

New directions in assisted breeding techniques for fish conservation

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Abstract. Fish populations continue to decline globally, signalling the need for new initiatives to conserve endangered species. Over the past two decades, with advances in our understanding of fish germ line biology, new *ex situ* management strategies for fish genetics and reproduction have focused on the use of germ line cells. The development of germ cell transplantation techniques for the purposes of propagating fish species, most commonly farmed species such as salmonids, has been gaining interest among conservation scientists as a means of regenerating endangered species. Previously, *ex situ* conservation methods in fish have been restricted to the cryopreservation of gametes or maintaining captive breeding colonies, both of which face significant challenges that have restricted their widespread implementation. However, advances in germ cell transplantation techniques have made its application in endangered species tangible. Using this approach, it is possible to preserve the genetics of fish species at any stage in their reproductive cycle regardless of sexual maturity or the limitations of brief annual spawning periods. Combining cryopreservation and germ cell transplantation will greatly expand our ability to preserve functional genetic samples from threatened species, to secure fish biodiversity and to produce new individuals to enhance or restore native populations.

Additional keywords: cryoconservation, gonad cryopreservation, cryopreservation, germ cell transplantation, fish biology, sterilisation.

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Introduction

Conservation status of fish in Australia

In 2015, the World Wildlife Fund (WWF) reported a 49% decline in global marine biomass, including a 50% decrease in fish harvested for local subsistence or commercial use (WWF 2015). In Australia, surveys of large fish numbers showed a 36% decrease in large marine fish biomass on fished reefs (Edgar *et al.* 2018). In inland waterways, Australia is home to 36 threatened freshwater fish (Cresswell and Murphy 2016), 26 of which reside only in the Murray–Darling Basin (MDB; Lintermans 2007). As for marine species, overfishing is a key contributor to declining freshwater fish numbers, but the MDB faces additional challenges, including poor water quality, cold water pollution due to damming and the introduction of invasive species (Lintermans 2007). Poor reproductive success, overfishing and competition with or predation by invasive species all contribute to declining fish populations, resulting in a predicted decline of 90% in MDB fish populations since European settlement (Lintermans 2007). The MDB also experienced several ‘mass death’ events in early 2019 due to toxic blue–green algae blooms related to droughts and the diversion of environmental

water flows from the system, primarily for agriculture (Murray–Darling Basin Authority 2019). This sudden loss of biodiversity in the MDB may have ongoing implications for the genetic health of already vulnerable populations. Until issues in the environment can be addressed there is risk of further declines in fish populations, suggesting that *ex situ* conservation strategies are now required to provide an insurance policy for the conservation of these species.

Issues with current methods of ex situ conservation

Common *ex situ* conservation methods include captive breeding and cryobanking of animal cells and tissues. For many species, the circumstances under which reproduction occurs is not known or cannot be easily replicated in captivity. Therefore, cryobanking of samples from endangered fish species has become a more convenient method to store their genetics. Cryopreserved cells and tissues can be stored indefinitely as a source of genetic material to resurrect a population in case of a stochastic event causing sudden loss of biodiversity or extinction. Semen, or milt, has been successfully cryopreserved in many fish species, including Murray cod *Maccullochella peelii*

peelii (Daly *et al.* 2008), brown trout *Salmo trutta* (Nynca *et al.* 2014), Atlantic salmon *Salmo salar* (Figuerola *et al.* 2015) and the zebrafish *Danio rerio* (Yang H *et al.* 2007). However, due to the large diversity in sperm morphology and function among fishes and the short reproductive periods in seasonal species, the optimisation process is often long and techniques may not be transferable across species, genera or families. Therefore, the time and resources required to preserve milt from a single endangered species are often unrealistic for use in conservation.

Even if spermatozoa can be stored, mature fish oocytes have not yet been successfully recovered after cryopreservation, limiting the use of cryopreserved semen samples to fresh fish eggs. Cryopreservation of fish oocytes and eggs presents more of a challenge because their large size limits the penetration of cryoprotectants, leading to fatal ice formation during cooling (Tsai 2009). Although cryopreservation of earlier-stage oocytes has been successful, subsequent *in vitro* culture and IVM failed to produce fertilisable eggs (Tsai 2009). When eggs of the same species are not available for fertilisation, androgenesis can produce offspring (androgenotes), using artificial techniques, in which the male is the only source of nuclear genetic material in the resulting individual (Schwander and Oldroyd 2016). While individuals produced in this way are usually homozygous and have low survivability (Pandian and Kirankumar 2003), androgenotes derived from preserved or cadaveric spermatozoa and surrogate eggs provide a foundation for the restoration of endangered fish species (Schwander and Oldroyd 2016). Although the combination of sperm cryopreservation and androgenesis is promising, it is limited in that it can only be used to reintroduce the genetic diversity of male individuals from which the genetic material is derived.

Embryos containing the genetic information from a male and a female make them an appropriate target for cryopreservation. However, like oocytes, embryos have a low surface to volume ratio, but they are also very complex, multicompartmented structures with a high lipid content and barriers to permeability that limit movement of the cryoprotectant and water across membranes (Hagedorn *et al.* 1997). Various strategies have been used to overcome these barriers, including direct injection of cryoprotectants into the embryo (Janik *et al.* 2000) and injection of gold nanoparticles that rapidly thaw the embryo when heated with a laser (Khosla *et al.* 2017). Despite this, a reliable cryopreservation method for fish embryos is yet to be established for most species.

New directions for the conservation of fish species

The lack of successful cryopreservation techniques for embryos and oocytes significantly reduces opportunities to bank genetics from endangered fish species and suggests the need for new directions to advance fish conservation. To develop these new directions, attention is turning to the use of earlier, undifferentiated cells, such as the primordial germ cells (PGCs), spermatogonia in males and oogonia in females. These cells have a relatively simple structure compared with their differentiated counterparts, namely spermatozoa and oocytes, which makes them an easier target for cryopreservation with the potential to standardise cryopreservation methods across species, and perhaps genera, reducing optimisation time and increasing

opportunities to preserve endangered fish species (Yamaha *et al.* 2007). Because these early stage cells are undifferentiated, methods to produce viable gametes, spermatozoa and oocytes are now being investigated. The most promising method is germ cell surrogacy via germ cell transplantation (GCT). Germ line stem cells are extracted from cryopreserved tissue and injected into a surrogate species, where they differentiate into viable spermatozoa or oocytes (Kobayashi *et al.* 2007; Saito *et al.* 2010; Yoshizaki *et al.* 2010); an overview of this is shown in Fig. 1. The primary benefit of the combination of these two techniques is that, particularly when gonadal tissue is available, it is not necessary to spawn the threatened species in captivity, which is often challenging. The combination of cryopreservation and cell transplantation offers a unique and adaptable method for the storage and subsequent reintroduction of important genetics into a population, which could be hugely beneficial to the conservation of fish.

This review summarises the mechanisms underpinning teleost germ line development and shows how this has led to the development of methods that manipulate the germ line in a way that will improve current conservation strategies for endangered fish species both in Australia and globally.

Key aspects of germ line development in fish

A detailed understanding of the basic biological mechanisms of germ cell development is needed to successfully manipulate reproduction in fish species. The teleost group of fishes is remarkably diverse; however, many aspects of germ cell formation, migration and differentiation are highly conserved. Biomedical model fish species, such as the zebrafish, are well studied and provide the key knowledge of germ cell formation, migration and differentiation. Although the timing of these events is species specific, the mechanisms are similar and can therefore be broadly applied to many species.

Germ cell formation

PGCs are formed early in embryogenesis. In zebrafish, PGCs are specified during the cleavage stage in the first 30 min of development via a process termed 'preformation', in which maternal deposits of PGC-specific mRNA transcripts and proteins found in the germplasm determine germ cell fate (Clelland and Peng 2009; Eno and Pelegri 2016). Ablating or disturbing the germplasm results in sterility or subfertility, and transplantation of the germplasm can induce the germ line fate at ectopic sites, demonstrating the importance of accurate germplasm formation (Hartung and Marlow 2014). Two mRNA transcripts, DEAD-box helicase 4 (*ddx4*) also known as *vasa*, and Nanos C2HC-Type Zinc Finger 3 (*nanos3* or *nos3*), found in the germplasm have been crucial to identifying primordial germ cells during development (Hartung *et al.* 2014). Maternal *vasa* mRNA, synthesised and deposited into the oocyte during oogenesis, localises to the cleavage furrows of the dividing embryo, creating four clusters that are eventually taken up by cells to become the first PGCs (Yoon *et al.* 1997; Extavour and Akam 2003). *Nanos3*, previously known as Nanos C2HC-Type Zinc Finger 1 (*nanos1* or *nos1*), follows a similar expression pattern to *vasa* but, unlike *vasa*, *nanos3* shows some expression

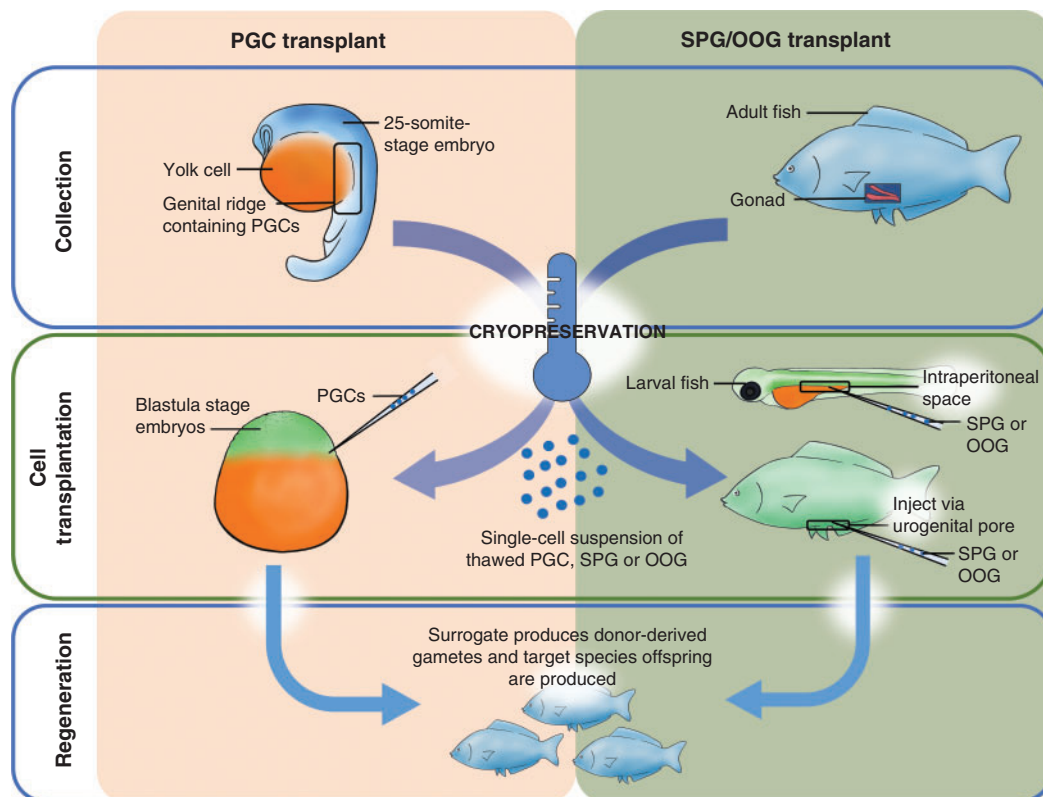


Fig. 1. Germ cell transplantation for the purposes of species conservation can be split into two groups depending on the tissue available, but the concepts are similar. First, tissue is collected from the donor species and cryopreserved; then, when required, the tissue is thawed and crushed into a single-cell suspension and injected into a surrogate, which results in donor-derived gamete production in the surrogate leading to the regeneration of the target species. Primordial germ cells (PGCs) can be isolated from cryopreserved embryos or the excised genital ridge tissue. Spermatogonia (SPG) and oogonia (OOG) can be isolated from cryopreserved testis or ovarian tissue respectively. Due to differences in developmental stage and cell receptivity to migratory cues, it is more common for PGCs to be transplanted into an embryo stage surrogate, whereas SPG and OOG are transplanted into more developed surrogates at the larval or adult stages.

in future somatic cells with post-transcriptional control mechanisms required to restrict expression to PGCs (Köprunner *et al.* 2001). When formed, PGCs are large, round cells with granular, light-staining cytoplasm, a large nucleus containing one or more dark-staining nucleoli, a distinct nuclear membrane and dense cytoplasmic inclusions, termed nuage, that contribute to the germplasm required for germ line specification in the next generation (Braat *et al.* 1999; Yoshizaki *et al.* 2002).

Germ cell migration

PGCs must migrate to the region where the gonad will eventually form, the genital ridge, for successful development of the gonad. In zebrafish, migration begins at the blastula stage around 4.5 h post fertilisation (h.p.f.) and ends around 24 h.p.f. Migration is guided by chemotaxis, specifically the chemokine stromal-cell derived factor-1a (SDF-1a) and its receptor chemokine receptor 4 (CXCR4b), which is found on the surface of PGCs. PGCs migrate dorsally from the marginal region of the blastodisc through intermediate targets between somite levels 1–3 at the 1–5 somite stage, and somite levels 5–7 at the 16 somite stage before localising to the gonadal region around

somite level 8 at 24 h.p.f. (Weidinger *et al.* 1999). In the presence of SDF-1a, CXCR4b releases calcium, resulting in myosin contractions on the leading edge of the PGC; these contractions result in a flow of cytoplasm responsible for migration (Blaser *et al.* 2006). PGCs with reduced CXCR4b activity are still capable of migration, but lack direction, often ending up in ectopic locations, whereas reduced SDF-1a activity results in fewer PGCs arriving at the gonad (Raz and Reichman-Fried 2006). During migration, PGCs are protected by the RNA-binding protein Dead end (*dnd*). The *dnd* gene is specifically expressed in the germplasm and PGCs in zebrafish and shares a similar expression pattern to *vasa* (Weidinger *et al.* 2003). Gene knockouts or knockdowns of *dnd* are commonly sterile, and so *dnd* is considered a critical factor for germ cell development in fish (Fujimoto *et al.* 2010; Wong and Zohar 2015; Li *et al.* 2016; Yoshizaki *et al.* 2016).

Differentiation and sexual development

Once reaching the gonadal ridge, the PGCs proliferate. Females appear to have a much faster rate of PGC proliferation than males in some species such as medaka (*Oryzias latipes*), and this

often marks the first distinction of sexual differentiation between males and females (Wootton and Smith 2014b). There is evidence to suggest that a threshold number of PGCs is required to induce female gonad formation (Dai *et al.* 2015). For example, knockout or knockdown of the *dnd* gene results in sterility but, in some species (e.g. zebrafish) treated embryos also develop to be phenotypically male, indicating that the loss of the germ line affects secondary sex characteristics (Dranow *et al.* 2013). In addition, in medaka, mutations to the male sex determining region, the *DM* domain gene on the Y chromosome, or *dmy* result in a 'female-like' PGC proliferation pattern in male medaka and, when *dmy* expression is extremely low, can result in male-to-female sex reversal (Matsuda 2003; Otake *et al.* 2006). However, although PGC number has been shown to affect sex ratios in zebrafish and medaka, gene knockout or knockdown experiments in other species, such as goldfish *Carassius auratus* (Goto *et al.* 2012) and loach *Misgurnus anguillicaudatus* (Fujimoto *et al.* 2010), have not shown a similar effect of PGCs on sexual development or sex ratios.

As the gonad develops, PGCs differentiate into spermatogonia in males and oogonia in females. These cells, although sex specific, are remarkably plastic in that they can reverse depending on the signals from surrounding tissue. For example, in rainbow trout, spermatogonia injected into female surrogate fish reverted and differentiated into oogonia capable of generating oocytes that could be fertilised and produce offspring (Lee *et al.* 2013). Spermatogonia and oogonia are distinguishable from other differentiating germ cells in the gonad because they are both large, round cells with a low nucleocytoplasmic ratio (Papah *et al.* 2013; Lacerda *et al.* 2014; Uribe *et al.* 2014, 2016; Viana *et al.* 2018). Because spermatogonia and oogonia in their primary undifferentiated stage are similar in structure and function, their ability to reverse cell type in response to changes in sex is most likely due to the effects of surrounding somatic cells on gametogenesis. Spermatogonia and oogonia undergo Type 1 mitotic divisions typical to stem cells and subsequent Type 2 divisions signify commitment to gametogenesis in which the cells divide and differentiate synchronously to eventually form mature spermatozoa or oocytes (Nishimura and Tanaka 2014).

Identification of germ line stem cells

Successful identification of PGCs, spermatogonia and oogonia *in vivo* is the first step in piecing together the reproductive development of a species and finding opportunities for *ex vivo* conservation methods. Although the use of transgenic species forms the basis of identification methods, non-transgenic methods, such as fluorescent tags or basic histological analysis, are currently the most accessible identification methods for endangered species.

Visualising PGCs during embryo development

Identifying PGCs and timing their migration during embryogenesis has been critical to the success of techniques such as GCT. Histologically, PGCs are big, circular cells with a large nucleus and electron-dense germplasm (McMillan 2007). Previously, PGC identification relied on these features alone, but the

development of fluorescent tagging methods has made the identification of PGCs in live embryos possible. Germ line-specific genes *vasa* and *nanos3* have been used in transgenic and non-transgenic methods to identify PGCs *in vivo*. The production of transgenic strains, such as rainbow trout *Oncorhynchus mykiss*, that produce green fluorescence in the germ line has been used to track germ cell migration and development (Yoshizaki *et al.* 2000, 2010; Takeuchi *et al.* 2002, 2003; Kobayashi *et al.* 2007; Okutsu *et al.* 2007; Lee *et al.* 2015). Data collected using transgenic embryos has been key to understanding the early formation and migration of PGCs; however, generating transgenic fish is currently not feasible when working with endangered species because of the need for a deeper knowledge of their genetics and the time required to produce transgenic animals.

Chimeric RNA transcripts containing a portion of germ line-specific mRNA adhered to a fluorescent marker such as green fluorescent protein (GFP) can be injected into fish embryos and result in germ line-specific fluorescence (Yoshizaki *et al.* 2005), as shown in Fig. 2. Two non-transgenic methods have been described using the 3' untranslated region (UTR) of *vasa* (Yoshizaki *et al.* 2005) and *nanos3* (Saito *et al.* 2006). Injection of chimeric mRNA results in temporary PGC-specific fluorescence. Although this technique does not allow for permanent identification of PGCs across multiple generations, as in transgenic models, it enables the identification of PGCs for days after injection; for example, GFP-*vasa* 3'UTR mRNA identified PGCs in rainbow trout 50 days after injection (Yoshizaki *et al.* 2005). In addition, the *vasa* and *nanos3* genes appear to be conserved between distantly related species, such that a chimeric RNA optimised in a common species could be used to tag PGCs in a distantly related endangered species. For example, *vasa* 3'UTR mRNA transcript originally isolated from Nibe croaker *Nibea mitsukurii* produced PGC-specific fluorescence in rainbow trout and zebrafish (Yoshizaki *et al.* 2005) and GFP-*nanos3* 3'UTR RNA identified PGCs across many different species: medaka, zebrafish, pearl danio *Danio albolineatus*, goldfish, loach, herring *Clupea pallasii* and ice goby *Leucopis petersii* (Saito *et al.* 2006), sturgeon (genus *Acipenser*; Saito *et al.* 2014), smelt *Hypomesus nipponensis* (Takahashi *et al.* 2017), barfin flounder *Verasper moseri* (Goto *et al.* 2015) and eel *Anguilla japonica* (Saito *et al.* 2011).

The use of transcripts from other species as PGC probes in less-studied fish species allows for easy identification of PGCs without the initial step of *vasa* or *nanos3* transcript isolation and sequencing. A disadvantage of using chimeric RNA is that inconsistencies in the injection technique between embryos may result in some PGCs not being identified, thereby lowering the number of cells available for transplant. For example, the number of PGCs identified in different species was extremely varied among embryos, ranging from 5 to 53 cells in zebrafish, 5 to 43 cells in pearl danio, 0 to 29 cells in loach, 6 to 90 cells in goldfish, 3 to 54 cells in medaka and 4 to 24 cells in the ice goby (Saito *et al.* 2006). Variation in PGC number is observed in transgenic species that presumably produce fluorescence in 100% of PGCs (Kobayashi *et al.* 2004), but observations of mRNA-injected embryos showed uneven fluorescence in the early blastodisc stages before PGC formation confirming

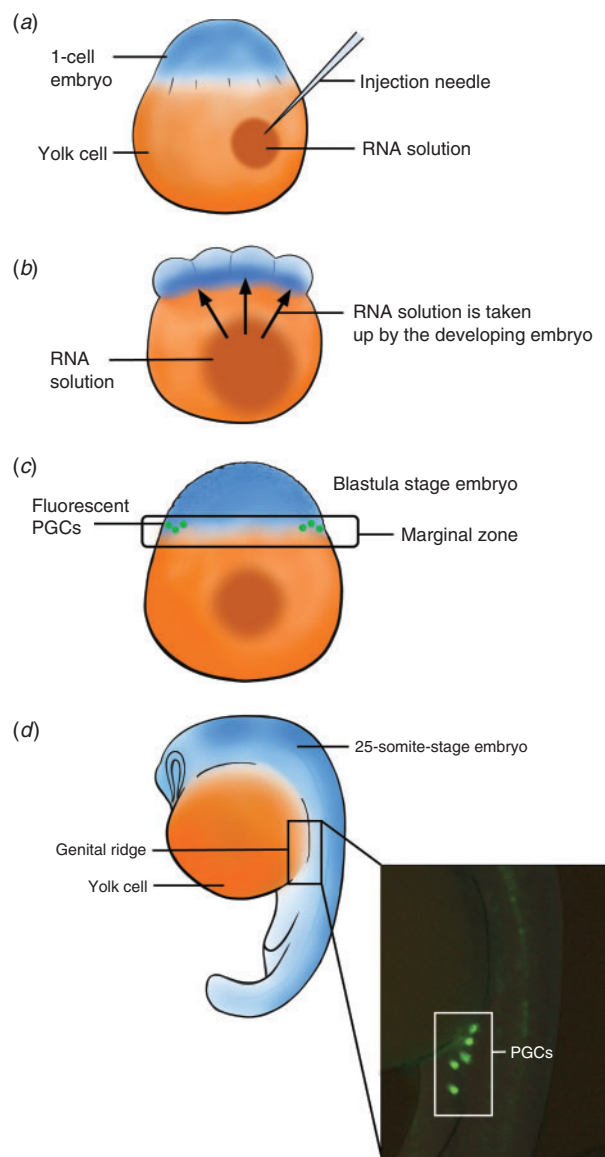


Fig. 2. Identification of primordial germ cells (PGCs) using chimeric RNA transcripts relies on the gradual segregation of the cytoplasm from the yolk during early embryo genesis. (a) In zebrafish, the RNA solution is injected into the yolk of the 1-cell embryo. (b) As the embryo develops and divides, it draws up the injected RNA (c), which is distributed throughout the embryo but readily degraded in somatic cells, leading to PGC-specific fluorescence (d) that allows tracking of PGCs throughout their migration to the genital ridge.

uneven mRNA distribution (Saito *et al.* 2006). In the absence of alternative non-transgenic methods, injection of chimeric RNA is currently the most effective way of gathering information on germ cell migration in endangered species.

Visualising spermatogonia and oogonia in the gonad

Spermatogonia and oogonia are embedded deep in the body cavity and cannot be easily visualised in living animals.

Histology is the primary method of identifying spermatogonia and oogonia during development. Spermatogonia can be identified by their large size, usually 10–15 μm (Kise *et al.* 2012), and multiple dark staining nucleoli (Wootton and Smith 2014a). Oogonia are similar in size to spermatogonia, but they are one of the smaller cells in the fish ovary because oocytes increase markedly in size during development. Similar to spermatogonia, oogonia are identified by their large nuclear to cytoplasmic ratio and nucleoli containing nucleus and more condensed chromatin compared with early stage oocytes (McMillan 2007). Using transgenic species of rainbow trout that produce fluorescent gonial cells can improve identification of spermatogonia and oogonia in juvenile fish during early stages of development when they are transparent. However, as fish develop, increased pigmentation of the skin and internal membranes can reduce visibility of the gonad. Although identification of spermatogonia and oogonia is challenging in live fish, there is an assumption that the cells are present in the gonad if the animal displays signs of sexual maturity, such as mature colouration and mating behaviour.

Reduced reproductive fitness in the wild often underpins the decline and eventual extinction of a species (Blomqvist *et al.* 2010). The identification and subsequent description of germ cell formation and differentiation mechanisms provide basic knowledge of reproductive development that can be manipulated to assist with species conservation management. New methods, such as the cryopreservation of early germ line cells in combination with cell surrogacy, mean that the genetic diversity of fish populations can be saved before they reach critical levels. This genetic diversity is then available for reintroduction, where needed, to alleviate the effects of inbreeding depression and to boost population numbers.

Cryopreservation

Collections of cryopreserved cells and tissues, often referred to as biobanks, cryobanks or ‘frozen zoos’, have been a popular method for safeguarding the genetics of endangered species for several decades (Clarke 2009). Because the physiological complexity of fish oocytes and embryos has prevented successful cryopreservation, targeting early stages of the germ line such as PGCs, oogonia and spermatogonia has become an alternative method of storing fish genetics.

Primordial germ cells

Despite problems recovering viable fish embryos after cryopreservation, extracting viable PGCs from cryopreserved embryos has been successful (Higaki *et al.* 2010, 2013). PGCs can also be cryopreserved as a single-cell suspension (Riesco *et al.* 2012) or extracted from cryopreserved genital ridge tissue from developing embryos (Kobayashi *et al.* 2007). Generally, extraction of PGCs from genital ridge tissue or whole embryos is preferable because it reduces the loss of cells and improves viability. PGCs cryopreserved as a single-cell suspension also have more DNA damage and a lower survival rate, around 25%, compared with 80% viability and less than 10% DNA damage in PGCs recovered from cryopreserved embryos or genital ridge tissue (Riesco *et al.* 2012).

Table 1. Reported cases of cryopreservation of fish gonadal tissue and embryos (for the purposes of sourcing primordial germ cells (PGCs)) with the most successful cryoprotectant and the highest post-thaw viability listedBSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EG, ethylene glycol; FBS, fetal bovine serum; PG, propylene glycol; *pvasa-Gfp*, green fluorescence produced in presence of *vasa* gene expression; PVP, polyvinylpyrrolidone

Species (common name)	Cell type	Post-thaw cell viability (%)	Cryoprotectant	References
<i>Acipenser ruthenus</i> (sterlet)	Testicular tissue	~85.5	1.5 M EG, 50 mM glucose, 0.5% BSA	Golpour <i>et al.</i> (2016)
<i>Acipenser baerii</i> (Siberian sturgeon)	Testicular tissue	~98	1.5 M EG, 50 mM glucose, 0.5% BSA	Pšenička <i>et al.</i> (2015)
	Dissociated testicular cells	~62	1.5 M EG, 50 mM glucose, 0.5% BSA	
	Ovarian tissue	~93	1.5 M EG, 50 mM glucose, 0.5% BSA	
	Dissociated ovarian cells	~66	1.5 M DMSO + propanediol, 50 mM glucose, 0.5% BSA	
<i>Asterropteryx semipunctata</i> (goby)	Testicular tissue		1.3 M DMSO, 0.1 M trehalose, 1.5% BSA	Hagedorn <i>et al.</i> (2018)
<i>Brachymystax lenok</i> (Manchurian trout)	Testicular tissue	81 ± 1.3 ^B	1.3 M methanol, 0.2 M trehalose with 10% egg yolk	Lee and Yoshizaki (2016)
<i>Danio rerio</i> (zebrafish)	PGCs (in embryos)	92.3 ± 13.9 ^C	5 M DMSO, 1 M EG, 4% PVP	Riesco <i>et al.</i> (2012)
	PGCs (in yolk-depleted embryos)	81	Pretreatment solution: 2 M EG, 1 M DMSO Vitrification solution: 3 M EG, 2 M DMSO	Higaki <i>et al.</i> (2013)
<i>Gnathopogon caeruleus</i> (cyprinid honmoroko)	Dissociated testicular cells	Adult: 64.7 ± 9.1 ^A Juvenile: 52.2 ± 9.5 ^A	2 M DMSO, 1 M acetamide, 3 M PG	Higaki <i>et al.</i> (2017)
<i>Oncorhynchus mykiss</i> (<i>pvasa-Gfp</i> transgenic rainbow trout strain)	Testicular tissue	33.5 ± 7.1 ^B	1.3 M DMSO, 0.1 M trehalose, 10% v/v egg yolk	Lee <i>et al.</i> (2013)
	Ovarian tissue	72.9 ± 6.2 ^B	1.0 M DMSO, 0.1 M trehalose, 10% egg yolk	Lee <i>et al.</i> (2016)
	PGCs (in genital ridge)	73	1.8 M EG, 5.5 mM D-glucose, 0.5% BSA	Kobayashi <i>et al.</i> (2003)
<i>Salmo trutta</i> (brown trout)	Ovarian tissue	40.34	Equilibration solution: 1.5 M MeOH, L-15 medium supplemented with 10% FBS, 25 mM HEPES, 0.5 M trehalose Vitrification solution: 3 M PG, L-15 medium supplemented with 10% FBS, 25 mM HEPES, 0.5 M trehalose	Lujić <i>et al.</i> (2017)
<i>Takifugu rubripes</i> (tiger puffer)	Testicular tissue	61.2 ± 2.7 ^B	1.3 M DMSO, 0.1 M trehalose, 10% egg yolk	Yoshikawa <i>et al.</i> (2018)
<i>Tinca tinca</i> (tench)	Testicular tissue	57.69 ± 16.85 ^A	1.5 M glycerol, 50 mM D-glucose, 0.5% BSA	Linhartová <i>et al.</i> (2014)

^AMean ± s.d.^BMean ± s.e.m.^CNot specified.

In addition to post-thaw viability and low DNA damage, the migratory capacity of PGCs must be maintained after cryopreservation if these cells are to be used for GCT. During GCT, PGCs must be able to navigate and migrate through the surrogate embryo to the developing gonad to be able to colonise and differentiate into viable gametes. Pseudopodial activity is a critical indicator of the migratory ability of the PGCs. In fish embryos, cryopreservation of PGCs is known to be inhibited by the yolk sac, and partial removal of yolk before cryopreservation has been observed to improve pseudopodial movement in zebrafish PGCs after thawing (Higaki *et al.* 2013). Although depletion of the yolk did not result in viable embryos after thawing, yolk-depleted, 14- to 18-somite-stage embryos were twice as likely to have PGCs with intact membranes. In addition, post-thaw PGCs from seven of 12 embryos showed pseudopodial movement, indicating they retained migratory ability, which would be imperative for successful GCT (Higaki *et al.* 2010, 2013).

The combination of PGC cryopreservation and subsequent cell transplantation has produced viable gametes in transgenic

(*pvasa-Gfp*) rainbow trout (Kobayashi *et al.* 2007), demonstrating the feasibility of combining cryopreservation and transplantation to regenerate fish populations. Although long-term post-thaw viability of fish embryos remains elusive, PGCs collected from cryopreserved embryos or the genital ridge present an achievable target for fish germplasm banking in endangered species.

Spermatogonia and oögonia

Access to embryos from endangered species may be limited if only one sex of the species is available or if breeding is not possible due to age or declining reproductive fitness. In these cases, gonadal tissue from juvenile or adult fish can be collected and cryopreserved. This tissue contains sex-specific derivatives of PGCs, spermatogonia in males and oögonia in females. Spermatogonia and oögonia have been isolated from cryopreserved tissues in various species, as indicated in Table 1. Past literature is skewed towards the cryopreservation of spermatogonia, although in the past 5 years more evidence

of successful oogonial cell cryopreservation has been published (Lujic *et al.* 2017).

Similar to PGCs, cell viability is improved when spermatogonia or oogonia are cryopreserved within gonadal tissues as opposed to in a single-cell suspension (Hagedorn *et al.* 2018). Cryomedia are often composed of a mixture of permeating and non-permeating cryoprotectants, as well as protective additives, including egg yolk, bovine serum albumin and fetal bovine serum. Varying concentrations of dimethyl sulfoxide, trehalose and egg yolk tend to outperform other cryoprotectants across multiple species, including rainbow trout (Lee *et al.* 2013, 2016), Nile tilapia *Oreochromis niloticus* (Lacerda *et al.* 2010) and tiger puffer *Takifugu rubripes* (Yoshikawa *et al.* 2018). Because spermatogonia and oogonia are more differentiated when they are collected, they do not possess the migratory ability of PGCs; however, these cell types are usually transplanted directly into the gonad of sterile adult fish or into the intraperitoneal cavity of juvenile fish. For this reason, it is not necessary for these cells to retain any migratory ability, so these samples can be collected at any stage of adult fish life. In addition, because these cells are present from the beginning of gonadal development, there is more flexibility for sample collection compared with spermatozoa and oocytes, which may only be available during seasonal spawning periods.

The cryopreservation of early germ cells gives conservation biologists a feasible means of storing genetic material from endangered species with relatively high viability compared with targeting differentiated cells types, such as spermatozoa and especially oocytes. Because these cells are already committed to the germ cell fate, they are easier to differentiate than cells collected from somatic tissue, such as fin clips, which have been used in somatic cell nuclear transfer in zebrafish but did not result in live adult fish (Siripattarapavat *et al.* 2009).

Sterilisation of the surrogate

Prior to injection of donor germ cells, a recipient must be sterilised to ensure the exclusive production of donor gametes. Although there are many different methods of sterilisation, all have their drawbacks and must be carefully considered on a species-by-species basis. The most effective method of sterilisation usually depends on germ cell formation and the age at which the sterilisation occurs; for example, sterilisation at the embryonic stage before germ cell development will likely require a different approach to sterilisation in mature adult fish that have established gametogenesis. Sterilisation at the embryo stage is often focused on genetic manipulation, such as gene knockout or knockdown therapies, that prevent the germ line from forming, whereas sterilising adult fish requires treatment with chemotherapeutic drugs that target germ cells based on their rapid proliferation.

Transgenic and non-transgenic gene expression manipulation

During embryogenesis, key genes are activated that are associated with germ cell development and survival. These genes include *vasa*, *nanos3*, *dnd* and critical elements of the migration pathway. Genes can be knocked out in transgenic strains or

knocked down by the injection of antisense morpholino oligonucleotides (AMO), such as *dnd*-AMO, which results in disruption to the signalling pathways, leading to PGC death or ectopic migration, leaving the future gonad devoid of germ cells and the adult fish unable to reproduce.

The use of *dnd*-AMO for the knockdown of *dnd* expression has resulted in sterility in many species, including medaka (Li *et al.* 2016), zebrafish (Slanchev *et al.* 2005; Siegfried and Nusslein-Volhard 2008), loach (Fujimoto *et al.* 2010), rainbow trout (Yoshizaki *et al.* 2016) and goldfish (Goto *et al.* 2012). Generally, *dnd*-AMO is injected directly into the yolk or cytoplasm of the early stage embryo, where it is absorbed and can result in 100% germ cell depletion. However, injecting individual embryos can be time consuming. Embryos immersed in media containing *dnd*-AMO have shown similar results to direct injection of *dnd*-AMO but, to achieve 100% ablation in this case, the embryos must be produced by IVF, not natural spawning, indicating that the timing of treatment affects success (Wong and Zohar 2015). As mentioned previously, knockdown of *dnd* can result in phenotypically male populations in some fish species (Dai *et al.* 2015), but sex-reversal can be induced by treatment with oestrogen to produce sterile, phenotypically female fish (Slanchev *et al.* 2005).

Although the use of *dnd*-AMO has been successful in many fish species, preparing a host embryo using this method requires the development of additional techniques, such as artificial fertilisation or specifically timed mating, to ensure embryos are available for injection within a specific time frame. The method of microinjection is also determined by the egg structure and early embryogenesis of the selected host species. For species conservation, it is possible that the biology of host species selected as surrogates based on their relatedness to the donor species may be relatively undescribed. Such hosts will require substantial optimisation before the successful application of this method of sterilisation.

Altering chromosome number

Sterility can also be induced by altering the chromosome number, or ploidy, after fertilisation by preventing extrusion of the second polar body, resulting in the retention of a third set of chromosomes by the embryo (Hammed *et al.* 2010). By inducing sterility this way, many embryos can be treated together with relatively high success. Heat shock, cold shock and pressure shock can be used to induce polyploidy, specifically triploidy. Although triploidy in other animal groups is often fatal, fish tolerate the triploid state but are sterile (Pearson 2001).

Applying immense mechanical pressure to embryos early after fertilisation prevents exclusion of the polar body. This method has been used in large farmed species such as rainbow trout (Chourrout 1984; Couture *et al.* 2007). Pressure shock in the range of 34 000–76 000 kPa, depending on the species (Benfey and Sutterlin 1984; Cassani and Caton 1986; Malison *et al.* 1993; Peruzzi and Chatain 2000; Huergo and Zaniboni-Filho 2006), is often used by large aquaculture facilities to induce triploidy and prevent sexual maturation in food fish. However, the equipment needed to apply hydrostatic pressure is not always available for smaller operations, and alternative methods, such as thermal shock, are used. The use of cold shock

or heat shock can depend on the natural rearing temperature of the species. For example, cold-water species such as the Atlantic salmon responded poorly (0–4% triploidy) to cold shock but respond favourably to heat shock (66–100% triploidy; [Peruzzi et al. 2007](#)). However, this may only apply to some species. In the blue tilapia *Oreochromis aureus*, a warm-water species, heat shocking at a mean (\pm s.d.) temperature of $39.5 \pm 0.2^\circ\text{C}$ for 3.5–4 min resulted in 100% triploidy with 61% survival ([Don and Avtalion 1986](#)). In contrast, the red tilapia, a hybrid of blue tilapia and Mozambique tilapia (*Oreochromis mossambicus*) and a close relative of *O. aureus*, responded to cold shock for 30 min at 9°C with a 98.7% triploidy rate and 75.8% survival to the yolk sac stage ([Pradeep et al. 2014](#)).

Inducing DNA damage in the germ line

Irradiation with ultraviolet (UV) light can result in DNA damage that prevents germ cell formation but must be germ cell specific to prevent genetic abnormalities and high mortality in the embryos. In many species this may not be possible because the germplasm is embedded within the embryo. However, it is possible in sterlet *Acipenser ruthenus* because, in this species, the germplasm localises at the vegetal pole in the early stages of embryogenesis. Dechorionated sterlet embryos suspended in water naturally orientate animal pole up, with the germplasm on the opposite pole underneath. Because of this orientation, UV irradiation underneath the embryos results in disrupted germplasm and subsequent sterilisation without damage to the animal pole ([Saito et al. 2018](#)). Other species with similar germplasm localisation could also be sterilised using this method.

Treatment with chemotherapeutic agents

In some species, spermatogonia and oogonia proliferate in response to increasing temperature and can be targeted by cytotoxic drugs such as busulfan. Treatment with multiple injections of busulfan combined with increased tank temperature has induced sterility in Nile tilapia ([Lacerda et al. 2010](#)), Patagonian pejerrey *Odontesthes hatcheri* ([Majhi et al. 2014](#)) and yellow tail tetra *Alestotepersius caudalis* ([de Siqueira-Silva et al. 2015](#)). Although sterilisation using this method has been successful, some species had higher female mortality due to increased ulceration after injection, which was not present in males ([Majhi et al. 2014](#)). Not all species respond in this way to changes in temperature, so this method is usually restricted to warm-water species ([Saito et al. 2018](#)). Regardless of its potential toxicity, busulfan is the only method of sterilisation for adult fish and is therefore a commonly used method of sterilisation because intervention at the embryo stage is often not feasible for species that take many years to reach sexual maturity.

Isolating germ cells for downstream applications

To improve germ cell transplant success, the suspension of germ cells to be injected into a surrogate should be enriched with a high concentration of the target cells (spermatogonia, oogonia or PGCs). Therefore, successful GCT requires the development of robust methods to isolate these cells from surrounding gonadal tissue.

Visual selection of cells

Isolation and identification of PGCs from the surrounding cells in a developing embryo is relatively simple if they produce fluorescence, such as in embryos injected with GFP-*nos3* 3'UTR or transgenic embryos. Embryos are crushed or enzymatically digested using a mixture of collagenase, trypsin and sodium citrate, and the resulting single-cell suspension is then assessed under a fluorescent microscope and the fluorescing PGCs are selected ([Saito et al. 2008](#)). However, this process can be time consuming because only a few PGCs are present in the embryo, and these must be identified and visually isolated from a suspension of cells. In contrast, spermatogonia and oogonia are often present in large numbers, so more high-throughput techniques of isolating these cells are required.

Density gradient-based separation of cells

Separation of cell suspensions by density gradient centrifugation is a basic but effective method of size-based cell separation. Gradients consisting of layers of increasing densities of Percoll act as a 'molecular sieve', allowing smaller cells to pass through while capturing larger cells in the top layers. Successful separation of cells produces multiple 'bands' within the gradient, with each band corresponding to a different population of cells. Because spermatogonia and oogonia are large, density gradient-based sorting has been used successfully to isolate large germ cell populations in several fish species, including sturgeon *Acipenser baerii* ([Pšenička et al. 2015](#)), Nile tilapia ([Lacerda et al. 2010](#)), rainbow trout ([Bellaiche et al. 2014](#)), common carp ([Franěk et al. 2019](#)), Patagonian pejerrey ([Majhi et al. 2014](#)), tench *Tinca tinca* ([Linhartová et al. 2014](#)) and catfish *Ictalurus furcatus* ([Shang et al. 2018](#)). Isolation of gonial cells by gradient centrifugation is cost effective, but lacks specificity because it sorts only on size and cannot exclude somatic cells, such as Sertoli cells and Leydig cells in the testis. It also requires a large number of cells to be successful, with protocols requiring tissue from many animals. This limits its application in smaller endangered species, which may not have enough cellular content to be visible in the gradient.

Cell sorting by flow cytometry

Flow cytometry allows for the analysis of individual cells in a suspension based on their light-scattering properties and, if available, fluorescence. By analysing each individual cell in a suspension, populations of rare cells can be easily detected and separated. Isolation is relatively straightforward when using transgenic species because gonial cells can be separated based on fluorescence alone ([Takeuchi et al. 2002](#); [Kobayashi et al. 2004](#); [Okutsu et al. 2006a](#); [Yoshizaki et al. 2010](#)). However, the forward scatter (FSC) and side scatter (SSC) properties of spermatogonia from transgenic species can be extrapolated across cell suspensions from non-transgenic animals. For example, by gating events based on the FSC and SSC profiles of transgenic rainbow trout spermatogonia, cells from wild-type rainbow trout, Japanese char *Salvelinus leucomaenis* and masu salmon *Oncorhynchus masou* were isolated ([Kise et al. 2012](#)). Subsequent transplantation of these cells into surrogates resulted in significantly higher colonisation rates (mean (\pm s.d.)

78.3 ± 8.0%, 80.0 ± 19.0% and 48.8 ± 1.0% for cells from rainbow trout, masu salmon and Japanese char respectively) compared with unsorted cells, for which colonisation rates ranged from 0% to 29.0 ± 4.0%, demonstrating that the gate successfully captured an enriched sample of spermatogonial cells (Kise *et al.* 2012). Gating events in a high FSC region also successfully isolated spermatogonia in Pacific blue fin tuna *Thunnus orientalis* (Ichida *et al.* 2017). Given that FSC correlates with the size of the cell, size-specific beads have been used to model the scatter properties of certain cell sizes. In species where the size of the spermatogonial cells is known, a size-specific gate is set based on the FSC of the beads with the assumption that events captured by this gate will contain the target cells. This method has been used successfully to isolate spermatogonia from the starry goby *Asterropteryx semipunctata* (Hagedorn *et al.* 2018); however, aside from injecting these isolated cells into a recipient fish to produce mature spermatozoa, validation of the presence of the target germ cells in the isolated population is difficult. Therefore, the isolated cell suspensions are often referred to only as 'large cells' with a presumption that spermatogonia are present.

Transplantation

GCT is the primary method for differentiating cryopreserved gonial cells into functional gametes that can give rise to donor-related offspring. Compared with other methods, such as *in vitro* germ cell differentiation, using a surrogate has the benefit of germ cell production throughout a reproductive lifespan. Although testicular cell cultures from the cyprinid honnoroko *Gnathopogon caeruleus* have been able to produce motile spermatozoa, production declined within months (Higaki *et al.* 2017). For endangered species with a proven regression in reproductive success, the ability to 'offload' reproductive function onto a more fecund species is a unique method to propagate new individuals and improve population numbers. Although many publications have described successful migration and gametogenesis, GCT resulting in the production of viable donor offspring has only been reported in a few species, namely zebrafish, pearl danio, loach, goldfish (Saito *et al.* 2008) and rainbow trout (Takeuchi *et al.* 2003; Kobayashi *et al.* 2007). The available literature indicates that several factors are important for transplantation success, including the appropriate timing of GCT in the donor and surrogate species, the transplantation method used and the effect of recipient sex on germ cell differentiation.

Timing of PGC migration affects transplantation success

The mechanisms behind PGC migration are highly conserved, as demonstrated by the successful migration of zebrafish PGCs in the distantly related Japanese eel (Saito *et al.* 2011). However, the migratory period of PGCs is species specific and must be carefully considered to ensure adequate migration and colonisation of the gonad. For example, PGCs collected from 10- to 15-somite-stage pearl danio embryos colonised the genital ridge in 50% of recipient zebrafish embryos when injected at the blastula stage (Saito *et al.* 2008). However, in transplants where PGCs were isolated later at the 25 somite stage, no colonisation

occurred but some embryos did have PGCs localised along the normal migration route, indicating some migratory capacity was maintained at this stage but not sustained long enough for complete migration (Saito *et al.* 2008). Similar findings were reported in rainbow trout, with a migratory period occurring around 20–35 days post fertilisation (d.p.f.; Takeuchi *et al.* 2003). Cells collected from the blastula (2.5 d.p.f.) and gastrula (6 d.p.f.) stages and injected into 35-d.p.f. hatchlings were unable to colonise the genital ridge, whereas cells transplanted from 35-d.p.f. hatchlings into the same age had a colonisation rate of 21.6%, indicating that earlier stage cells were not yet receptive to migratory cues in the host. The host environment plays an equally important role because cells from 35-d.p.f. trout had declining colonisation rates when transplanted into older hosts, with 10% success in 40-d.p.f. hosts and 0% in 45-d.p.f. hosts (Takeuchi *et al.* 2003).

Because these mechanisms are so highly conserved, the efficiency of migration is not related to the phylogenetic distance between the host and donor. In some cases, transplants between distantly related species result in higher colonisation success. For example, in a study comparing PGC colonisation rates between phylogenetically diverse groups, zebrafish to zebrafish PGC transplants had a colonisation rate of 28.9%, whereas PGCs from goldfish localised to the gonadal region in 63.5% of zebrafish hosts (Saito *et al.* 2010). This may be attributed to differences in the length of migration in different species. PGCs collected from species with a relatively long migratory period may have more opportunity to respond to the cues in the recipient embryo, thereby increasing the chance of colonisation (Saito *et al.* 2010; Goto and Saito 2019).

Currently the evidence suggests that knowledge of the commencement, conclusion and length of PGC migration in both the donor and host is critical to transplantation success. The use of GFP-*nos3* 3'UTR mRNA and GFP-*vasa* 3'UTR mRNA transcripts has made the mapping of PGC migration in different species more easy and accessible for endangered species. Although troubleshooting of poor colonisation rates often focuses on migration, many other aspects of germ cell development may affect transplant success, such as the effects of genetic or hormonal cues that are not conserved across species. These mechanisms are rarely studied in the context of GCT and represent a significant gap in our knowledge of how introduced germ cells may navigate an unknown environment.

Transplantation techniques after the embryo stage

Because spermatogonia and oogonia are more differentiated than PGCs, they have reduced migratory ability and must be injected directly into the gonad or the region where the gonad will form in juvenile or adult fish. Cells can be tagged with fluorescent markers before injection to assist with visualisation during and after injection. For example, PKH26 is a red-fluorescing cell membrane label that allows cell tracking *in vivo* for over 100 days. PKH26 has been used to tag cells in various fish species, including tiger puffer *T. rubripes* (Hamasaki *et al.* 2017), Chinese sturgeon *Acipenser sinensis* (Ye *et al.* 2017) and Nile tilapia (Farlora *et al.* 2014). Spermatogonia and oogonia can be transplanted into the peritoneal cavity of juvenile fish, where they will migrate towards the

genital ridge, which lies along the dorsal wall (Yoshizaki and Lee 2018). Embryo and larval-stage surrogates lack a developed immune response (Yoshizaki *et al.* 2012). The fish immune system is similar to that of mammals, including a cell-specific immune rejection driven by the thymus and T cell-like cells (Chilmonczyk 1992). It is therefore possible that adult surrogates could produce an immune response to the transplanted cells, resulting in poor transplantation efficiency. Thus, the use of immunosuppressed or immunodeficient surrogates may avoid low success rates due to immune rejection (Okutsu *et al.* 2006b), but generating these animals may be difficult for the purposes of working with endangered species. However, there is evidence that regions of the gonad are immune privileged (i.e. they are isolated from the blood supply and therefore unable to initiate an immune response; de Siqueira-Silva *et al.* 2018). Other than poor cell colonisation rates, there are few published data relating to the immune response in cell transplant recipients. This suggests that further investigation into the immunological physiology of surrogates is warranted to determine specific mechanisms that could affect transplant efficiency.

In adults, cells can be introduced directly into the gonad by surgical or non-surgical methods. Pejerrey *Odontesthes bonariensis* spermatogonia injected into Patagonian pejerrey *O. hatcheri* testes via surgical intervention colonised the gonad in two of the three fish assessed 4 weeks after transplantation, and 20% of recipients produced donor-derived spermatozoa 8 months after transplantation (Majhi *et al.* 2009). Immune rejection and reabsorption of the transplanted cells may have accounted for the lack of gamete production in a further 16 treated fish but, as mentioned previously, there is little evidence to confirm this. A non-surgical method using a catheter to inject *Jundia* catfish spermatogonia into Nile tilapia testes via the spermatic duct resulted in donor-derived sperm production in all surrogates (Silva *et al.* 2016). Although surgical transplantation methods have shown success, they do pose an increased risk to the surrogate because of the higher anaesthetic dose required and the greater risk of infection after surgery; because of this, non-surgical interventions are more popular.

Species relatedness and the importance of host sex in transplantation success

GCT is possible between distantly related species, but there appears to be a strong effect of the sex of the host on the success of GCT, with more successful results from male surrogates. PGCs isolated from goldfish and loach differentiated into spermatozoa after transplantation into a sterile zebrafish embryo. Despite divergence between goldfish and zebrafish approximately 50 mya and divergence between loach and zebrafish 133.4 mya, the mechanism of spermatogenesis is conserved (Saito *et al.* 2008). However, this was not observed in female chimeras. Goldfish or loach PGCs transplanted into female zebrafish did not undergo oogenesis (Saito *et al.* 2008). Because oogenesis requires more maternal input from surrogates, in particular the synthesis and deposition of lipids to form yolk, it is possible that the lack of success of these GCTs reflects interspecies differences in oogenesis (Lubzens *et al.* 2010). However, in the goldfish and loach transplants, no germ cells were found even in the early stages. This was also noted in

zebrafish PGC transplants into hybridised zebrafish and pearl danio recipients. Female chimeras had no identifiable germ cells and the gonads were underdeveloped and threadlike (Wong *et al.* 2011). However, it is important to indicate that in that study control hybridised zebrafish and pearl danio females also had poor ovary structure compared with hybridised males (Wong *et al.* 2011). Hybridisations in carp have also resulted in poor female outcomes; in common carp and goldfish, oogenesis was observed in hybrids but not beyond the yolk globule stage (Yamaha *et al.* 2003).

Although less reported, there have been some transplant successes in female fish. For example interspecies oogonial cell transplants between Patagonian pejerrey and pejerrey resulted in oocytes capable of producing viable offspring (Majhi *et al.* 2014), as did allogenic transplants in rainbow trout, (Yoshizaki *et al.* 2010; Lee *et al.* 2016). In Chinese sturgeon, oogonial cells successfully colonised the gonad in 40% of recipients ($n = 10$) compared with 70% for spermatogonia ($n = 10$), but assessment of oogenesis was not performed (Ye *et al.* 2017). There is a skew in the literature regarding GCT in males versus females. Although it is difficult to ascertain whether this is due to low success rates using female recipients or a lack of research, what is evident from the available publications is that oogenesis is more sensitive to genetic abnormalities and interspecies differences.

Next-generation effects of GCT

Few data are available on the downstream effects of GCT on future generations.

Although the gametes that result from PGC transplantation represent the donor species, the gonadal environment in which those gametes develop and mature is controlled by the surrogate, so there is potential for interspecies differences to arise. For example, pearl danio PGCs transplanted into a zebrafish resulted in oocytes the same size as zebrafish eggs and embryos that were smaller than average pearl danio embryos. However, these embryos had pigmentation that was similar to pearl danios and matured into fertile pearl danio adults (Saito *et al.* 2008). Although these differences in size are small and did not affect the fertility of the offspring, they clearly show that the surrogate can influence gamete production in ways that alter normal embryo development. Further investigation into the extent of the effect of the surrogate on offspring outcomes is paramount if these techniques are to be considered for mass application in endangered species.

Practical applications for conservation and concluding remarks

Targeting early germ line cells as a means of securing endangered fish genetics has greater application for long-term conservation strategies. Compared with conservation methods that aim to store gametes such as spermatozoa, cryopreservation of PGCs, spermatogonia and oogonia has a clear advantage in that each individual cell has the potential, when transplanted into the surrogate, to generate gametes for the lifetime of that individual. As broodstock, these surrogates can be used to establish breeding programs for threatened species and potentially

produce millions of larvae for restocking programs. The collection of these early germ line cells is therefore far more efficient than the cryopreservation of spermatozoa or milt, because these samples are limited by the sperm population in a single ejaculate. In the context of conservation, the use of germ cell surrogacy is better suited to producing large numbers of offspring because, by selecting highly fecund surrogates, the number of gametes available can be greatly increased compared with the reduced reproductive capabilities of the threatened donor species. Considering that conservation programs often require thousands of new individuals to be produced over many years to successfully support dwindling populations, the application of germ cell surrogacy is arguably better suited to support large-scale fish conservation efforts than the cryopreservation of spermatozoa, eggs and embryos.

A great challenge in applying these methods to threatened species is that their reproductive biology is often poorly understood. Most of the previous research is based on farmed species, such as salmonids, and although many aspects of germ cell development in fish are conserved, the translation of these methods into endangered species will require optimisation. Australia in particular is home to many endemic species that, due to the dry climate, have evolved in isolation in restricted freshwater river systems. Although previous studies suggest that spermatogenesis is well conserved in the bony fishes, the production of donor eggs via germ cell surrogacy will most likely require the use of host species that are closely related to the donor. Because no germ cell surrogacy has yet been attempted in an Australian fish species, the development of techniques, particularly in relation to host sterilisation and cell transplantation, will require a significant research effort to determine which species will make suitable hosts for early germ cells from Australian fish.

In the absence of alternative *ex situ* conservation methods, cryopreservation of gonadal tissue combined with GCT is a unique, effective and adaptable strategy for the conservation of endangered fish. Unlike fish oocytes and embryos, early stage germ cells have relatively high viability rates after cryopreservation and offer a tangible and simple method of storing valuable fish genetics for future use. Germ cell transplantation has proven to be a feasible method for the production of gametes from donor cells in some species (Takeuchi *et al.* 2003; Kobayashi *et al.* 2007; Saito *et al.* 2008) and the introduction of non-transgenic methods make it more accessible than ever before for endangered species (Saito *et al.* 2006). However, there are many gaps in our understanding of fish reproductive biology, such as the effect of endocrine and paracrine signalling during gametogenesis, which may account for low reporting of oocyte production in female surrogates. Further investigation into the underlying mechanisms of oogenesis and how this process differs among species may improve gamete production in female surrogates. Nevertheless, optimisation of the GCT method does not negate cryopreservation of gonadal tissue as an achievable means of storing the genetics of important fish species now before species and genetic diversity are lost forever. A changing climate and increasing environmental pressures make *ex situ* conservation methods an essential approach for safeguarding endangered species from potentially toxic and

unsustainable environments. With fish populations in decline, it is imperative that all possible conservation strategies are investigated to ensure a continuing global biodiversity of fish populations.

Conflicts of interest

The authors declare no conflicts of interest.

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