



NOTE

Conservation of coral reef fishes: a field-hardy method to cryopreserve spermatogonial cells

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Abstract The biodiversity of marine fishes is threatened globally by climate change and other anthropogenic activities, particularly in coral reef ecosystems. We present a simple, field-hardy method to cryopreserve marine fish gonads, targeting spermatogonial cells (undifferentiated diploid germ cells) with the ultimate goal of permitting recovery of threatened species and populations via gonadal diploid germ cell transplantation technologies. The use of a simplified cryopreservation extender based on L-15 medium resulted in minimal decline in spermatogonial cell viability post-thaw. Moreover, we compared post-cryopreservation viability of sperm and spermatogonial cells from gonads cryopreserved with freshly prepared cryoprotectant and with cryoprotectant prepared in advance and stored at -20°C . We found no significant difference, suggesting that these solutions may be prepared in advance and frozen, ready for later use. We urge conservation, academic, and regulatory agencies to cryobank fish gonads as part of their sample collection processes to support the biodiversity and security of valuable marine fish resources, alongside other restoration efforts.

Keywords *Asterropteryx semipunctata* · Collection protocol · Cryopreservation · Primordial germ cells · Testes · Vouchers

Introduction

Marine and freshwater fishes worldwide provide vital ecosystem functions such as a source of food, nutrient cycling, food web dynamics regulation, and increased resilience in coral reef ecosystems (Hughes 1994; Holmlund and Hammer 1999; Bellwood et al. 2004). Yet the world's ~35,000 recognized species of fishes in freshwater and marine ecosystems are under severe threat from habitat fragmentation and degradation, introduction of exotic species, pollution, overfishing, and climate change (Arthington et al. 2016). In particular, coral reefs support over a quarter of all marine life in the oceans, but reef habitats are severely threatened (GCRMN 2021). As these reef habitats decline, so does coral reef fish diversity and abundance (Jones et al. 2004; Pratchett et al. 2008).

We focus on a simple field-hardy method to secure the biodiversity and genetic diversity of fishes using modern reproductive technology: spermatogonial cell cryopreservation. These cells are early stage male germ cells that are still diploid, having not yet undergone meiosis, and retain the ability to undergo both oogenesis and spermatogenesis. This method is based on the pioneering work of Dr. Yoshizaki et al. (2010, 2011); Lee et al. (2013, 2016), who revolutionized fish conservation by defining the parameters of fish spermatogonial cell cryopreservation, including thawing and implantation into sterile hosts (for details see Yoshizaki et al. 2011; ESM 1, Fig. S1). The extraordinary power of this process resides in the ability of the spermatogonial cells to readily withstand the cryopreservation process and to

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migrate and produce donor gonads in the sterile host. Given these spermatogonial cells are able to differentiate into either testes or ovaries depending on the gender of the host fish they are transplanted into, both male and female gametes are recovered, thus facilitating reproduction and the production of donor offspring. Conveniently, the same species is not needed as the host, so extinct species could potentially be resurrected (Takeuchi et al. 2004; Okutsu et al. 2007; Yoshizaki and Yazawa 2019).

Much of the work of Yoshizaki et al. was accomplished in freshwater fishes, which may easily be cultured and reared to allow for extensive transplantation studies (Okutsu et al. 2007; Lee et al. 2016; Octavera and Yoshizaki 2020). Because the lifecycle of most marine fishes has not been closed in captivity (Chen et al. 2020), cryopreserved and thawed marine fish spermatogonial cells cannot yet be tested for their efficacy to produce donor offspring. Therefore, post-thaw survival of the cells, determined by live/dead staining and flow cytometry, is one of the few alternative metrics to test the cryopreservation success of spermatogonial cells from marine fish species (Figueroa et al. 2016). The broad survival and use of these thawed spermatogonial cells in freshwater species support the notion that frozen and thawed marine spermatogonial cells could be successfully transplanted (Okutsu et al. 2006; Yoshizaki et al. 2011; Yoshizaki and Lee 2018), thus producing an effective conservation tool once the supportive marine husbandry methods have been devised (de Siqueira-Silva et al. 2018; Yoshizaki and Lee 2018).

Cryopreservation is a low cost, practical method to help conserve aquatic species diversity (Tiersch and Green 2011; Magnotti et al. 2018). We focus on a simple, rapid and straightforward method to cryopreserve marine fish spermatogonial cells. The method can potentially help restore fish populations from the samples stored for many years (Lee et al. 2013), thereby reducing extinction pressures on fish populations. Recently, coral sperm cryopreserved for over 10 yrs was used to facilitate assisted gene flow between populations of a threatened species, *Acropora palmata* (see Hagedorn et al. 2021), suggesting that these frozen assets, securely stored in biorepositories, will be valuable both on short and long time scales for restoration. Long-term success will obviously depend on whether the impacts to fishes and their habitats worldwide are being addressed and managed appropriately, but this method provides the option to revive fish populations and species at any time in the future.

Here, we present our modification of the methods of Yoshizaki et al. (2010, 2011); Lee et al. (2013, 2016) and Hagedorn et al. (2018) to accommodate coral reef fishes. Previously, the buffers used to cryopreserve spermatogonial cells were complex and had to be freshly made in the laboratory thereby limiting remote field use. We considered whether using standard solutions that could be easily

purchased worldwide, and freezing these solutions ahead of time, would reduce the efficacy of the cryopreservation process. Our goal was to simplify the cryopreservation process so that these methods could be incorporated into the workflow of museums, federal agencies and non-governmental organizations involved in field collections of marine fishes.

Methods

Fish collection

Twenty-four male *Asterropteryx semipunctata* (Starry goby) were collected live from a sandy reef flat on the north east of Moku o Lo'e (Coconut Island, GPS: 21.435070° N, 157.786901° W) in southern Kāne'ohe Bay, O'ahu, Hawai'i, over several days in November 2019, and October–December 2020 (see ESM 2 for further details). Fish were identified and sexed visually after being captured in small hand nets. Females were released, and males were transported within 5 min to the laboratory at the Hawai'i Institute of Marine Biology (HIMB). Maintenance and handling of live fish met the animal care standards of the National Institute of Health (NIH). Full details of the study approval are listed with the Smithsonian Institution, USNM IACUC (approval ID #2018-03) and the University of Hawai'i IACUC (protocol ID# 12-1491). Fish were collected under permit SAP-2020-25 and SAP-2021-33 from the Department of Land and Natural Resources, Hawai'i.

Specimen preparation

Live fish were immersed in a 0.01% solution of buffered tricaine methanesulfonate (MS-222; Sigma-Aldrich, Inc., MO) dissolved in seawater until gill movement and response to mechanical stimulation ceased (~5 min). Individuals were rinsed with seawater and placed dorsal surface down on a damp sponge or paper towel. Size was recorded for each individual (Standard Length: the straight-line distance from the tip of the snout to the base of the caudal fin; ESM 2). The gonad of male gobioid fishes comprises the testis and accessory gonadal structures, following Miller (1984, 1992). The testis has a spermatogenic function, whereas the accessory gonadal structures have a secretory function (Cole and Parenti, 2022). We surgically removed the gonads from the adult male specimens, following a standard protocol (Hagedorn et al., 2018). Briefly, an incision was made with micro-dissecting scissors to expose the abdominal cavity. Once the gonads were visible, they were gently peeled off, leaving behind the main testicular blood vessel and the mesorchium. The entire dissection was performed under a stereomicroscope (Wild M5). Each gonad was weighed and placed

into a labelled 1.8 ml cryovial with 500 μ l of L15 medium (Sigma-Aldrich L1518-500ml) and held at 0 $^{\circ}$ C, on ice. Each pair of testes and accessory gonadal structures was then used in one of two experiments that analyzed the efficacy of the field protocol: (1) determine the difference in viability between fresh and cryopreserved testicular cells (both sperm and spermatogonial cells) using freshly made cryoprotectants, or (2) determine whether there was a difference in cell viability when cryopreserved tissues were exposed to freshly made or previously frozen cryoprotectants. If successful, this second experiment would ensure that solutions could be made ahead of time, frozen and taken into the field to help standardize and expedite the cryopreservation process.

Cryopreservation, bio-banking, and thawing

Gonads were treated as defined in Fig. 1. The intact gonads in 500 μ l L15 medium destined for cryopreservation received 500 μ l of double strength cryoprotectant (CPA) and were left on ice for an hour of equilibration during which some water left the gonad cells and some CPA penetrated the cells. The CPA solution was prepared at double strength for a final concentration of 0.1 M trehalose, 9.2% v/v DMSO (1.3 M), 10% v/v Fetal Bovine Serum (FBS), in L15 medium after the initial 1:1 dilution with the sample. The CPA was prepared fresh or prepared in advance and frozen at -20° C until use. (The preparation used is mentioned for each result.) Cryovials were then placed in a passive cooling device with a

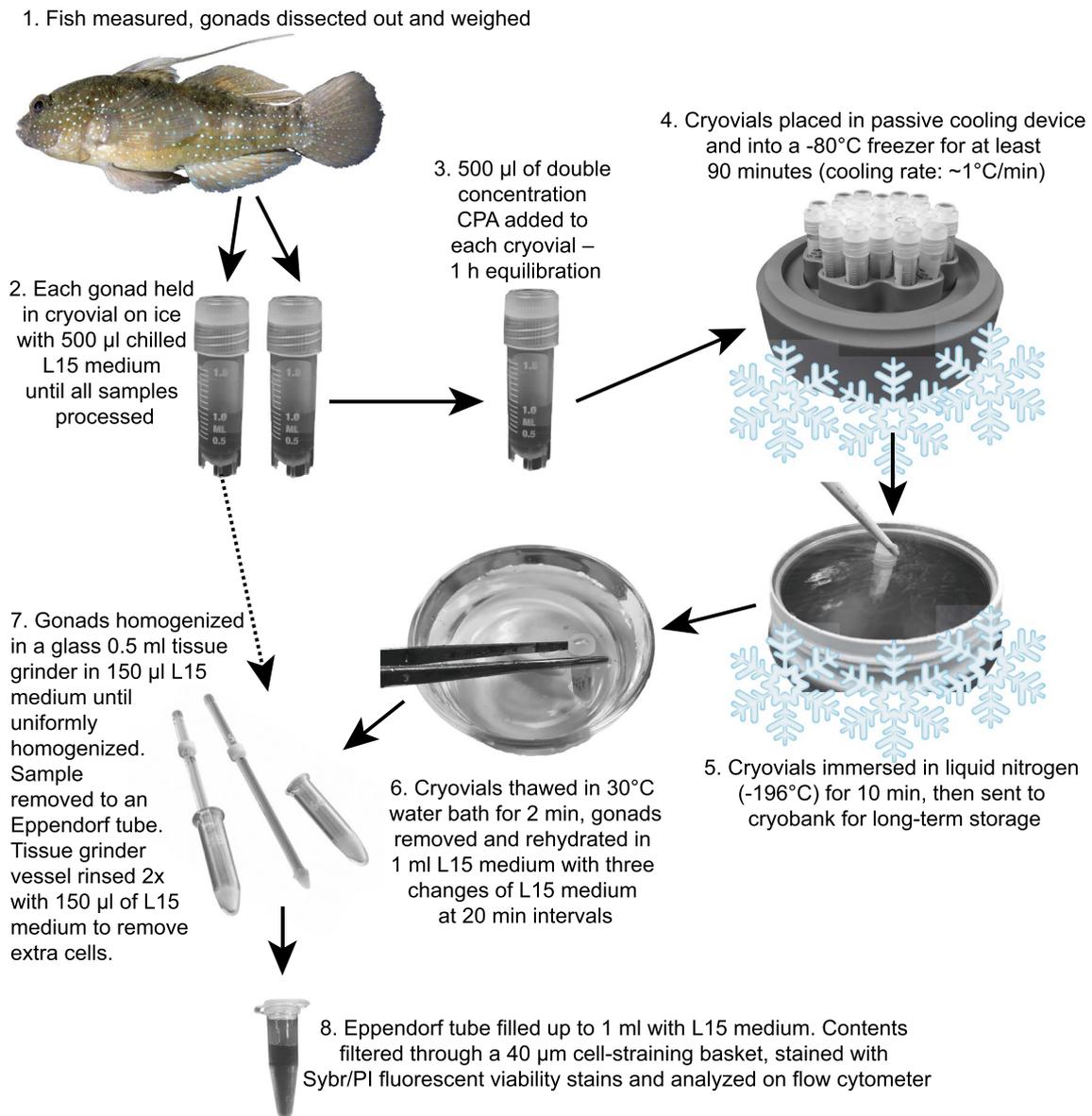


Fig. 1 Flow diagram for the fish gonad cryopreservation and preparation for flow cytometer analysis

cooling rate of 1 °C per minute (BioCision Cool Cell, Bath, the UK) and moved to a -80 °C freezer until they reached -80 °C (about 80 min). The samples were then quenched in liquid nitrogen and transferred to a liquid nitrogen Biobank at -196 °C for at least 24 h.

Samples to be thawed were retrieved from the Biobank and transferred to a small cryogenic Dewar containing liquid nitrogen. They were thawed and assessed one at a time. Each vial was transferred into a water bath at 30 °C and gently stirred until completely thawed (about 2 min). The intact gonad was then transferred to a new tube containing 1 ml L15 medium, which was renewed every 20 min until an hour had passed. The gonad was then ready for dissociation and assessment.

Cell dissociation and assessment of sperm and spermatogonial cell viability

Gonads contain sperm cells and spermatogonial cells that are the largest present (~ 10 μm) and that can be sorted from other cells by their size (forward scatter) and granularity (size scatter), visualized with a flow cytometer (Kise et al. 2012; Ichida et al. 2017; Hagedorn et al. 2018; Rivers et al. 2020). Sperm and spermatogonial cells were either assessed fresh or post-thaw after cryopreservation. The fresh or cryopreserved and thawed gonads were dissociated in L15 medium with a 0.6-ml glass homogenizer until the solution appeared homogenous without any visible clumps. The dissociated cells were then filtered through a 40- μm cell-straining basket, diluted in L15 medium for a total of 990 μl , stained with 5 μl SYBR-14 and 5 μl propidium iodide (PI) fluorescent stains (live/dead sperm viability kit, Invitrogen, Carlsbad, CA), and incubated in the dark, at room temperature. The stained dissociated cells were then analyzed with flow cytometry (BD Accuri C6 Plus Flow Cytometer equipped with a 488 nm excitation laser; Fig. 1) at a maximum rate of 10^4 cell counts per second. The cell concentration for flow cytometry analysis was maintained below 5×10^6 cells/ml, with samples diluted and re-analyzed if needed. A size vs. granularity profile of spermatogonial and sperm cells was plotted in the BD Accuri C6 Plus software in a forward scatter vs. side scatter plot and measured against standard-sized beads (2, 5 and 10 μm in diameter) run through the flow cytometer. The forward scatter profile of the 10- μm beads and the lower values of the side scatter profile was used to set a gate corresponding approximately with type A spermatogonial cells (Schulz et al. 2010; Kise et al. 2012; Rivers et al. 2020), which are the target cells for transplantation, and separating them from sperm cells, which made up $> 90\%$ of the cells present from analyses. Within each category (spermatogonial or sperm cells), live cells were distinguished from dead cells according to their fluorescent staining with SYBR-14 and PI, detected with

the flow cytometry filters 533/30 (FL1) and 670 LP (FL3), respectively.

Statistical analyses

Gonads from eight fish specimens were used to determine the difference in viability between fresh and cryopreserved testicular cells (with one gonad used for each treatment), and gonads from another eight fish specimens were used to determine whether there was a difference in cell viability when cryopreserved tissues were exposed to freshly made or previously frozen cryoprotectants. All data are expressed as cell concentration means (cells per ml) \pm standard error. Due to variation in standard length and gonad size among males, cell concentrations were normalized against gonad weight prior to statistical analyses. All statistical analyses were conducted in R (R Core Team 2019) with the packages ggplot2, rcompanion, Rmisc, scales. Cell concentrations were log-transformed to fulfil normality assumptions. Cell concentrations from fresh and cryopreserved gonads were compared using a paired *t*-test, with 95% confidence interval ($\alpha = 0.05$).

Results and discussion

Simplified cryopreservation protocol

The success profile for these experiments is defined in Fig. 2. The total number of all dissociated testicular cells (defined

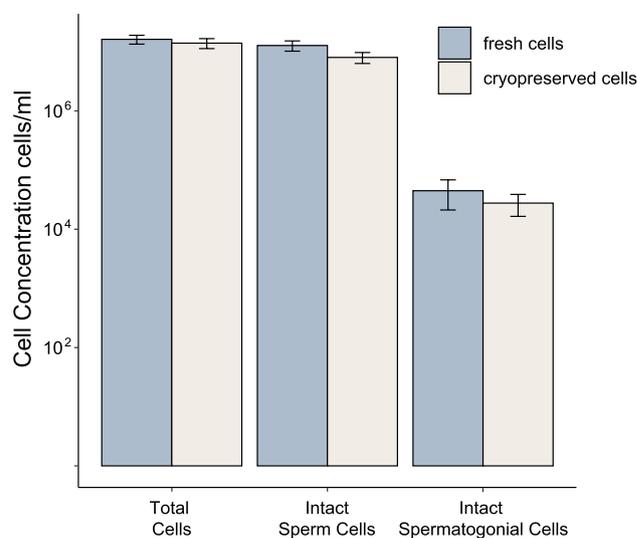


Fig. 2 Fresh versus cryopreserved gonads: difference in concentration of total testicular cells (sperm cells + spermatogonial cells, including intact, damaged, and dead cells), intact sperm cells, and intact spermatogonial cells. The y-axis is plotted on a log scale. Error bars represent standard error of the mean

as all sperm cells and spermatogonial cells, including intact, damaged, and dead cells) was similar between the fresh and cryopreserved gonads (paired t -test, $t_{(15)} = 1.13$, $p = 0.28$; Fig. 2; ESM 3, Table S1), indicating no overall loss of cells from the cryopreservation process. When analyzing sperm and spermatogonial cells separately, the number of intact sperm cells was slightly reduced after cryopreservation (paired t -test, $t_{(15)} = 2.41$, $p = 0.03$), but the number of intact spermatogonial cells was unaffected by the cryopreservation (paired t -test, $t_{(15)} = 0.77$, $p = 0.46$; Fig. 2). On average, spermatogonial cells from fresh gonads comprised only a small fraction of the total testicular cells (0.39%; ESM 3, Table S1), but cryopreservation seemed to have little impact on these overall numbers. These results suggest that the simplification of the solution using standard buffers, such as L15 medium, had little impact on the resulting number of successfully cryopreserved and thawed spermatogonial cells. Given its simplicity, this method may be readily implemented in the field by biologists after a brief period of training. This will allow for greater standardization of the cryopreservation process. The gonads may be cryopreserved as whole and the testicular cells dissociated later, after thawing. For later transplantation purposes, the spermatogonial cells would then need to be isolated from the other cells to maximize transplantation success, for example, using cell sorting with flow cytometry light scattering (Kise et al. 2012; Ichida et al. 2017; Rivers et al. 2020).

Cryoprotectant solution prepared in advance and frozen

The use of frozen cryoprotectant solutions prepared in advance yielded no difference in the concentration of total sperm and spermatogonial cells (paired t -test, $t_{(7)} = -0.02$, $p = 0.99$), intact sperm cells (paired t -test, $t_{(7)} = -0.11$, $p = 0.92$), or intact spermatogonial cells (paired t -test, $t_{(7)} = -0.52$, $p = 0.62$), (Fig. 3, ESM 3, Table S2) compared to freshly prepared cryoprotectant solutions. Therefore, for field campaigns on boats or in remote locations, cryoprotectants can be prepared in advance and frozen until needed in the field. Additionally, this means the solutions can be prepared safely in the laboratory, by laboratory-trained personnel.

Implications for marine fishes conservation

Regardless of the aquaculture and larviculture husbandry challenges still facing marine species (Vadstein et al. 2018; Chen et al. 2020; Groover et al. 2021), conservation of fishes must be considered a high priority due to the severe extinction pressure on many species and populations. This is a crisis for biodiversity as well as worldwide food resources (Srinivasan et al. 2010; McCauley et al. 2015; Yan et al. 2021). Fishes in coral

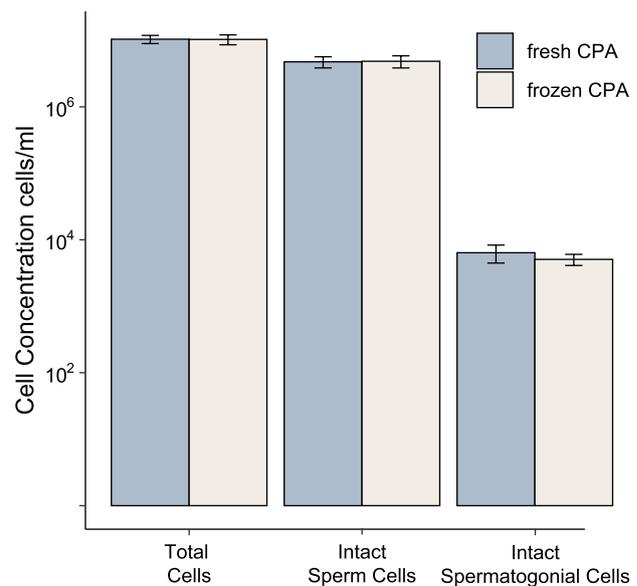


Fig. 3 Cryopreservation using freshly prepared versus frozen cryoprotectant (CPA): difference in the concentration of total testicular cells (sperm cells + spermatogonial cells, including intact, damaged, and dead cells), intact sperm cells, and intact spermatogonial cells. The y-axis is plotted on a log scale. Error bars represent standard error of the mean

reef ecosystems are at even higher risk, due to the increasing pressure of climate change on the reef-building corals that support these ecosystems (Wilson et al. 2008). The challenge is to get people mobilized to begin this conservation process now, to incorporate it into collection workflow processes (Hagedorn et al. 2018), and to create additional space and support for national cryorepositories. If proper metrics, voucher samples, high-resolution images, and DNA samples are included in this process, natural history museums may be willing to hold these frozen assets for their country of origin. It is important to cryopreserve and bank these reproductive spermatogonial cells now, while they are available, even though current larviculture and aquaculture techniques in most marine fishes are not yet sufficiently advanced to proceed with restoring these populations following the methods by Yoshizaki et al. (2011). Once culture techniques allow for the full life cycle of marine fishes to occur in a controlled captive environment, frozen material will be available to verify that spermatogonial cell transplantation works equally well for marine fishes as it does for freshwater fishes, which will allow for active fishes restoration efforts to be initiated using already the cryopreserved spermatogonial cells. With rapidly advancing research, this may be forthcoming. For example, groupers are ecologically and commercially valuable fishes in coral reef ecosystems, and many are listed as threatened due to overfishing and threats to their habitat (Olsen and LaPlace 1979; Sadovy de Mitcheson et al. 2020). Some of the key challenges in grouper aquaculture include its life history parameters such as longevity and late sexual maturity.

Yet, the brown-marbled grouper *Epinephelus fuscoguttatus*, a relatively well-studied and widely cultured grouper, lives well in aquaculture and can reach sexual maturity within three years in captivity (Sugama et al. 2012; Mustafa et al. 2015; Boonanuntasarn et al. 2016; Villanueva et al. 2021). This makes it a suitable surrogate candidate for transplantation by cryopreserved spermatogonia from other grouper species with higher longevity and later sexual maturity. Techniques for transplantation of cryopreserved spermatogonial cells into a surrogate host involve transplantation into the host during its larval stage, when the endogenous primordial germ cells are migrating and before they are completely surrounded by gonadal somatic cells (Takeuchi et al. 2009; Yazawa et al. 2010; Morita et al., 2015). This timing has recently been established in the brown-marbled grouper using *vasa* gene expression during larval development, with an identified transplantation window between 9 and 21 days post-hatching (Boonanuntasarn et al. 2016). The next step will be to investigate techniques to micro-inject transplanted spermatogonial cells into the grouper larvae, which are known to be generally fragile and difficult to raise (Boonanuntasarn et al. 2016).

Without critical actions taken to secure the biodiversity of fishes, fish populations and some of the more vulnerable fish species will continue to dwindle and head towards extinction. We urge conservation, academic, and regulatory agencies to take up this challenge and secure fish genetic and species diversity, alongside the other measures being undertaken to reduce the anthropogenic pressures on these organisms, both on a global and regional scale.

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Declarations

Conflict of interest On behalf of all the authors, the corresponding author declares that there is no conflict of interest.

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