently been achieved in sablefish (Luckenbach et al., unpublished data) via pressure or thermal shock. However, more research is needed to optimize methods and evaluate the performance of triploid sablefish in aquaculture.

3.1.2 | Interspecific hybridization

Interspecific hybridization has been used in some fish species to obtain hybrid progenies with potentially improved production traits (e.g., fast growth, high survival, increased disease resistance) inherited from both parent species.[125–127] Hybrids from interspecies crosses are, in some cases, sterile or gametogenesis-impaired, which can be used for sterile fish farming. However, most of the hybrids evaluated did not prove beneficial for aquaculture, and many of them were found to be fertile and under-performing.[128]

In salmonids, Chevassus[129] reviewed interspecific hybridization in 15 species from three genera of *Salvelinus*, *Salmo* and *Oncorhynchus* and discussed the use of their hybrids for fish farming and water management to mitigate the mingling of gametically compatible allopatric species that can produce fertile hybrids. Many of these crosses either resulted in non-viable embryos or low survival rates or produced hybrids with fertilizable gametes. For instance, no individuals reached the hatching stage from coho salmon female × chum salmon (*Oncorhynchus keta*) male crosses, but fertile male hybrids were observed when crossing Chinook salmon females with coho salmon males. Only a few specific combinations created sterile hybrids with performance higher than or equal to their purebred counterparts, for example, brown trout female × brook trout (*Salvelinus fontinalis*) male.[130] The limited number of crosses that can produce sterile hybrids with acceptable performance constrains the feasibility of using hybridization to widely produce sterile salmonids for aquaculture. In some species, interspecific hybridization remains underexploited, while challenging for other species. In the case of sablefish, it is one of only two species in the family *Anoplopomatidae*, [along with skilfish (*Erilepis zonifer*)], and no studies have been conducted on the possibility of hybridization. Producing sterile fish by interspecific hybridization is highly species-specific, and it needs intensive investigations to validate the fertility and fitness of hybrid progenies. Although interspecific hybrids are potentially sterile, the plasticity of reproduction and the questionable performance of hybrids diminish the practical use of this approach for producing sterile fish in aquaculture.

3.2 | Modern approaches

3.2.1 | Genetic engineering

The wide availability of complete genome sequences and revolutionary progress in genetic engineering techniques have significantly advanced our knowledge and understanding of many fundamental

biological processes, including reproduction, in the past two decades. The introduction of desired traits can be achieved through genetic modification (GM) by introducing recombinant DNA into the recipient genome or genome editing (GnEd). GnEd creates an insertion, deletion, or substitution of one or more nucleotides at a specific targeted site in the recipient genome using zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats associated with Cas9 protein (CRISPR/Cas9). These approaches are robust enough to conduct either gene functional studies or create new genetically-engineered fish lines with unique characteristics for both basic and applied science.[131–134]

A common consideration for using genetic engineering methods to improve aquaculture stocks is that desirable traits can be transmitted via the germline into subsequent generations through conventional breeding. However, when sterility is the objective, it is contradictory to such breeding, as sterility terminates the lineage. As a result, there are challenges with making reproducible sterility hereditary. The strategies that can resolve this 'heritable sterility paradox' and continuously produce sterile progeny include inducible, controllable and reversible/rescuable sterilization. Inducible sterilization refers to turning sterility induction on only when a particular treatment is applied, such as a prodrug[135,136] or heat-shock treatment.[137] Controllable sterilization is realized by special breeding regimes such as crossing two transgenic lines[138] or using a specific genotype as a dam.[139] A reversible/rescuable strategy can be achieved by exogenous hormone[140] or mRNA[141] compensation or through surrogate propagation[142] after gene knockout.

The major disadvantages of genetic engineering methods, when applied in foodfish, are the extensive regulatory scrutiny processes (or even prohibition in some countries) and potential long-term consumer resistance even after regulatory approval. Getting these technologies through the regulatory process and widely accepted by the public may be even more challenging than overcoming the technical hurdles required for commercialization. As noted above, it took over 20 years for 'AquAdvantage' transgenic salmon to be approved for human consumption.[20] This experience has led to hesitancy among stakeholders to invest heavily in the development of genetically engineered foodfish. As GnEd enables precise changes equivalent to what conventional breeding techniques could obtain, updating regulatory policies to embrace these GnEd breeding technologies, which are different from classical GM methods, has been broadly requested by researchers, investors, developers and breeders. With the advancing regulatory developments in Japan, Argentina, Brazil, the United States and other countries in the past 4 years, a consensus seems to be building toward more progressive regulatory approaches related to the use of GM methods.[143]

Among the many emerging genetic engineering methods to sterilize fish, the HPG axis, PGC development and gametogenesis are the three most prominent processes being targeted (summarized in Figure 2). However, the plasticity of the target gene functions in different fish would typically require detailed validations on a species-by-species basis before transferring the methodology from one fish to another. With all the target genes involving the HPG axis, PGC development and gametogenesis, *dnd* is the most functionally conserved gene. Disruption of *dnd* could universally lead to sterility across a variety of species. Other target genes are indispensable for fertility in one specific sex and/or certain species (examples below).

Targeting the HPG axis

The HPG axis is the seminal neuroendocrine and endocrine system in vertebrates, including fish, that, among other functions, controls reproduction.[161] The hypothalamus releases stimulatory and inhibitory neurohormones, in particular, gonadotropin-releasing hormone (Gnrh), to control the biosynthesis and secretion of pituitary gonadotropins, namely, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). Fsh and Lh further stimulate the differentiation, growth and maturation of the gonads in males and females.[162,163]

Gnrh is the highest-level stimulator in the HPG axis and has been targeted for sterility induction in many species, with different results. In model fish systems, disruption of *gnrh1* (hypophysiotropic form) by TALEN led to female sterility due to anovulation in medaka,[146] while the loss of *gnrh3* (hypophysiotropic form) by TALEN-mediated mutagenesis in zebrafish did not affect reproductive performance.[147] Moreover, a *gnrh3*/*gnrh2* double mutant[148] or a *kiss1*/*kiss2* double mutant[149] had no prominent phenotype for reproduction in zebrafish. Although it could be explained by compensation or redundancy,[132,164] these findings challenge the dogma of the pivotal role of Gnrh in fish reproductive regulation. Disruption of Gnrh has also been exploited in farmed fish like common carp and salmonids through transgenic antisense *gnrh* RNA expression.[150,151,165] Xu et al.[151] found that 38 of 102 (37%) transgenic common carp had abnormal or missing gonads at 4 years old. In rainbow trout, the antisense RNA induced down-regulation of Gnrh, but did not decrease plasma concentrations of Fsh or Lh nor induce sterility in transgenic fish.[150] Thus, targeting Gnrh to induce sterility needs to be validated in different species to ensure the disruption of the HPG axis and reproductive function.

Fsh, Lh and their receptors (Fshr and Lhcgr) are also of interest in fish endocrinology studies.[146,152–155] Disruption of Fsh and Lh signalling generated diverse and complex results depending on species, sex and target gene(s). For example, in model species zebrafish and medaka, a single knockout of *fshb*, *lhb*, *fshr* or *lhcgr* mainly affected ovarian rather than testicular development. In medaka, the knockout of either *lhb* or *fshb* by TALEN led to sterility in females.[146] On the other hand, in zebrafish, the *fshb*-deficient females were fertile despite exhibiting delayed ovarian development, whereas knockout of *lhb* by TALEN resulted in impaired ovulation and thus sterility in females.[152–154] In the case of gonadotropin receptors (*fshr* and *lhcgr*), their disruption led to opposite results to that of ligands (*fshb* and *lhb*) in zebrafish. Folliculogenesis was arrested entirely at the primary growth stage in the *fshr* mutant causing sterility in female zebrafish, while the deletion of the *lhcgr* gene alone caused no obvious phenotypes.[153–155] Interestingly, double knockouts of *fshb* and *lhb* or *fshr* and *lhcgr* led to sterile, all-male fish with significantly retarded spermatogenesis in zebrafish.[152,153,155] In farmed fish, *lhb* editing has been achieved using ZFN in channel catfish (*Ictalurus punctatus*). The reproductive ability of mutant

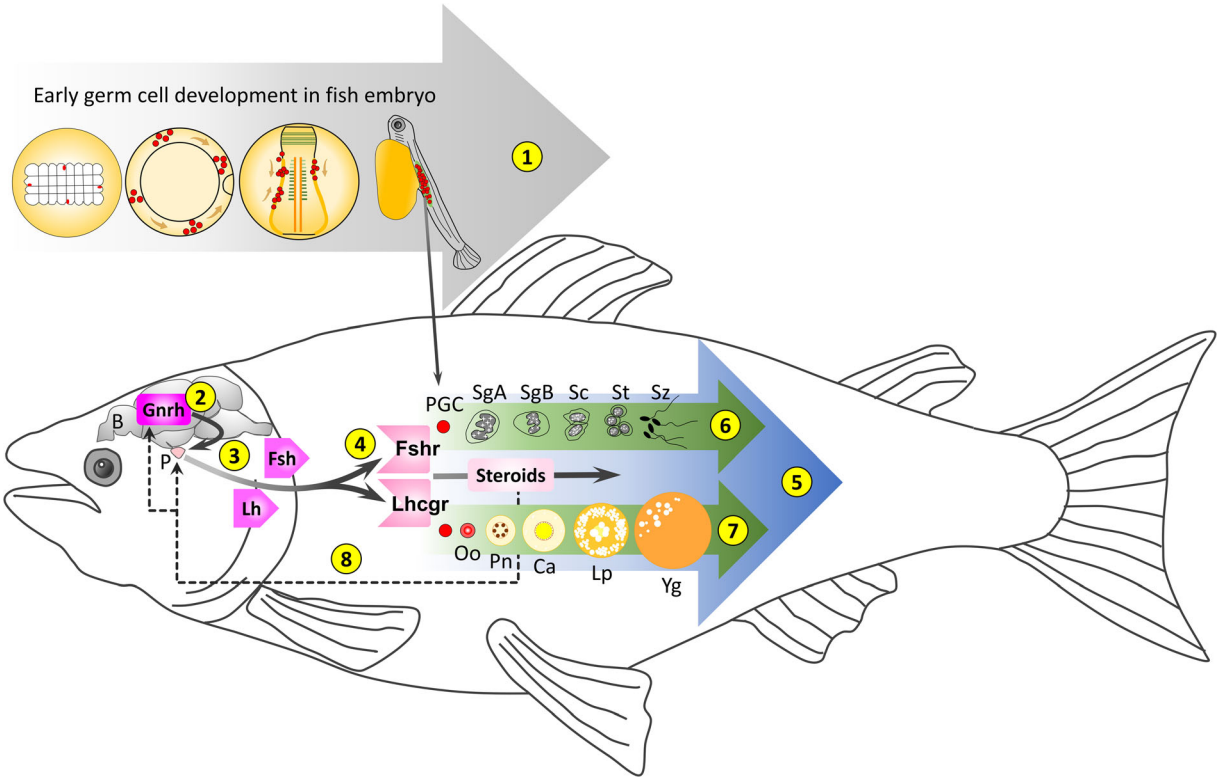

FIGURE 2 Schematic overview of the developmental processes or genes that different methods target to induce reproductive sterility in fish. The numbers in the circles represent different target processes or genes and are annotated as follows with representative references: 1. PGC (red circles) development[22,25–27,137–139,144,145]; 2. *gnrh* genes[146–151]; 3. *fshb* and *lhb* genes[146,152–154]; 4. *fshr* and *lhcgr* genes[153–157]; 5. Gametogenesis[15,95–98,106,130,145]; 6. Spermatogenesis only[136,158,159]; 7. Oogenesis only[135,159,160]; 8. Steroid feedback to the pituitary and brain.[80,89,90] Androgen treatment induces sterility presumably through negative-feedback inhibition of gonadotropin release, but that remains to be determined. B, brain; Ca, early cortical alveolar oocyte; Lp, lipid droplet stage oocyte; Oo, oogonia; P, pituitary; Pn, perinucleolar oocyte; Sc, spermatocyte; SgA, spermatogonia A; SgB, spermatogonia B; St, spermatid; Sz, spermatozoa; Yg, yolk globule stage oocyte.

fish was thought greatly impaired since no eggs were obtained when mating mutant males with mutant females. Still, no assessment of gonadal development was provided.

Loss-of-function studies for genes involved in the HPG axis are still primarily focused on model fish species. In light of the plasticity of gene function among different species, functional investigation of genes involved in the HPG axis needs to be carefully carried out in a species-by-species manner before targeting them for sterilization in aquaculture fish. In addition, differences in gene function between males and females should be taken into consideration (more examples below). To reverse sterility in these strategies targeting Gnrh or Fsh and Lh, exogenous administration of corresponding hormones during the specific developmental period should be established if any promising and universal sterility methods are to be achieved. Another rescuable strategy was demonstrated in medaka by surrogate propagation.[157] In medaka, *fshr*-mutant males are fertile, while female mutants are sterile due to the disabled somatic supporting cells in their ovaries.[166] When germ cells from *fshr*-deficient mutants were isolated and transplanted into hybrid sterile recipient females carrying normal Fshr, donor ($fshr^{-/-}$)-derived germ cells were rescued by functional gonadal supporting cells in the recipients and developed into fully functional eggs carrying $fshr^{-/-}$. These $fshr^{-/-}$ eggs can be fertilized by masculinized neomale (XX) $fshr^{-/-}$ sperm or go through gynogenesis to produce large numbers of genetically sterile ($fshr^{-/-}$) all-female populations. In this case, one more step to make $fshr^{-/-}$ neomales (XX) is needed for producing all-female sterile fish since the $fshr^{-/-}$ males are fertile. In principle, once a somatic supporting cell mutation causing both male and female sterility is identified, it would be possible by directly mating surrogate broodstock to mass-produce genetically sterile offspring.[142]

Targeting PGC development

PGCs are embryonic precursors of the gametes and are specified and set apart from the somatic cells during early development. Then, they must maintain their specification and migrate to and reside in the gonadal ridge to give rise to sperm in males and eggs in females.[167] Thus, successfully disrupting PGC development can affect fertility in both sexes. Several genes involved in PGC development have been identified, including *nanos*,[168] *dnd*,[23] *sdf1*[169] and *cxcr4*.[170] Among these factors, *dnd* is one of the most-studied genes, revealing its conserved and indispensable role in PGC development in various fishes.[23,25–28,171] Therefore, *dnd* represents the best target gene for sterilization in finfish aquaculture species currently. One of the recent promising outcomes from Atlantic salmon corroborates its evolutionary conservatism, as the biallelic *dnd*-knockout Atlantic salmon were

completely germ-cell free.[144] These sterile mutant fish failed to up-regulate gonadal sex steroid production and did not undergo subsequent puberty,[86] which is beneficial to the cultured fish from a health and welfare perspective.

CRISPR/Cas9 employed in the study to generate *dnd* knockout Atlantic salmon has been considered a game-changer in the field of reverse genetics because of its versatility and high efficiency, which allows for biallelic disruption.[172] The immediate mutant phenotypes achieved in the founder generation are especially beneficial for non-model fish with prohibitively lengthy maturation processes (e.g., some Pacific salmonids and sablefish). Continuous production of these homozygous sterile fish ($dnd^{-/-}$), on the one hand, can be obtained by crossing heterozygous mutants. However, it is cumbersome to do genotyping, and only 25% of homozygous mutants would be produced. On the other hand, Güralp et al.[141] proposed a rescue approach by co-injecting a wild-type salmon *dnd* mRNA together with CRISPR/Cas9 targeting *dnd*. They showed that rescued *dnd* Atlantic salmon mutants have either spermatogonia or primary oocytes at up to 1 year old. It is known that PGC formation can be fully rescued by *dnd* mRNA co-injection when MO is used to knockdown *dnd* in zebrafish[173] and medaka.[28,174] However, it is uncertain whether fish permanently lacking *dnd* (i.e., by knockout in this case), even after the early rescue, will fully develop gonads and produce fertilizable gametes. The Dnd protein may function in later germ-cell development in zebrafish.[138] Thus, the complete rescue of the gonads upon later sexual maturation needs to be confirmed in *dnd*-knockout Atlantic salmon to validate this rescuable strategy. Furthermore, as a major drawback associated with CRISPR/Cas9, the potential off-target and pleiotropic effects[175,176] should be carefully evaluated.

In addition to direct knockout, other studies employed antisense strategies to block *dnd*. Zhang et al.[138] took advantage of the GAL4/UAS system in two separate zebrafish transgenic lines to drive transcription of antisense *dnd* as a controllable sterilization strategy. When crossing the two fertile parent lines, endogenous *dnd* in their offspring was down-regulated by the antisense RNA, which rendered them sterile or reduced their fertility. This strategy avoids the problem of producing heritable sterility in offspring. However, two parental transgenic lines must be created and maintained, which might be less favourable for the aquaculture industry. Su et al.[145] reported a reversible transgenic sterilization system using a copper-responsive promoter to drive short hairpin RNA (shRNA) and complementary DNA expression to target *dnd* and *nanos* genes in channel catfish. When exposed to an ambient copper concentration in the water, the transgenic constructs will be expressed to block PGC development, resulting in sterile fish, while when treated with an elevated concentration of copper, the construct will be repressed, and embryos can develop into fertile broodstock. In this study, some of the constructs knocked down PGC marker gene expression, and reduced sexual maturity was achieved, measured by the expression of secondary sexual characteristics. However, no assessment of gonadal development was provided. As a primary proof of principle for the approach of transgene-mediated reversible sterility, the study offered a strategy to repress sterility for maintaining broodstock when needed. However, optimization of this system will be required for further application, as the authors stated.

Targeting other genes related to PGC development and a PGC elimination strategy also achieved successful sterilization. Inducible sterilization has been implemented by heat-shock protein promoter-driven overexpression of Sdf1a in zebrafish embryos when incubated at an elevated temperature.[137] The transgenic overexpression of Sdf1a signalling disrupted the normal PGC migration pattern and yielded sterile fish. Fertile broodstock could be maintained by rearing them at a standard rearing temperature without heat induction. Alternatively, maternal sterility technology (MST) targets the elimination of PGCs by a pro-apoptotic factor.[139] The maternal-specific promoter and germ cell-specific *cis*-acting element ensured the unique expression of pro-apoptotic proteins only in PGCs, eliminating the germ cells by programmed cell death. This controllable MST approach only produces PGC-eliminated sterile fish when a transgenic female is employed as a dam, while the fertile transgenic male maintains the transgenic line.

Targeting gametogenesis

Other steps of germ-cell development, such as spermatogenesis and oogenesis, have been targeted for sterility induction. A few loss-of-function studies in Nile tilapia revealed genes responsible for the gonadal development of one sex but not the other. CRISPR/Cas9-mediated loss of Nanos2 in male and Nanos3 in female tilapia resulted in germ cell-deficient gonads, but not vice versa.[159] Knockout of *foxh1* by CRISPR/Cas9 resulted only in arrest of oogenesis and female sterility without affecting males,[160] whereas *eEF1A1b* knockout in tilapia led only to arrest of spermatogenesis and reduced male fertility without affecting females.[158] In addition, a nitroreductase/prodrug system has been developed in zebrafish to eliminate germ cells in either sex specifically.[135,136] Nitroreductase was driven by either testis-specific or ovary-specific genes in separate transgenic lines. Sterilization could be induced only when treating larvae with a prodrug, by which cytotoxic metabolites converted specifically in male or female germ cells caused DNA crosslinks and germ-cell death through apoptosis. Prodrug treatment can be omitted to obtain fertile broodstock. The main downside is that arrest of germ-cell development can be accomplished only for one sex at a time, which would be detrimental for large-scale applications.

3.2.2 | Other methods

As an alternative to genetic engineering, other approaches, including vaccines against gonadal development, have been attempted.[156,177] The vaccination methodology can be considered inducible sterilization since the fish not receiving the vaccine could serve as broodstock. However, significant optimization should be performed before further application considering the potential transient effects[156] and highly variable results among treatments and individuals.[177] In addition, a small molecule compound, called primordazine, that selectively ablates PGCs by repressing translation was identified through chemical screening in zebrafish embryos.[178] The delivery of this small molecule

via direct bath immersion may represent a practical strategy to induce sterility in large-scale operations. However, one or more PGCs surviving the primordazine treatment often populated the adult gonads and rendered the approach ineffective. Besides, the effect of primordazine on inhibition of PGC translation in other fish species has not been investigated.

4 | NOVEL IMMERSION-BASED GENE SILENCING TECHNOLOGY FOR FISH STERILIZATION

In light of the disadvantages and potential technical and regulatory hurdles described above, alternative strategies are needed to sterilize farmed fish in a manner that is amenable to both commercialization and market acceptance.

4.1 | Development and advantages of the emerging approach

We previously developed an innovative technology using bath immersion to induce temporary gene silencing with the molecular transporter, Vivo, conjugated to MO to induce sterility without introducing any genetic modifications to fish.[22] MOs are chemically modified nucleic acid analogues serving as antisense oligonucleotides. They bind with target mRNAs to inhibit gene expression.[179] This blocking of gene function by MOs is a transient process that does not alter any genome sequences, which can circumvent public concerns over food safety and environmental sustainability. Since a bath immersion is used to administer MOs instead of microinjection, it can easily be scaled up to simultaneously treat many eggs/embryos. The immersion strategy is versatile in a manner suitable for either bulk treatment of many individuals being incubated *en masse* or for high-throughput screening with a large number of chemicals. For example, Jin et al.[178] screened 7000 compounds by direct immersion of zebrafish embryos and found a novel small compound called primordazine (M.W. of form A: 433.5; M.W. of form B: 367.4) that blocks the maintenance of PGCs in zebrafish. However, direct uptake through the chorion is restricted to small molecules because there is a limitation of chorion permeability.[180] The molecular transporter, Vivo, used in the immersion method was shown to penetrate the chorion and promote uptake of *zfdnd*-MO by PGCs.[22]

A flow chart of this method is shown in Figure 3. Vivo was conjugated to the MO targeting zebrafish *dnd* mRNA (*zfdnd*-MO-Vivo) to administer *zfdnd*-MO by bath immersion. The *zfdnd*-MO-Vivo penetrated the chorion of embryos and entered target cells, which effectively knocked down Dnd expression, disrupted PGC development, and subsequently resulted in germ cell elimination and reproductively sterile fish. Under optimal conditions, 100% sterility was achieved when zebrafish embryos were treated immediately after fertilization. This novel sterilization technology has many advantages, including: (1) the targeted gene and developmental pathway are highly conserved among fishes, (2) it achieves sterility by transient gene silencing without genetic modification, (3) it is feasible for mass production of sterile fish in an efficient manner that is practical for commercialization and (4) sterilization is inducible and achieved only when the treatment is applied.

PGCs are progenitor cells of both male and female germline cells, impaired development of which will lead to individuals devoid of any gametes. Also, as mentioned above, Dnd is evolutionarily conserved and plays an indispensable role in PGC development during fish embryogenesis.[181] In *zfdnd*-MO-Vivo treated fish, all PGCs migrated to ectopic locations other than the gonadal ridge. In addition, many of them differentiated into different types of cells[22] (see Figure 3), which ultimately resulted in adults devoid of any germ cells. In addition to zebrafish,[23] blocking Dnd expression also led to sterility in many other teleost fish, including pond loach (*Misgurnus anguillicaudatus*),[25] goldfish (*Carassius auratus*),[26] salmonids,[29,144] medaka[28] and sterlet (*Acipenser ruthenus*).[171] Hence, targeting *dnd*, a highly functionally conserved gene, to disrupt PGC development is a reliable strategy to produce sterile individuals for all sorts of farmed fish.

In addition, this novel approach achieves sterility in an inducible manner; namely, sterility will only be achieved by active treatment, while untreated individuals could serve as future broodstock. This strategy also makes it convenient, straightforward and easy to immediately integrate sterilization procedures into seed production with established superior aquaculture strains by simply treating their eggs. It is procedurally different from genetic modification strategies that require additional efforts to create new transgenic lines and maintain fertile broodstock.

4.2 | Application in coho salmon and sablefish

As explained above, the evolving method targeting *dnd* to induce sterilization in zebrafish is transferable to other fishes, and the immersion strategy has enormous potential for large-scale aquaculture production. Therefore, as examples herein, we applied this immersion-based technology to commercially important aquaculture species, including coho salmon and sablefish.

Whether bath immersion is applied to eggs (pre-fertilization) or embryos (post-fertilization) depends on the characteristics of the eggs and embryos in different fish species. Zebrafish eggs can only be held briefly before fertilization. Fertilization or survival rates were shown to decrease with the prolonged holding of zebrafish eggs in other studies as well.[182] Accordingly, we immersion-treated the fertilized embryos for sterility induction. To promote a homogeneous concentration throughout the immersion medium and enhance the uptake of *zfdnd*-MO-Vivo, slight agitation/mixing was applied to developing zebrafish embryos during bath immersion. However, some fish embryos are susceptible to movement, particularly during early embryogenesis, making post-fertilization treatments that involve agitation impractical. On the other hand, unfertilized eggs can withstand mechanical agitation without affecting hatching or development rates in many fish, such as common carp.[183] Thus, as an alternative to treating embryos, pre-fertilization immersion treatment of 'green' eggs can

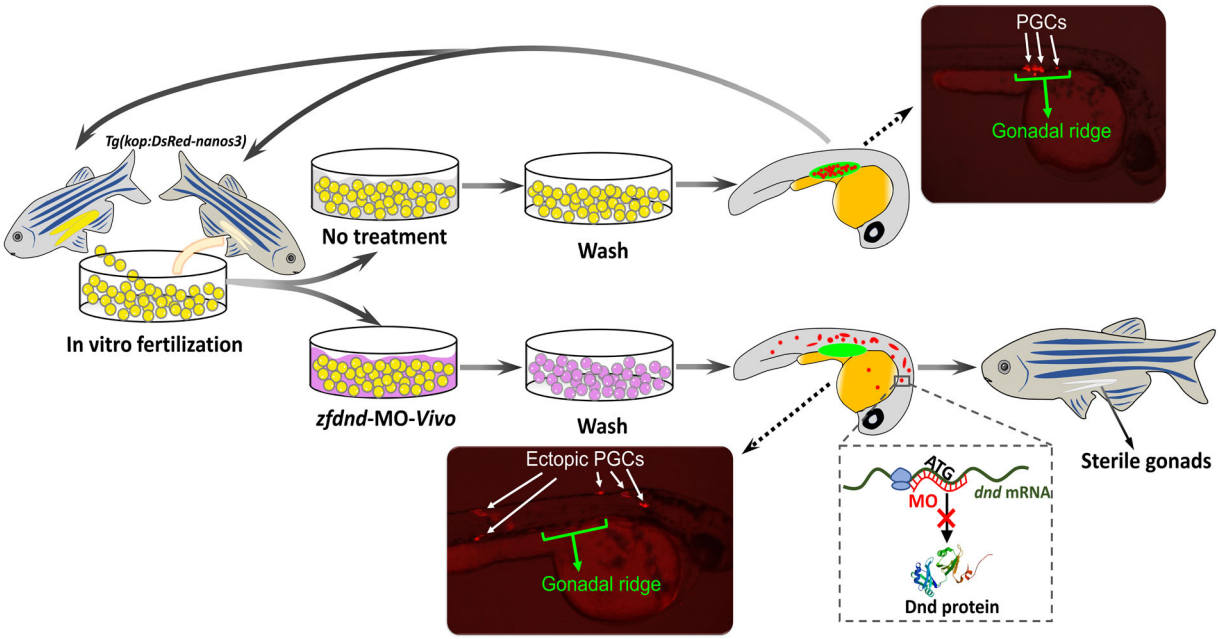

FIGURE 3 A flow chart of the novel immersion-based gene silencing sterilization technology developed in zebrafish. The *Tg(kop:DsRed-nanos3)* transgenic line expressing red fluorescent protein (RFP) specifically in PGCs was employed for monitoring PGC development during embryogenesis. For sterility induction, immediately after in vitro fertilization, embryos were subjected to bath immersion treatment in Vivo-conjugated Morpholino oligomer (MO) against zebrafish *dnd* (*zfdnd*-MO-Vivo) solutions. PGCs (indicated by RFP expressing cells) of treated fish were shown to mis-migrate to ectopic locations other than the gonadal ridge (the green area inside the fish larva), and some PGCs differentiated into somatic lineages indicated by their cell shape. With optimal conditions, after treatment, all fishes developed into reproductively sterile adults. These results indicated that the immersion treatment could deliver MO into developing PGCs and disrupt *dnd* translation. Fertile broodstock can be obtained simply by omitting immersion treatment, so the PGCs and subsequent gonad would develop as normal.

be adopted for those species (e.g., many salmonids), and the eggs can be kept in a suitable medium like ovarian fluid before fertilization for hours to a few days without affecting their viability. Delivering MO into unfertilized eggs has an advantage in that the MO is already in place inside the eggs and is ready to block *dnd* translation upon the onset of PGC development after fertilization. Therefore, we immersion-treated unfertilized coho salmon and sablefish eggs instead of embryos for sterility induction.

The bath-immersion treatments of coho salmon and sablefish eggs were adapted and modified from our published protocol developed in zebrafish.[22] In brief, eggs and milt were collected separately by strip-spawning from mature female and male broodstock. Unfertilized eggs were bath immersion-treated (pre-fertilization treatment) at 4°C for 24 h in a medium containing 35% ovarian fluid and 65% fertilization diluent (85 mM NaCl, 50 mM glycine and 20 mM Tris base) with 15–20 μM *dnd*-MO-Vivo targeting the *dnd* gene of each species. Ovarian fluid collected from strip spawning was briefly centrifuged to remove debris before use. After the treatment, eggs were washed with medium and fertilized in vitro. Later, fish from the treatment and control groups were dissected to examine gonadal development when the gonads had developed at least to the point that they could be visually distinguished by their respective size.

When coho salmon reached 14-months-old, female fish were recognized by two apparent ovarian bulbs. Although smaller, two lobes of the testes were easily observed in male fish attached to and spanning the length of the roof of the abdominal cavity (Figure 4a,b). In contrast, gonad development was absent except for two pieces of thin filament-like tissue (Figure 4c) in 4%–20% of treated fish, depending on the *dnd*-MO-Vivo concentration. These individuals were defined as sterile, since no germ cells were found in their gonads. When the ovaries, testes and sterile gonads were excised from the fish, there was a marked size difference between fertile and sterile gonads (Figure 4a–c). For sablefish, treated and control fish were cultured for as long as two-and-a-half years before the final assessment of gonadal development. Of the *dnd*-MO-Vivo treated sablefish at 1 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 two-and-a-half years of age, 11% of treated fish were completely sterile. The ovaries, testes and sterile gonads from these sablefish are shown in Figure 5. 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.

4.3 | Future directions for refinement and application

In our initial sterility induction trials in coho salmon and sablefish, we demonstrated a proof of principle for this new approach for the

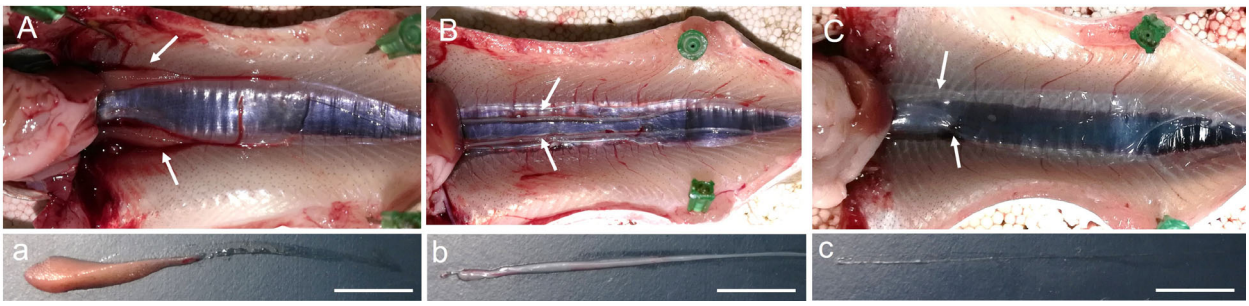

FIGURE 4 Gross morphology of the gonads in 14-month-old treated sterile and control coho salmon. (A) A control female; (B) a control male; (C) a treated, sterile fish. Arrows in (A–C) point to the gonads. The lower images (a–c) show the gonads excised from (A–C), respectively. The ovarian bulbs were present in control female fish (A and a). Gonads of sterile fish (C and c) are filament-like and much smaller than testes (B and b), which can be easily distinguished. Scale bar = 1 cm in a, b and c.

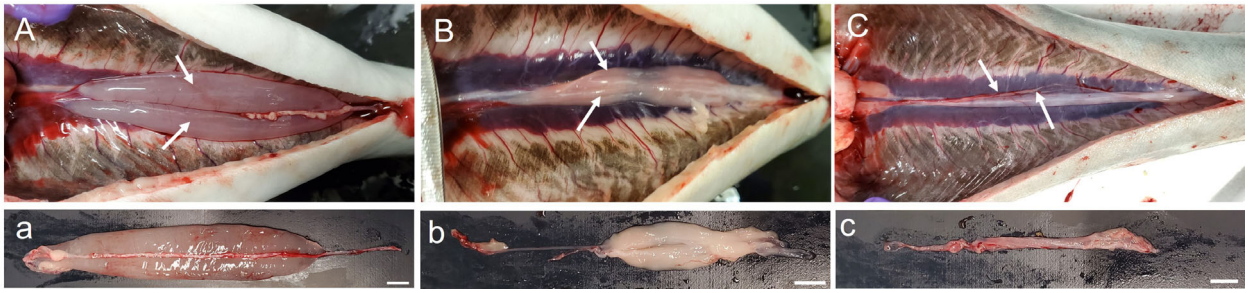

FIGURE 5 Sablefish reared up to two and a half years were sacrificed to evaluate gonadal development for treated and control groups. (A) Fertile female had prominent and plump ovaries. (B) Fertile male had two firm testes. (C) sterile gonads were soft, smaller in size and less structured. Arrows in (A–C) point to the gonads. Lower images (a–c) show ovaries, testes and sterile gonads that have been excised from the body, respectively. Scale bar = 1 cm in a, b and c.

production of sterile fish for aquaculture. The future optimization of sterility induction rates and fish survival rates after treatment will further boost the potential of this technology.

The sterile coho salmon and sablefish that we obtained further supported the view that the *dnd* gene and PGC developmental mechanism are evolutionarily conserved in teleost fishes. Applying this novel technology to coho salmon and sablefish to produce sterile animals mitigates the challenges associated with undesired maturation and reproduction. As we continue to build upon these results by optimizing the methodology and scaling up the treatment, farming reproductively-sterile native fish in the Pacific Northwest can be achieved in the near future. This approach will promote ecologically and economically sustainable aquaculture development. The advancement from model species, like zebrafish, to aquaculture species may expand this non-genetically engineered, non-chromosome set manipulation sterilization technology to other commercially important species in which stripping gametes and in vitro fertilization is possible. A schematic diagram for the potential application of immersion-based sterilization technology in aquaculture is illustrated in Figure 6.

For coho salmon and sablefish, since unfertilized eggs were immersion-treated before fertilization, eggs must be incubated in an appropriate medium to maintain the viability of the eggs. The immersion medium is a crucial component for egg/embryo survival. A common practice to preserve eggs before fertilization and prevent their activation is to keep them in ovarian fluid, which imitates the surroundings from which they originated.[183,184] Therefore, ovarian fluid was included as a major component of the immersion medium during the treatment. Alternatively, a well-defined medium with similar components and physicochemical properties as ovarian fluid may be beneficial for establishing reliable and consistent treatment conditions that generate highly viable eggs and embryos when it is challenging to obtain enough fresh ovarian fluid and maintain its consistency.

When this technology was developed in zebrafish, a transgenic line *Tg*(*kop:DsRed-nanos3*) was adopted to visualize and monitor PGC development. Likewise, PGC labelling through microinjection of fluorescent protein-encoding mRNA[185–187] or transgenesis[188] would allow tracking PGC development in a timely manner. With that, sterility effectiveness indicated by PGC development can be obtained as early as hatching, which would substantially accelerate technology refinement, especially in species that have prohibitively long reproductive cycles.

In the case that PGC labelling is challenging or not possible in such species, an alternative way to receive timely feedback is through MO labelling to monitor immersion efficacy. MOs are routinely attached to different fluorescence moieties for labelling. However, Vivo is not compatible with fluorescence-linked MOs, according to the vendor, GeneTools (Philomath, OR). An alternative molecular transporter compatible

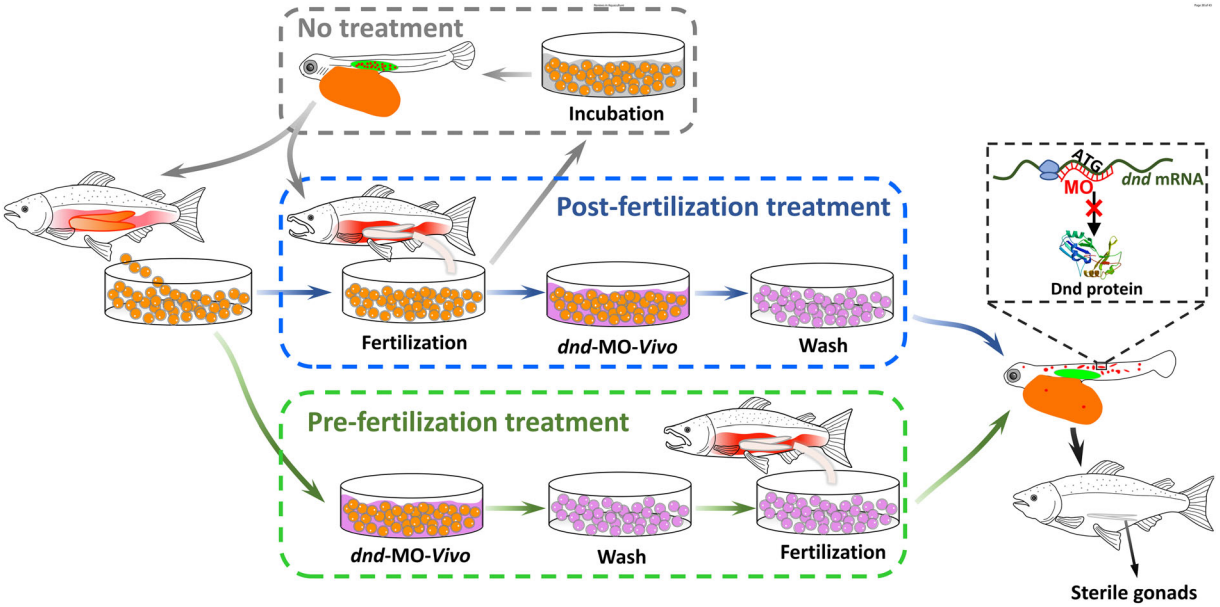

FIGURE 6 A schematic diagram illustrating the application of immersion-base sterilization technology in aquaculture. Two different types of bath immersion, either post-fertilization (blue dotted box) or pre-fertilization (green dotted box), can be adopted for sterilization depending on the characteristics of eggs and embryos in different species. Species-dependent *dnd*-MO-Vivo, administered by bath immersion, disrupts PGC (red circles) development and migration to the developing gonads (the green area inside the fish larva), which results in the production of reproductively sterile fish. Fertile broodstock can be generated by simply skipping the immersion with *dnd*-MO-Vivo and incubating embryos routinely (grey dotted box)

with fluorescence groups would make real-time monitoring of MO uptake achievable. Treating eggs/embryos with fluorescent-labelled MOs would allow MO uptake tracking immediately after immersion treatment. The fluorescence intensity in the eggs is a theoretical indicator for knockdown efficacy and consequent sterility. Further, in conjunction with advanced fluorescence screening equipment, it is possible to screen and retain only individuals with a certain degree of MO uptake implied by fluorescence intensity inside eggs/embryos and to obtain 100% sterile populations for later culture.

Even if sterility rates were not 100% from the immersion-based method and fish escape were to occur, the fact that this method does not involve genetic engineering ensures that there would be no potential spread of modified genomes to wild fish populations. This approach circumvents the negative perceptions of genetically engineered aquaculture products that may face complex regulatory oversight and consumer resistance. That being said, the pharmaceutical and toxicological properties of species-dependent MO-Vivo should be assessed comprehensively before applying this novel sterilization technology in aquaculture. Since the US Food and Drug Administration has approved a MO-based antisense therapy for human muscular disease,[189] we expect a relatively smooth transition of MO-based technologies from the bench to aquaculture applications.

5 | CONCLUSIONS

Aquaculture is playing an increasingly important role in achieving global food security. Minimizing ecological impacts and increasing production should both be considered as critical goals during aquaculture expansion and advancement. Undesired maturation and reproduction are major challenges in aquaculture in terms of escape events and precocious maturation, which can have profound ecological and economic impacts. Farming reproductively sterile fish is a useful mitigation strategy to address these challenges and promote environmentally and economically sustainable aquaculture practices.

Here, we comprehensively reviewed and summarized potential approaches for sterility induction with their applications in different fish species. Among all methods mentioned, triploidization is currently considered the most effective and practical sterilization method for large-scale aquaculture. However, triploid fish are generally more sensitive and may exhibit reduced performance attributes than diploid counterparts under suboptimal rearing conditions that commonly occur and that are expected to occur more often in open water in the future due to climate change. Modern approaches primarily take advantage of genetic tools to overexpress or knock out target genes for inducing sterility. However, the main limitations of genetic engineering methods—regulatory complexity and potential consumer resistance—may deter their further applications in foodfishes.

The emerging immersion-based approach targeting *dnd* to sterilize fish without introducing any genetic modifications presents an encouraging solution for overcoming the limitations and disadvantages of triploidization and GM approaches. Furthermore, the successful application of this approach to sterility induction of both coho salmon and sablefish corroborates the conservatism of the *dnd* function and PGC development in fishes, facilitating the future transfer and application of sterilization technology to other commercially important aquaculture species. Farming sterile coho salmon, steelhead, or sablefish in the Pacific Northwest and elsewhere would

represent progress toward phasing out the need to use triploidy or other sterilization methods, promoting cost-effective and ecologically responsible aquaculture practices and serving as a model for expanding sustainable aquaculture globally.

AUTHOR CONTRIBUTIONS

Lan Xu: Conceptualization; investigation; visualization; writing – original draft; writing – review and editing. **Mingli Zhao:** Visualization; writing – original draft; writing – review and editing. **Jun Hyung Ryu:** Writing – review and editing. **Edward S. Hayman:** Investigation; writing – review and editing. **William T. Fairgrieve:** Funding acquisition; investigation; resources; writing – review and editing. **Yonathan Zohar:** Funding acquisition; methodology; project administration; resources; supervision; writing – review and editing. **J. Adam Luckenbach:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing – review and editing. **Ten-Tsao Wong:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting this article are openly available in this manuscript's text, tables, and figures.

ETHICS STATEMENT

All experimental protocols involved in this study were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine IACUC# 0521013.

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