Received: 5 January 2023 | Revised: 26 May 2023 | Accepted: 18 July 2023

DOI: 10.1111/1755-0998.13868

MOLECULAR ECOLOGY RESOURCES WILEY

OPINION

Fish germ cell cryobanking and transplanting for conservation

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Funding information
Ministry of Business, Innovation and Employment Endeavour Programme, Grant/Award Number: CAWX2101; Te Pūnaha Hihiko: Vision Mātauranga Capability Fund, Grant/Award Number: C11X1913; The New Zealand Government via the Ngā Pou Rangahau platform; The Royal Society of New Zealand Catalyst Seeding General Contract, Grant/Award Number: CSG-PAF1803; The University of Canterbury; Tokyo University of Marine Science and Technology

Handling Editor: Alana Alexander

Abstract

The unprecedented loss of global biodiversity is linked to multiple anthropogenic stressors. New conservation technologies are urgently needed to mitigate this loss. The rights, knowledge and perspectives of Indigenous peoples in biodiversity conservation—including the development and application of new technologies—are increasingly recognised. Advances in germplasm cryopreservation and germ cell transplantation (termed 'broodstock surrogacy') techniques offer exciting tools to preserve biodiversity, but their application has been underappreciated. Here, we use teleost fishes as an exemplar group to outline (1) the power of these techniques to preserve genome-wide genetic diversity, (2) the need to apply a conservation genomic lens when selecting individuals for germplasm cryobanking and broodstock surrogacy and (3) the value of considering the cultural significance of these genomic resources. We conclude by discussing the opportunities and challenges of these techniques for conserving biodiversity in threatened teleost fish and beyond.

KEYWORDS
assisted reproductive technologies, broodstock surrogacy, *Galaxias*, germplasm biobanking, Mātauranga Māori, sperm

1 | INTRODUCTION

Anthropogenic environmental change and associated stressors are rapidly transforming environments and pose a major threat to species and ecosystems (Halsch et al., 2021). These changes are predicted to be a primary cause of biodiversity loss, with an abrupt disruption of ecological assemblages expected within the next decades (Trisos et al., 2020). Mitigating biodiversity loss necessitates an interdisciplinary and coordinated approach across multiple temporal and spatial scales that stretches from genes to ecosystems (Bonebrake et al., 2018). Indigenous-managed land represents over a quarter of the world's land surface (Garnett et al., 2018) and hosts high levels of biodiversity (Schuster et al., 2019), thus opening opportunities for the development and implementation of mitigation strategies that

are led or co-led by Indigenous Peoples (Brondízio et al., 2021; Henri et al., 2021; Reid et al., 2021). For many Indigenous cultures, species and ecosystems are inextricably linked (Ens et al., 2016; Gadgil et al., 1993; Goolmeer et al., 2022), and such perspectives enable more holistic approaches to species conservation (e.g. Collier-Robinson et al., 2019; Rayne et al., 2022).

For the world's most imperilled species, there is growing interest in (1) in vivo approaches to safeguard biodiversity (e.g. Bolton et al., 2022), (2) increasing the efficacy and efficiency of in situ approaches (Howell et al., 2021, 2022) and (3) approaches to reintroduce lost genetic diversity to the wild (Fritts, 2022). Each of these necessitates careful protocols and processes for the collection, storage, documentation and use of material, especially when working with culturally significant species (Hudson et al., 2016, 2020, 2021). In mammals, gametes (sperm and eggs) and, in many cases, reproductive tissues (testes and ovaries) as well as embryos can often be readily cryopreserved (Comizzoli, 2018; Holt & Comizzoli, 2022). In fish, however, the situation is more complicated as only sperm, but not eggs, can be successfully cryopreserved (Cabrita et al., 2010; Diwan et al., 2020). Recently, significant advances in the cryopreservation and downstream transplantation of undifferentiated germ cells in fish (termed 'broodstock surrogacy') have opened a new window to preserve biodiversity in this group; however, the uptake of these new technologies has been slow.

Although a number of studies have summarised the applications of cryopreservation and transplantation of spermatogonia in fish previously (e.g. Goto & Saito, 2019; Lacerda et al., 2013; Robles et al., 2017; Yoshizaki & Lee, 2018; Yoshizaki & Yazawa, 2019), our perspective goes beyond these contributions by connecting technological advancements with practical applications for conservation and by describing the valuable insights that come from the incorporation of genomic information to support sample selection processes and cultural perspectives in this work. Here, we showcase these recent advancements and highlight their untapped potential to help mitigate biodiversity loss using teleost fishes as an exemplar group. We do this by first outlining the methodological breakthroughs in cryopreservation and broodstock surrogacy technologies. Second, we discuss that an understanding of genome-wide genetic variation and its distribution across a species' range is necessary to ensure representative sampling. Third, we use examples of threatened freshwater fish species in Aotearoa-New Zealand (NZ) to illustrate how cryopreservation and broodstock surrogacy technologies could enhance in situ and ex situ conservation efforts and how Indigenous perspectives can be embedded from research inception to implementation. The latter Indigenous perspectives are grounded in Ngāi Tahu (a Māori tribe of NZ) values, which is the NZ Indigenous authors' connected ancestry (MJW, JK and KR). We argue that the application of these technologies has been underappreciated and, consequently, underused globally to curb biodiversity loss. We further argue that future efforts should draw upon these technologies more often to preserve and enhance captive and wild natural diversity.

1.1 | Emerging technologies for cryobanking and transplanting reproductive cells

Fish sperm can be successfully cryopreserved, and several species-specific protocols have been developed to store sperm in the long term (Cabrita et al., 2010). Fish eggs differ and, due to their large size (>0.5 mm in diameter), large amounts of yolk and lipids are impossible to cryopreserve (Diwan et al., 2020). However, recent advancements in the cryopreservation of spermatogonia and oogonia, that is undifferentiated germ cells, have provided exciting opportunities to cryobank both paternal and maternal genomes (Yoshizaki & Lee, 2018).

Success in the cryopreservation of immature testes and ovaries in rainbow trout (*Oncorhynchus mykiss*; Lee et al., 2013; Lee, Iwasaki, et al., 2016; Lee, Katayama, et al., 2016) demonstrated the possibility of preserving undifferentiated germ cells semipermanently. The next discovery was the production of functional eggs and sperm from cryopreserved spermatogonia and oogonia, and this was achieved via the transplantation of 'donor' cells from one species into fertile recipients of another species (termed 'surrogate broodstock'; Yoshizaki & Yazawa, 2019). To achieve this, testes and ovaries from donor fish were enzymatically dissociated and the resulting cell suspensions were then intraperitoneally transplanted into newly hatched larvae of the surrogate species (Okutsu et al., 2006; Yazawa et al., 2010; Yoshizaki et al., 2010; Figure 1). It was previously known that hatchlings have an undeveloped immune system and a low capacity for rejecting foreign substances (Manning, 1996). Accordingly, some of the transplanted germ cells migrated through amoebic movement to the immature gonads of the recipients, where cells were successfully incorporated (Yoshizaki et al., 2012). Furthermore, study results confirmed that donor-derived germ cells proliferated in recipient gonads, eventually differentiating into functional gametes (Okutsu et al., 2006; Yoshizaki et al., 2010). It should be noted that donor germ cells—whether spermatogonia or oogonia—differentiated into eggs in female recipients and sperm in male recipients (Okutsu et al., 2006; Yoshizaki et al., 2010). Thus, for threatened species that are difficult to obtain, even if only one sex of donor individuals is available, their undifferentiated germ cells could be transplanted into both male and female recipients to produce eggs or sperm. Such transplantation procedures can be performed using cryopreserved undifferentiated germ cells to produce individuals (Lee et al., 2013; Lee, Katayama, et al., 2016).

When undifferentiated germ cell transplantation is applied to fertile recipients, both donor-derived gametes and the recipient's own gametes are produced. To obtain surrogate broodstock that produce only donor-derived gametes, sterilised recipients are required. When germ cell transplantation was first established, triploid infertile fish were used (Okutsu et al., 2007). Although these triploids show meiotic abnormalities and do not produce their own gametes, they retain functional gonadal somatic cells to nurture the development of donor-derived germ cells and produce large numbers of functional eggs and sperm derived solely from the

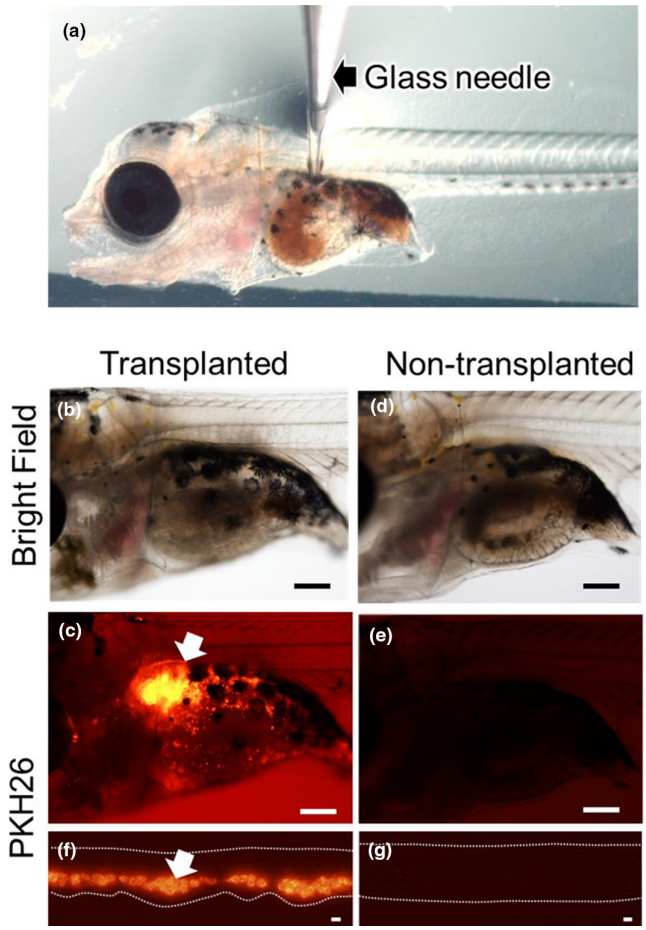

FIGURE 1 Example of a technique established by Yazawa et al. (2010) for the transplantation of donor-derived undifferentiated germ cells from Nibe croaker (*Nibea mitsukurii*) into the larva of a 'surrogate broodstock' species (chub mackerel, *Scomber japonicus*). (a) Intraperitoneal transplantation of donor cells into an anaesthetised larva; (b) bright-field view and (c) fluorescent-field view of donor-cell transplanted larva; (d, e) bright-field and fluorescent views of larvae nontransplanted with donor cells, respectively (Scale bars = 200 μm). Fluorescent views of PKH26 in the excised gonad of transplanted (f) and nontransplanted (g) larvae after 3 weeks (scale bars = 10 μm). Location of transplanted PKH26-stained germ cells indicated by white arrowhead.

transplanted donor species (Okutsu et al., 2007). It is also possible to produce germ cell-less recipients by inhibiting the function of the *dead end* gene (*dnd*), a gene essential for primordial germ cell survival (Yoshizaki et al., 2016). A recent study confirmed that germ cell-less recipients created through genome editing—using CRISPR/Cas9 targeting *dnd*—efficiently nurture donor-derived germ cells to functional gametes (Fujihara et al., 2022). Next-generation individuals produced by genome-edited recipients are wild type and have not themselves been genetically modified. Therefore, CRISPR/Cas9 can be theoretically applied to conserve species, but its application may be subject to legal and regulatory restrictions (e.g. Everett-Hincks & Henaghan, 2019).

What makes this method even more significant is that interspecies germ cell transplantation can be applied to different donor and recipient species. Rainbow trout germ cells can be transplanted into triploid masu salmon (*Oncorhynchus masou*), and the resulting masu salmon recipients then produce rainbow trout gametes (Okutsu et al., 2007). The same approach has also produced tiger puffer (*Takifugu rubripes*) gametes in a small-sized and closely related species, grass puffer (*Takifugu niphobles*; Hamasaki et al., 2017). Recently, goldfish (*Carassius auratus*) recipients have produced carp (*Cyprinus carpio*) gametes (Franěk et al., 2021). Functional gametes as well as next-generation individuals have also been successfully produced through transplantation of cryopreserved germ cells in Chinese rosy bitterling (*Rhodeus ocellatus ocellatus*; Octavera & Yoshizaki, 2020) and medaka (*Oryzias latipes*; Seki et al., 2017). Thus, if species are phylogenetically close enough, both eggs and sperm can be produced through interspecific transplantation (Yoshizaki & Yazawa, 2019).

Application of these technologies is not without challenges, however. For threatened species, the number of individuals to supply donor germ cells may be limited—or the species itself may be small-bodied. In these cases, transplanting enough undifferentiated germ cells to recipients can be difficult. To overcome this challenge, in vitro expansion of undifferentiated germ cells can be a powerful strategy (e.g. Iwasaki-Takahashi et al., 2020). A second challenge is that while producing gametes from parental fish is achievable by controlling their rearing environment or administering exogenous hormones (Mylonas et al., 2010), the in vivo proliferation (rather than maturation) of undifferentiated germ cells in gonads of donor fish for downstream transplantation has not been successful to date.

In the next sections, we will outline the importance of linking knowledge about genome-wide genetic diversity into the sample selection process. Following that, we will provide some practical examples of how such an integrated approach can be applied to preserve the declining freshwater diversity of teleost fishes endemic to NZ.

1.2 | The importance of understanding the genomic context

To best capture genetic diversity for cryobanking, the collection of donor cells should be guided by a solid understanding of factors that shape genetic variation in wild populations, making it fundamental to bridge the fields of reproductive biotechnology and conservation genomics. The recent advent in cost-effective sequencing technologies, alongside improved bioinformatic workflows for large datasets (Segelbacher et al., 2021), allows genome-wide data at a scale useful for conservation to be generated. While genetic approaches have long been used to inform conservation (Allendorf et al., 2010), the vast increase in genomic data now provides extraordinary opportunities for unravelling the demographic and adaptive patterns and processes that form the basis of adaptive evolutionary management (Bernatchez, 2016).

First, key for any conservation practices is understanding the geographic clustering of populations (Coates et al., 2018). The increased resolution from genomic data facilitates the detection of

subtle population clusters based on both neutral and adaptive variation and for inferring the relative importance of different evolutionary processes (gene flow, drift and selection) across populations. Of particular importance for conservation and for informing germplasm cryobanking is the notion of adaptive genetic variation and how this is counteracted by the extent and direction of gene flow (Bernatchez, 2016). This knowledge helps preserve species-wide genetic diversity, which bolsters the adaptive potential of a species and, therefore, its ability to evolve in response to environmental change (Hoffmann & Sgrò, 2011). Consequently, failing to account for the spatial scale at which adaptive variation exists and its distribution relative to spatially heterogeneous selection in conservation plans can erode the population of interest and ecosystem functioning (Blanchet et al., 2020). This is particularly relevant when studying species across fragmented landscapes, as it is often the case in freshwater fish inhabiting lotic environments (e.g. Brauer et al., 2018).

Second, the effective population size is a key parameter in conservation biology because the rate of inbreeding, and thereby the change in genetic heterozygosity, is related to this. Genomic data can assist to maintain large and representative effective population sizes of captive species so that they can serve as a reservoir for genetic material to re-establish or reinforce wild populations (Segelbacher et al., 2021). These considerations are important not only during the establishment of captive populations but also during subsequent generations in captivity, especially if these populations remain small and cannot be periodically bolstered with new genetic material (Galla et al., 2020). Genomic data can also be used to measure genetic load, particularly in isolated, inbred populations characterised by a low effective population size (e.g. Dussex et al., 2021 but see Guhlin et al., 2023), but how to use these measures to inform conservation management remains uncertain (Grueber & Sunnucks, 2022).

Third, the relative frequencies of genetic variants—including single nucleotide polymorphisms (SNPs) and structural variants (SVs; rearrangements >50 base pairs)—their size, linkage and compatibility are of importance. For example, negative effects on fitness because of outbreeding depression are expected in situations where genetic incompatibilities between alleles from foreign and recipient sampling populations may occur (e.g. Bateson–Dobzhansky–Müller incompatibilities). These can be revealed by admixture between diverged populations, or when species or subpopulations differ in their genomic architectures; genomic haplotype data can help to characterise the genomic mosaic of local ancestry and inform sampling strategies. Genomic regions underlying incompatible architectures can be caused by differences in large structural variants, for example the presence–absence of large chromosomal inversions, which can lead to recombination suppression and the production of inviable gametes (Mérot et al., 2020; Wellenreuther & Bernatchez, 2018). Recent work is increasingly acknowledging the need to incorporate the full spectrum of genetic variants to estimate genomic fitness and incompatibilities, including the epistatic interactions between loci and genome structure (Wellenreuther et al., 2019; Wold et al., 2021). This is in part driven by the fact that the effects of a given genetic variant will depend on its linkage disequilibrium with other genetic variants (SNPs or SVs) and its frequency in the population (Leitwein et al., 2020). Therefore, incorporation of both SNPs and SVs can provide improved insights into important processes to inform conservation efforts, including population structure and adaptive variation in the short term, as well as fitness consequences for both ex situ and in situ management in the long term.

1.3 | Mitigating biodiversity loss of culturally significant species: NZ threatened teleost fishes as a case study

Innovative technologies are increasingly applied in NZ to support aquaculture selective breeding programmes of native fishes (Valenza-Troubat, Davy, et al., 2022; Valenza-Troubat, Hilario, et al., 2022), but extension of such approaches to threatened species has only started to be considered. Accumulating evidence suggests that biobanking and surrogacy can be effective tools to enhance conservation outcomes for animals (e.g. Holt & Comizzoli, 2022; Sandler et al., 2021; Yoshizaki & Lee, 2018), but to our knowledge, none of these have been considered from an Indigenous perspective.

Māori (Indigenous Peoples of NZ) are intrinsically connected to the environment through whakapapa, which connects ancestral lineages, genealogical connections, relationships and links to ecosystems. This familial kinship connects tāngata whenua (people of the land) to the environment and with this connection comes responsibilities to maintain the balance and appropriate relationship with the environment for future generations (Roberts et al., 1995).

Fisheries are important in the cultural identity of Māori. For Ngāi Tahu, this is captured in the term *mahinga kai*, which describes the customary gathering of food and natural materials and the places where those resources are gathered (Ngāi Tahu Claims Settlement Act, 1998). Mahinga kai is not just about the harvested species but also includes the knowledge transmission, cultural practice and access to the landscape (Panelli & Tipa, 2007, 2009; Waitangi Tribunal, 1991). As such, the expression *tino rangatiratanga mō tātou, ā, mō kā uri ā muri ake nei*, which captures the ability of Māori to sustain biodiversity and ecosystems for generations to come, is a fundamental aspect of Māori environmental management through the use of mātauranga (knowledge) and tikanga (customary protocols; Ataria et al., 2018; Palmer et al., 2020).

In NZ, 76% of native freshwater fish species (39 of 51) are either threatened with, or at risk of, extinction. Of these, 82% belong to the Galaxiidae family (Ministry for the Environment & Stats NZ, 2020)—a group of amphidromous galaxiids locally known as 'whitebait' and nonmigratory galaxiids (Genus: *Galaxias*) and mudfish (Genus: *Neochanna*). Many of these species are taonga (treasured) species and mahinga kai to Māori. Factors contributing to their decline include habitat destruction, environmental change, predation and competition with introduced species (Williams et al., 2017). Efforts have been made to develop captive breeding techniques for some native galaxiids (Dunn & O'Brien, 2018; Mitchell, 1989), including taiwharu (giant kōkopu; *Galaxias*

argenteus; Wylie et al., 2016), an endemic species and promising candidate for surrogacy due to its large body size (Figure 2). However, there is limited knowledge about population genomic structure and genome-wide diversity below the species level for most galaxiid species, and no long-term genomics-based breeding programmes exist. Consequently, there is increasing urgency to document the spatial and temporal population genomic structure of galaxiids in NZ and to then use this information to guide the sample design for cryobanking reproductive material from members of the family Galaxiidae, with the ultimate goal to preserve genomic resources and improve the management of this taonga species group. This has to go hand in hand with efforts to mitigate environmental impacts on the species. Habitat restoration and the improvement of water quality is a priority for Ngāi Tahu people (Ngāi Tahu 2025, 2001). In parallel to this, initiatives led or co-led by Ngāi Tahu in the development and implementation of these technologies will enable tino rangatiratanga (self-determination) and provide an essential insurance policy for these taonga (and beyond).

DNA from taonga species is seen by Māori as a physical expression of whakapapa, and therefore, cultural values may also apply to the study of the DNA itself (Collier-Robinson et al., 2019). This extends to the way DNA and tissues are stored and managed, and who has access rights and decision power over the samples, the genomic data generated and how these data are reused (Carroll et al., 2021; Hudson et al., 2021; Mc Cartney et al., 2022). Thus, similar notions will guide the application and development of germ cell cryobanking and transplantation technologies by embedding cultural considerations around the collection of taonga species—as well as the storage, use and reuse of samples and data collected from them. As such, by embedding Māori principles such as whakapapa, whakawhanaungatanga (the process of building relationships), ki uta ki tai (holistic resource management), taonga tuku iho (intergenerational protection of taonga, passed across generations), te ao tūroa (intergenerational concept of resource sustainability timeframes/vision) and tino rangatiratanga, a unique opportunity is provided to guide an Indigenous approach to fish conservation. For example, understanding how the whakapapa connection of reproductive material that is cryobanked and/or cultured in vitro is maintained with its place of origin and respective kaitiaki/tāngata tiaki (guardians) is an important consideration. Such considerations ensure that tissues initially collected are also used for their intended purpose at the outset of the project and that these agreed principles are followed over the lifetime of the project and beyond. Such discussions would also help explore how an appropriate governance structure can be established to enable and guide change of thought or circumstance over the generations.

FIGURE 2 Adult taiwharu (giant kōkopu; *Galaxias argenteus*), a large-bodied and culturally significant species endemic to Aotearoa-New Zealand. Scale bar = 1 cm. Photo credit: Ron Munro.

2 | CLOSING REMARKS

Protection of biodiversity necessitates an interdisciplinary and coordinated approach. This includes the preservation of adaptive genetic variation in natural, captive and domesticated populations to enhance species resilience (Hoffmann et al., 2017). For native or endemic species, cultural perspectives and considerations can provide important contexts for Indigenous-led or co-led conservation plans and can guide how cryobanking and reproductive technologies can be developed, implemented and managed.

Germ cell transplantation technologies are promising innovations to curb biodiversity loss, but both have been underutilised in conservation efforts for fishes so far. Specifically, the bipotent nature of germ cells to enable the production of eggs and sperm from donor species once the surrogacy system has been developed—and in some cases the ability to span this technology across species or even genus borders to produce interspecies surrogates—is advantageous to conserve paternal and maternal genomes. Another benefit comes from the ability to develop in vitro cultures to increase cell abundance prior to transplantation. This is particularly relevant when the threatened species are of small size, as is the case for many galaxiids of NZ. Furthermore, surrogacy and cryopreservation techniques are of crucial importance to support aquaculture, the former for developing assisted reproductive technologies and the latter for the maintenance of broodstock genetic diversity. Cryobanking has significant utility in preserving sperm from elite breeders; however, it should be noted that the reproductive diversity of fishes and the associated diversity in gamete biology necessitates the design of species-specific cryopreservation protocols.

Despite recent advances, critical knowledge gaps remain, such as how long do germ cells (and sperm) of fishes remain viable in long-term frozen storage and the effect this has on the epigenome. Furthermore, while induction of sterility in the recipient is desirable for surrogacy systems, achieving this is challenging unless methods like the CRISPR/Cas 9 systems are used. However, due consideration of the social, cultural, ethical and legal implications of the application of such techniques will be necessary (e.g. Everett-Hincks & Henaghan, 2019; Hudson et al., 2019), especially if culturally significant species were to be considered as surrogates (e.g. taiwharu). Future efforts should thus strive to actively invest in both the knowledge—and the social licence—needed for implementation. Prime species are those of economic or cultural interest, which can be bred in captivity and where the rebuilding of depleted or genetically impoverished populations is a high priority. Holistic approaches that support these innovations, together with habitat restoration, can provide an essential insurance policy

for threatened taonga species and can help to promote tino rangatiratanga and enhance mahinga kai opportunities for future generations.

ACKNOWLEDGEMENTS

This work was funded by the Royal Society of New Zealand Catalyst Seeding General contract CSG-PAF1803 'Enabling breeding of indigenous aquaculture species by overcoming reproductive dysfunctions' (to MW and MJW), Te Pūnaha Hihiko: Vision Mātauranga Capability Fund (Contemporary kaitiakitanga of freshwater taonga in Aotearoa—exploring and demystifying cryopreservation and surrogacy tools to aid native fish conservation, C11X1913, to MJW and MW) and by the New Zealand Government via the Ngā Pou Rangahau platform, a research framework developed by the New Zealand Institute for Plant and Food Research Limited and supported by a Strategic Science Investment Fund grant from the Ministry of Business, Innovation and Employment (MBIE). JK was supported by the 'Fish Futures' MBIE Endeavour Programme (grant number CAWX2101). This work was also funded by the University of Canterbury (TES) and the Tokyo University of Marine Science and Technology (GY and RY). We thank Hemi Cumming for translating our abstract into Te Reo Māori.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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How to cite this article: Wylie, M. J., Kitson, J., Russell, K., Yoshizaki, G., Yazawa, R., Steeves, T. E., & Wellenreuther, M. (2023). Fish germ cell cryobanking and transplanting for conservation. *Molecular Ecology Resources*, *00*, 1–8. https://doi.org/10.1111/1755-0998.13868