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Integrating biobanking could produce significant cost benefits and minimise inbreeding for Australian amphibian captive breeding programs

Lachlan G. Howell[A,B,I], *Peter R. Mawson*[C], *Richard Frankham*[D,E], *John C. Rodger*[A,B], *Rose M. O. Upton*[A,B], *Ryan R. Witt*[A,B], *Natalie E. Calatayud*[F,G], *Simon Clulow*[H] *and John Clulow*[A,B]

[A]School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.
[B]FAUNA Research Alliance, Kahibah, NSW 2290, Australia.
[C]Perth Zoo, Department of Biodiversity, Conservation and Attractions, PO Box 489, South Perth, WA 6951, Australia.
[D]Department of Biological Sciences, Macquarie University, Sydney, NSW 2019, Australia.
[E]Australian Museum, Sydney, NSW 2010, Australia.
[F]San Diego Zoo Institute for Conservation Research, San Pasqual Valley Road, Escondido, CA 92027, USA.
[G]Conservation Science Network, 24 Thomas Street, Mayfield, NSW 2304, Australia.
[H]Centre for Conservation Ecology and Genomics, Institute for Applied Ecology, University of Canberra, Bruce, ACT 2617, Australia.
[I]Corresponding author. Email: lachlan.howell@newcastle.edu.au

Abstract. Captive breeding is an important tool for amphibian conservation despite high economic costs and deleterious genetic effects of sustained captivity and unavoidably small colony sizes. Integration of biobanking and assisted reproductive technologies (ARTs) could provide solutions to these challenges, but is rarely used due to lack of recognition of the potential benefits and clear policy direction. Here we present compelling genetic and economic arguments to integrate biobanking and ARTs into captive breeding programs using modelled captive populations of two Australian threatened frogs, namely the orange-bellied frog *Geocrinia vitellina* and the white bellied frog *Geocrinia alba*. Back-crossing with frozen founder spermatozoa using ARTs every generation minimises rates of inbreeding and provides considerable reductions in colony size and program costs compared with conventional captive management. Biobanking could allow captive institutions to meet or exceed longstanding genetic retention targets (90% of source population heterozygosity over 100 years). We provide a broad policy direction that could make biobanking technology a practical reality across Australia's *ex situ* management of amphibians in current and future holdings. Incorporating biobanking technology widely across this network could deliver outcomes by maintaining high levels of source population genetic diversity and freeing economic resources to develop *ex situ* programs for a greater number of threatened amphibian species.

Keywords: anuran, artificial reproductive technologies, captive survival-assurance colonies, cryopreservation, frog, genome resource banking, IVF.

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Introduction

Ex situ conservation tools, particularly captive breeding and assisted reproductive technologies (ARTs) are becoming increasingly important for amphibian species recovery (Bishop *et al.* 2012; Kouba *et al.* 2012; Clulow and Clulow 2016; Browne *et al.* 2019; Clulow *et al.* 2019). Australian animals face significant *in situ* declines across multiple taxa due to expanding and persistent anthropogenic threats, policy failings and funding neglect (Woinarski *et al.* 2017; Howell and Rodger 2018; Wintle *et al.* 2019). Threatening processes have driven some Australian species to an increasing reliance on *ex situ* management across the country's network of institutions holding captive collections

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(Harley *et al.* 2018). This is especially true for amphibians, which face considerable *in situ* declines globally (Scheele *et al.* 2019) and domestically (Scheele *et al.* 2017) due to infectious fungal diseases (Bower *et al.* 2017). For example, the amphibian chytrid fungus *Batrachochytrium dendrobatidis* alone has resulted in the decline or extinction of more than 500 species in just a few decades (Scheele *et al.* 2019), with little that can be done to combat ongoing declines in the wild (although see Clulow *et al.* 2018*a*). Several Australian amphibian species are at immediate risk of extinction (Skerratt *et al.* 2016; Gillespie *et al.* 2020), some of which rely on *ex situ* conservation tools (particularly captive breeding) as a last line of defence (Skerratt *et al.* 2016; Harley *et al.* 2018).

Amphibians account for >27% of all native species held in historic and on-going captive breeding programs in Australia, representing 11 of the 40 species held in significant captive programs between 1980 and 2018, including six on-going amphibian programs (a modest number compared with the quantum of species facing *in situ* declines; Harley *et al.* 2018; Gillespie *et al.* 2020). The modest number of programs (particularly on-going programs) can be attributed to the logistical challenges and financial costs of captive breeding (Mawson and Lambert 2017; Harley *et al.* 2018), particularly the high average annual costs (>A$200 000) that are often required for on-going multiyear (or multidecade) programs (Harley *et al.* 2018). External multisectoral funding support is often required to meet these high costs (Mawson and Lambert 2017; Harley *et al.* 2018) and, in the absence of external funding, captive breeding is often self-funded by the institution responsible for the program (Harley *et al.* 2018). As a result of limited funding, it has been estimated that there are only resources to hold approximately 50 amphibian species globally in captive programs, which is in stark contrast to the at least 3000 amphibian species currently threatened with extinction (Gagliardo *et al.* 2008; Zippel *et al.* 2011; Bishop *et al.* 2012; Clulow *et al.* 2014; Murphy and Gratwicke 2017; González-del-Pliego *et al.* 2019).

Aside from economic challenges, captive breeding programs face significant challenges managing genetic diversity within captive collections. Captive breeding programs are criticised for the theorised or realised deleterious genetic effects associated with sustained captivity (Snyder *et al.* 1996), including domestication and adaptation to captivity (Frankham 2008), inbreeding depression (Ralls *et al.* 1988), reduced reproductive fitness and success (Farquharson *et al.* 2018) and loss of fitness when reintroduced or translocated (Robert 2009). There are various examples of these genetic problems occurring within Australian captive breeding programs across multiple taxa. Even some of Australia's most well-resourced captive programs (e.g. Tasmanian devils *Sarcophilus harrisii* and orange-bellied parrots *Neophema chrysogaster*) have theorised or realised genetic issues (Farquharson *et al.* 2017; Grueber *et al.* 2017; Morrison *et al.* 2020). There is a clear focus within many programs on understanding and maximising genetic diversity (Hogg 2013), including for captive amphibians (Lees *et al.* 2013). Genetic diversity is consistently a key consideration and desired performance outcome when planning and running captive breeding programs (Harley *et al.* 2018).

A suggested global target for captive breeding programs is to retain 90% of source population heterozygosity (H_t/H_o) for 200 years (Soulé *et al.* 1986), later adjusted to 90% for 100 years (Frankham *et al.* 2010). However, it is unlikely this target has been met within any captive breeding program due to the large colony sizes and resources that would be required to avoid heterozygosity loss through inbreeding and genetic drift (Howell *et al.* 2020). Howell *et al.* (2020) provided a strong argument based on modelling that biobanking technology could address the challenges of high costs and genetics common to captive breeding programs and allow captive programs to meet (and surpass) the suggested genetic retention benchmark (<10% H_t/H_o loss after 100 years).

Biobanking technology (frozen living cell repositories combined with ARTs) has long been proposed as a complementary tool that may reduce space and holding requirements, as well as labour and other resources, required to run captive breeding programs (Holt *et al.* 1996; Clulow and Clulow 2016; Ananjeva *et al.* 2017; Silla and Byrne 2019). Howell *et al.* (2020) quantified the potential cost and genetic benefits of biobanking technology using genetic and economic modelling and demonstrated that supplementing captive-bred populations of Oregon spotted frogs *Rana pretiosa* with frozen founder spermatozoa through IVF every generation could result in massive short- and long-term cost reductions, minimise inbreeding and greatly reduce required live colony sizes. That modelling suggested that the reductions in inbreeding were potentially significant enough to exceed the genetic retention benchmark of 90% of source population heterozygosity within a captive amphibian population, and even allow 95% or 99% heterozygosity targets to be realistically achieved. Howell *et al.* (2020) proposed that amphibians are an ideal taxon to demonstrate the synergies and benefits of biobanking in captive breeding due to a combination of ideal life history traits (e.g. minimal post-fertilisation parental support, short generation length and high fecundity; Bloxam and Tonge 1995), comparatively low *ex situ* holding costs for individual animals (Conde *et al.* 2015) and recent advances in the underlying reproductive sciences in amphibians (Kouba and Vance 2009; Kouba *et al.* 2013; Clulow *et al.* 2014, 2018*b*, 2019; Clulow and Clulow 2016).

A consensus on the potential merits of biobanking technology and ARTs is not universal among the Australian conservation community (Skerratt *et al.* 2016), and uptake of the technology has been largely absent in Australia and elsewhere (Monfort 2014; Clulow *et al.* 2019). No examples yet exist of biobanking technology leading to measurable outcomes in an Australian amphibian captive breeding program, and there are few, if any, examples globally (Howell *et al.* 2020). Without a paradigm shift from practitioners, the benefits of biobanking will not be realised in Australian amphibian conservation despite clear economic and genetic fitness benefits. Nevertheless, stretched budgets for conservation, the challenges of captive breeding with standard husbandry and the persistent *in situ* decline of amphibians provide Australian *ex situ* conservation practitioners and institutions with a strong impetus to develop and adopt cost-effective biobanking and ART tools for the *ex situ* management of Australian amphibians.

This study provides support for integrating biobanking technology into Australian amphibian captive breeding programs by testing economic and genetic models targeted towards the network of Australian captive breeding institutions. Here, we model the end-stage cost benefits, reductions in required colony sizes and genetic diversity outcomes achieved with biobanking technology in modelled populations of orange-bellied frogs *Geocrinia vitellina* and white-bellied frogs *Geocrinia alba* using cost data from the Perth Zoo Native Species Breeding Program. As in Howell *et al.* (2020), we modelled the economic cost and required colony sizes to meet different heterozygosity (H_t/H_o) targets (90%, 95% and 99% of source population H_t/H_o) under scenarios of no biobanking and biobanked colonies of *G. vitellina* and *G. alba* where the captive populations are supplemented with frozen founder spermatozoa using existing or developing ARTs at every generation.

We also provide a roadmap to incorporate biobanking technology into existing and novel *ex situ* amphibian captive breeding programs in Australian institutions. We propose required inputs (through economic resources, strategic partnership generation, infrastructure usage and/or leverage from existing programs) across a set of actions for *G. vitellina* and *G. alba* that would also be applicable to other Australian amphibian species. We provide budgets and timelines for significant actions along the pathway, including closing species-specific knowledge gaps and biobanking protocol development for candidate amphibian species. Finally, we outline a new paradigm for the *ex situ* management of Australian amphibians under which biobanking technology could free economic resources to generate an *ex situ* network in Australia with the flexibility and resource capacity to allow the inclusion of a greater number of species in captive programs in response to the continued *in situ* declines of Australian amphibians.

Materials and methods

Study species

Australia's orange-bellied frog *G. vitellina* and white-bellied frog *G. alba* are small, terrestrial breeding frogs endemic to a small area of south-west Western Australia (Clulow and Swan 2018). The two species are listed as vulnerable and critically endangered respectively under the International Union for the Conservation of Nature (IUCN) Red List (Hero and Roberts 2004; Roberts and Hero 2004), and *Australia's Environment Protection and Biodiversity Conservation (EPBC) Act 1999* Threatened Species List (https://www.environment.gov.au/cgi-bin/sprat/public/publicthreatenedlist.pl). Both species are facing combined persisting threats of disease (chytridiomycosis), land use and habitat alterations, climate change (Hoffmann *et al.* 2021) and invasive species (Wardell-Johnson *et al.* 1995; Roberts *et al.* 1999). Each species is reduced to small fragmented metapopulations within narrow home ranges, and both have documented *in situ* genetic issues (Driscoll 1997, 1998, 1999; Roberts *et al.* 1999; Conroy 2001). We selected *G. vitellina* and *G. alba* as case study species due to the availability of the primary data required for the modelling presented in Howell *et al.* (2020), *G. vitellina* and *G. alba* effective population sizes (N_e; Table 1; Driscoll 1997) and access to captive breeding costs and colony sizes from Perth Zoo's Native Species Breeding Program (Supplementary Tables S1–S3). *G. vitellina* and *G. alba* are currently maintained as part of a successful population supplementation program in which spawn are collected from the wild, raised to metamorphosis with a high survival rate and returned to supplement (head-start) the wild source population (with only a small component of *ex situ* breeding). In the present study we use data for the cost of husbandry derived from these captive holdings, but model a more conventional captive breeding colony in which there is a single founder event from a source population and subsequent generations are derived from the F_0 founders.

Models

We have adopted the methodologies presented in Howell *et al.* (2020) to model the potential cost and genetic benefits of incorporating biobanking technology into the captive breeding of *G. vitellina* and *G. alba*. We incorporate actual captive breeding program costs (2017–18 financial year breeding season of *G. vitellina* and *G. alba* within Perth Zoo's Native Species Breeding Program) and genetic modelling (Howell *et al.* 2020) to model the long-term cost benefits and genetic outcomes achieved if biobanking were successfully implemented and could be incorporated with minimal additional infrastructure investment and labour costs into existing funded programs.

We modelled various 100-year captive breeding cost scenarios in which the size of live *G. vitellina* and *G. alba* captive colonies reflects the colony sizes required to maintain different proportions of the source population heterozygosity (90%, 95% and 99% H_t/H_o retention), with or without the use of biobanking technology, as proposed in other studies (Soulé *et al.* 1986; Frankham *et al.* 2010; Howell *et al.* 2020). For these hypothetical populations, we model biobanking strategies in which cryopreserved founder spermatozoa of *G. vitellina* and *G. alba* are used to reduce the size of live colonies while meeting genetic retention targets by the reintroduction of founder genes at each generation via IVF up to 100 years after the colony is established. We assume 4-year generational intervals for *G. vitellina* and *G. alba* (Table 2).

We adjusted the genetic back-crossing model presented in Howell *et al.* (2020) to account for the different life history traits, required back-cross frequency and N_e of *G. vitellina* and *G. alba*. Our model determines the census size (N) of the captive colony required to maintain various levels of genetic diversity (with heterozygosity values derived from inbreeding coefficients) under two scenarios: (1) non-back-crossed populations representing the minimum number of individuals required to meet heterozygosity targets in a conventional captive breeding program without biobanking; and (2) populations with back-crossing to founder genotypes every generation at 4-year intervals sourced from biobanked founder spermatozoa. For back-crossed populations, N_e was generated by random substitution into iterative genetic models until the 100-year H_t/H_o met desired genetic retention targets. We derived a range of colony size numbers (N) using various published and assumed N_e/N ratio estimates for *G. vitellina* and *G. alba* (Table 1). Modelled N_e/N ratios included: (1) a

Table 1. Effective population size (N_e) to census population size (N) ratios used for different modelling scenarios for modelled captive biobanked and non-banked populations of orange-bellied frogs *Geocrinia vitellina* and white-bellied frogs *Geocrinia alba*

The N_e/N ratios presented here were used to perform N_e to N conversions for modelled non-banked populations designed to meet different genetic retention targets (90%, 95% and 99% H_t/H_o retention)

Modelling scenario	Description and rationale	N_e/N ratios sampled	N_e/N value used in modelling	Source
1. Mean ratio for captive vertebrates	The mean N_e/N ratio for captive vertebrates across various taxa, used in the absence of N_e/N ratios for captive amphibians. We consider this modelling scenario to be the most realistic for biobanked populations among the published captive N_e/N values	Red-crowned crane *Grus japonensis* ($N_e/N = 0.45$) Grevy's zebra *Equus grevyi* ($N_e/N = 0.28$) Scimitar-horned oryx *Oryx dammah* ($N_e/N = 0.20$)	0.3	Mace (1986)
2. Median temporal and demographic N_e/N values for amphibians	The median N_e/N ratio from a range of published estimates of amphibian N_e/N values; these values are derived from wild populations and provide lower estimates than captive-derived estimates from other taxa	Cane toad *Rhinella marina* ($N_e/N = 0.052$) Crested newt *Triturus cristatus* ($N_e/N = 0.185$) European common frog *Rana temporaria* ($N_e/N = 0.705$) Marbeled newt *Triturus marmoratus* ($N_e/N = 0.185$) Natterjack toad *Bufo calamita* ($N_e/N = 0.097$) Red-spotted newt *Notophalmus viridescens* ($N_e/N = 0.073$)	0.141	Frankham (1995), Frankham *et al.* (2019)
3. Species-specific N_e/N values	Only available species-specific N_e/N ratios for *G. vitellina* and *G. alba*. Estimates are demographic and wild derived, and although these estimates do account for variables typically affecting N_e/N ratios discussed in Frankham (1995), the approach taken may overestimate N_e/N. In fact, these values are considerably higher than other estimates for amphibian species that account for the same variables (see Scenario 2). We present these estimates as published values for *G. vitellina* and *G. alba* while recognising they represent extremes of N_e/N estimates that generate much lower estimates of required census captive population sizes	Orange-bellied frog *G. vitellina* ($N_e/N = 1.17$) White-bellied frog *G. alba* ($N_e/N = 1.21$)	1.17 and 1.21	Driscoll (1999)

mean N_e/N ratio for captive vertebrate populations of 0.3 (presented in Mace 1986); (2) a median N_e/N value of 0.141 calculated using range of wild-derived temporal N_e/N estimates for amphibian species (Table 1; Frankham 1995; Frankham *et al.* 2019); and (3) species-specific wild-derived demographic N_e/N estimates for *G. vitellina* and *G. alba* of 1.17 and 1.21 respectively (Table 1; Driscoll 1999). The range of N_e/N values modelled here includes extremes of published values and assumptions that generate a wide range of census population sizes for captive *G. vitellina* and *G. alba*; we assume that true captive N_e/N values for *G. vitellina* and *G. alba* values would lie somewhere between the extremes modelled here. Derived N values were substituted into an economic costing model based on costs for the 2017–18 breeding season of *G. vitellina* and *G. alba* at Perth Zoo. This was used to estimate an economic *ex situ* holding cost per individual animal for each species and 100-year captive colony costs for each derived colony size (N) with or without biobanking technology in various modelling scenarios.

Cost modelling

All cost modelling was based on all fixed and variable program costs from Perth Zoo's Native Species Breeding Program for the 2017–18 breeding seasons of *G. vitellina* and *G. alba*. We used a microcosting approach whereby all fixed and variable costs (expressed in nominal Australian dollars) associated with the captive breeding of *G. vitellina* and *G. alba* were calculated or estimated using face-to-face interviews with personnel as well as access to financial, logistical and operations reporting.

Year 1 economic *ex situ* holding cost per individual (C) is given by Eqn 1:

$$C = ([I + G + E + L + U + F]/N) + M \quad (1)$$

where all costs are in nominal 2018 Australian dollars, and where I represents facilities costs (set-up and recurring maintenance), G represents fixed founder collection costs (field collection costs including vehicles, accommodation and food for staff), E represents minor equipment and consumables

Table 2. General genetic modelling assumptions and species-specific parameters used to adapt modelling approaches for captive colonies of orange-bellied frogs *Geocrinia vitellina* and white-bellied frogs *Geocrinia alba*

General genetic modelling assumptions and parameters for captive colonies

- Model assumes founder *G. vitellina* and *G. alba* males and females in the colony have $F_0 = 0$
- The captive colony would initially contain one live female per founder male (drawn at random from *G. vitellina* and *G. alba* source populations); under back-crossing scenarios, males produced each generation are redundant after founder spermatozoa are banked from the F_0 generation
- Colony census numbers (N) are managed at each generation to achieve the target N_e (N_e assumed constant)
- A mean 1:1 sex ratio is assumed, and an average of one female offspring per female contributed to subsequent generations (via management of the colony)
- Random pairing of males and females occurs each generation during back-crossing; live colony pairings are uncontrolled (to be consistent with assumptions for published N_e values)
- Each generation produced would entirely replace the previous captive generation with no overlap
- Females in each non-back-crossed generation have the same variability in offspring numbers as do wild females
- At each back-crossing event, one fertile female offspring, on average, is retained from each female in the colony; male offspring are redundant and removed
- All biobanked founder males are unrelated ($F_0 = 0$)

Species-specific genetic modelling assumptions and parameters for captive colonies of *G. vitellina* and *G. alba*

- The frequency of *G. vitellina* and *G. alba* females in the captive colony successfully breeding at 4 years of age has the same variability as wild females. All *G. vitellina* and *G. alba* females in the captive colony are sexually mature at 4 years of age, enabling IVF at 4-year generational intervals. This is assumed to represent the *ex situ* age at first breeding for wild *G. vitellina* and *G. alba* (Conroy 2001). Here, we assume that all individuals would be sexually mature and breeding by this age. We have selected these values for use in the models given that adult mortality in the wild is high and few adults survive to breed in more than one season (Conroy 2001)

costs, L represents labour costs (director and husbandry staff salaries), U represents utilities costs (electricity and water), F represents food costs, N is the captive colony size for *G. vitellina* and *G. alba*, and M represents fixed management costs (research and administration).

Year 1 program costs for non-back-crossed populations (P_c) and program costs for *G. vitellina* and *G. alba* populations incorporating biobanking technology (P_{bc}) are given by Eqns 2 and 3 respectively and were incorporated into iterative processes in Microsoft Excel 16.45 to model 100 years of captive management starting in 2019 (by adjusting 2017–18 values for inflation) based on Year 1 consolidated costs and assuming a 2.6% inflation rate (mean Australian inflation rate 1990–2016) going forward (https://tradingeconomics.com/australia/inflation-cpi):

$$P_c = C \times N \quad (2)$$

$$P_{bc} = (C + B) \times N \quad (3)$$

where C represents Year 1 economic *ex situ* holding costs for either *G. vitellina* or *G. alba* (Eqn 1), B represents biobanking costs (fixed basic additional infrastructure costs and variable outsourced labour costs for recurring IVF and Year 1 cryopreservation, summarised below) and N represents the number of adult frogs in the colony. Most costs recurred annually, excluding facilities set-up costs, which occurred in Year 1 and then at 50-year intervals.

We present a low-cost biobanking scenario where biobanking-specific costs include replacement of frozen storage infrastructure of dewars and freezers at 10- and 15-year intervals respectively, based on asset deprecation rates (https://www.ato.gov.au/law/view/document?docid=TXR/TR20195/NAT/ATO/00003). Founder collection costs for *G. vitellina* and *G. alba* were modelled in Year 1 based on costs for the Perth Zoo Native Species Breeding Program annual collection of egg clutches. Costs were fixed and assumed to cover field collection costs (vehicles, accommodation and food) and did not recur after Year 1. Biobanking-specific labour costs were modelled as outsourced labour to a team of two reproductive biologists at assumed hourly rates (A$40 h^{-1}). To estimate these costs, we generated theoretical protocols for cryopreserving *G. vitellina* and *G. alba* founder spermatozoa for long-term storage and thawing for use in IVF to provide variable estimates of full-time equivalent effort (person hours) for biobanking and genetic back-crossing. We generated theoretical protocols in line with existing amphibian reproductive technologies by adapting protocols for amphibian species of similar life history for cryopreservation and similar body mass for IVF protocols (Silla 2011, 2013; Silla and Roberts 2012). Our theoretical cryopreservation protocol is assumed to involve hormone induction of males and preparation and freezing of founder spermatozoa (an estimated 8 h to freeze and store founder spermatozoa from 15 founder males concurrently). Back-crossing (reintroduction of founder spermatozoa to captive females via IVF) sessions were assumed to involve hormone induction of females, thawing of spermatozoa and IVF (an estimated 8 h to perform IVF on 16 females, assuming staggered and concurrent IVF for four females every 2 h following a fixed 36-h time frame of preparatory tasks before the IVF procedures, such as hormone induction). Time frames and the number of animals biobanked and back-crossed in each session are based on author experience as well as relevant time frames reported in protocols used for modelling (e.g. peak spermiation and time between hormone induction and oocyte collection; Silla 2011, 2013; Silla and Roberts 2012).

Genetic modelling

We provide a summary of the genetic modelling methodology here. For detailed genetic modelling procedures, see Howell *et al.* (2020). We have adapted the methodologies in Howell *et al.* (2020) to account for different life history traits, back-cross frequency (due to generation length) and effective population size for *G. vitellina* or *G. alba* (Table 1).

The predicted rate of loss of heterozygosity was derived from the relationship between the inbreeding coefficient (F_t) and heterozygosity (H) in Eqn 4 (Crow and Kimura 1970):

$$H_t/H_0 = 1 - F_t \quad (4)$$

This allowed determination of residual heterozygosity (H_t/H_o) at each generation for which F_t was estimated. F_0 for founders was assumed to be 0.

The increase in inbreeding between generations in the captive colony without back-crossing was determined from the relationship in Eqn 5 (Frankham *et al.* 2010), which determines F_t (inbreeding coefficient, generation t) from N_e (effective population size) and F_{t-1} (inbreeding coefficient, generation t–1):

$$1 - F_t = (1 - 1/[2N_e]) \times (1 - F_{t-1}) \quad (5)$$

This allows sequential determination of F_t for any generation (t) up to the number required to reach 100-year heterozygosity benchmarks (25 generations for *G. vitellina* or *G. alba* assuming a generation interval of 4 years).

We used an iterative process, as in Howell *et al.* (2020), using Eqn 6 to determine the effect of recurrent back-crossing (each generation) using frozen founder spermatozoa on the rate of inbreeding for the hypothetical populations modelled in each of the three back-cross scenarios:

$$F_t = \left(1 - [1/2]^t\right)/2N_e \quad (6)$$

where N_e represents the effective number of founder males and t is the number of back-cross generations. For detailed derivation of Eqn 6, see Howell *et al.* (2020).

This genetic modelling involves a range of general assumptions presented in Howell *et al.* (2020) and species-specific assumptions for captive *G. vitellina* and *G. alba* populations, presented in Table 2.

Results

Holding captive colonies of *G. vitellina* and *G. alba* designed to maintain long-term genetic viability under increasingly rigorous genetic retention benchmarks (90%, 95% and 99% of source population H_t/H_o) would require increasing live animal numbers and therefore higher set-up and total program costs (Table 3). Populations with biobanked founder spermatozoa can meet these targets with greatly reduced colony sizes, and therefore reduced program costs (Table 3), compared with non-biobanked populations in captive colonies of *G. vitellina* and *G. alba* (Figs 1–3). Costs of current captive populations held into the future are also plotted in Figs 2 and 3 and are provided for comparative purposes only, but do not relate to the genetic or economic captive breeding models. There is no equivalence between the modelled scenarios and the current holdings that are not maintained as breeding colonies.

Captive breeding populations of *G. vitellina* ($N_e = 120$; $n = 400$ live individuals in the captive colony) and *G. alba* ($N_e = 120$; $n = 400$) designed to retain 90% of source population heterozygosity (<10% loss in H_t/H_o after 100 years) in a conventional captive colony without the use of biobanking technology would require significant Year 1 start-up costs of more than A$1.1 million for *G. vitellina* and more than A$718 000 for *G. alba*, followed by total 100-year program costs of more than A$466 million and more than A$284 million respectively (mean annual expenditure of more than A$4.6 million and more than A$2.8 million; Table 3). These hypothetical populations would meet 90% retention targets with residual H_t/H_o of 0.9009 after the captive period (Table 3; Fig. 1). The more ambitious target of 95% retention in hypothetical *G. vitellina* ($N_e = 244$; $n = 813$) and *G. alba* ($N_e = 244$; $n = 813$) breeding populations would require increased investment of more than A$945 million and more than A$576 million respectively across 100 years to maintain residual H_t/H_o of 0.9500 (Table 3; Fig. 1). The most ambitious genetic retention target presented here of 99% retention in *G. vitellina* ($N_e = 1245$; $n = 4150$) and *G. alba* ($N_e = 1245$; $n = 4150$) populations would require significantly increased investment of more than A$4.8 billion and more than A$2.9 billion across 100 years respectively to maintain residual H_t/H_o of 0.9900 (Table 3; Fig. 1).

Captive breeding populations of *G. vitellina* and *G. alba*, designed to meet the above heterozygosity retention targets under the conditions of our modelled populations, could reduce the required number of live individuals in each generation, and therefore program costs, by using biobanked *G. vitellina* and *G. alba* frozen founder spermatozoa to back-cross females in every generation to founder males.

Back-crossing every generation at 4-year intervals into *G. vitellina* and *G. alba* colonies designed to retain 90% source population heterozygosity ($n = 17$ founder males; same number of live females per generation) would each require A$948 000 in biobanking costs but would allow reductions of >380 individuals to live *G. vitellina* and *G. alba* census colonies of only 17 female individuals per generation. This would reduce *G. vitellina* and *G. alba* Year 1 program costs to A$68 000 and A$48 000 respectively and reduce total forecast 100-year program costs to more than A$21 million and more than A$13.3 million respectively to retain residual H_t/H_o of 0.9000 (Table 3; Figs 1–3).

Incorporating biobanking technology into hypothetical *G. vitellina* and *G. alba* populations designed to meet 95% and 99% genetic retention targets would result in comparatively higher program costs and colony sizes ($n = 33$ and 167 founder males; same number of live females respectively), but still considerably less than colony sizes required for non-back-crossed populations (Table 3). Populations of *G. vitellina* and *G. alba* designed to maintain 95% of initial source population heterozygosity ($n = 33$ founder males; same number of live females per generation) using biobanking technology to

Table 3. Genetic and cost analyses for hypothetical captive colonies of orange-bellied frogs *Geocrinia vitellina* and white-bellied frogs *Geocrinia alba* designed to meet different genetic retention targets under various genetic back-cross scenarios using an assumed N_e/N of 0.3, the mean ratio for captive vertebrate populations (Mace 1986)

Year 1 (start-up) and Year 2 costs are costs in Years 1 and 2 of the 100 years of colony life. 'Back-cross costs' are the estimates of costs of genetic back-cross events (i.e. the generation of offspring from cryopreserved founder spermatozoa) for each back-cross scenario based on the number of offspring to be generated. 'Total captive colony costs after 100 years' provide program costs without back-crossing. 'Total program cost after 100 years' include captive colony costs and expenditure for back-cross events. Back-cross scenarios tested were 90%, 95% and 99% heterozygosity retention with no back-cross and back-cross every generation. Effective population size (N_e) and colony numbers (N) are shown for all hypothetical colonies. Here, colony numbers (N) have been derived using an assumed N_e/N of 0.3, the mean ratio for captive populations (Mace 1986), to represent managed captive populations of *G. vitellina* and *G. alba* against the published values analysed in this study (1.17 and 1.21 for *G. vitellina* and *G. alba* respectively presented in Table 1; Table S4; Driscoll 1999) and against a mean of published wild-derived values derived using six amphibian species (Table 1; Table S5). The true N_e/N value for captive populations and the genetic and cost benefits are likely between the values presented here and in Table 1 and Tables S4 and S5. Inbreeding coefficients (F_t) and heterozygosity (H_t/H_0) are values at 100 years. All dollar amounts shown are in Australian dollars (A$), starting in 2019. n.d., not determined

Back-cross scenario	N_e	N	F_t		H_t/H_O after 100 years	Cost (A$)		Total captive colony cost (A$) after 100 years	Back-cross cost (A$; labour and set-up)	Total program cost (A$) after 100 years
			No backcross	Backcross		Year 1	Year 2			
G. vitellina										
90% heterozygosity retention										
No back-cross	120	400	0.0991	n.d.	0.9009	1 178 645	978 369	466 093 409	n.d.	466 093 409
Back-crossing every generation (4-year intervals)	n.d.	17	0.5	0.1	0.9	68 532	42 232	20 121 593	948 313	21 069 906
95% heterozygosity retention										
No back-cross	244	813	0.05	n.d.	0.95	2 391 590	1 984 422	945 379 952	n.d.	945 379 952
Back-crossing every generation (4-year intervals)	n.d.	33	0.5	0.05	0.95	116 206	81 207	38 689 595	1 032 383	39 721 977
99% heterozygosity retention										
No back-cross	1245	4150	0.01	n.d.	0.99	12 192 067	10 113 234	4 817 968 799	n.d.	4 817 968 799
Back-crossing every generation (4-year intervals)	n.d.	167	0.5	0.01	0.99	515 469	407 627	194 196 609	1 736 468	195 933 077
G. alba										
90% heterozygosity retention										
No back-cross	120	400	0.0991	n.d.	0.9009	718 954	597 088	284 449 915	n.d.	284 449 915
Back-crossing every generation (4-year intervals)	n.d.	17	0.5	0.1	0.9	48 996	26 027	12 401 745	948 313	13 350 057
95% heterozygosity retention										
No back-cross	244	813	0.05	n.d.	0.95	1 457 268	1 209 468	576 189 550	n.d.	576 189 550
Back-crossing every generation (4-year intervals)	n.d.	33	0.5	0.05	0.95	78 281	49 752	23 704 006	1 032 383	24 736 389
99% heterozygosity retention										
No back-cross	1245	4150	0.01	n.d.	0.99	7 422 777	6 157 441	2 933 417 543	n.d.	2 933 417 543
Back-crossing every generation (4-year intervals)	n.d.	167	0.5	0.01	0.99	323 548	248 442	118 360 450	1 736 468	120 096 918

back-cross every generation would each require more than A$1 million in biobanking-specific investment costs across 100 years. This would allow a reduction in the live colony size of 780 live individuals to a constant live colony size of only 33 *G. vitellina* and *G. alba* individuals (live females) in each program. This reduces total forecast 100-year program costs of A$945 million and A$576 million to A$39.7 million and A$24.7 million respectively in order to retain residual H_t/H_o of 0.9500 after the captive period (Table 3; Figs 1–3). Back-crossing every generation into *G. vitellina* and *G. alba* populations designed to maintain 99% source heterozygosity ($n = 167$ live females) would require A$1.7 million in 100-year biobanking-specific investment but would allow a reduction in required census captive colony numbers of 3983 individuals to 167 live *G. vitellina* and *G. alba* females in each program. This results in total forecast 100-year program costs of A$195.9 million and more than A$120 million respectively in order to retain residual H_t/H_o of 0.9900 after the captive period (Table 3; Figs 1–3).

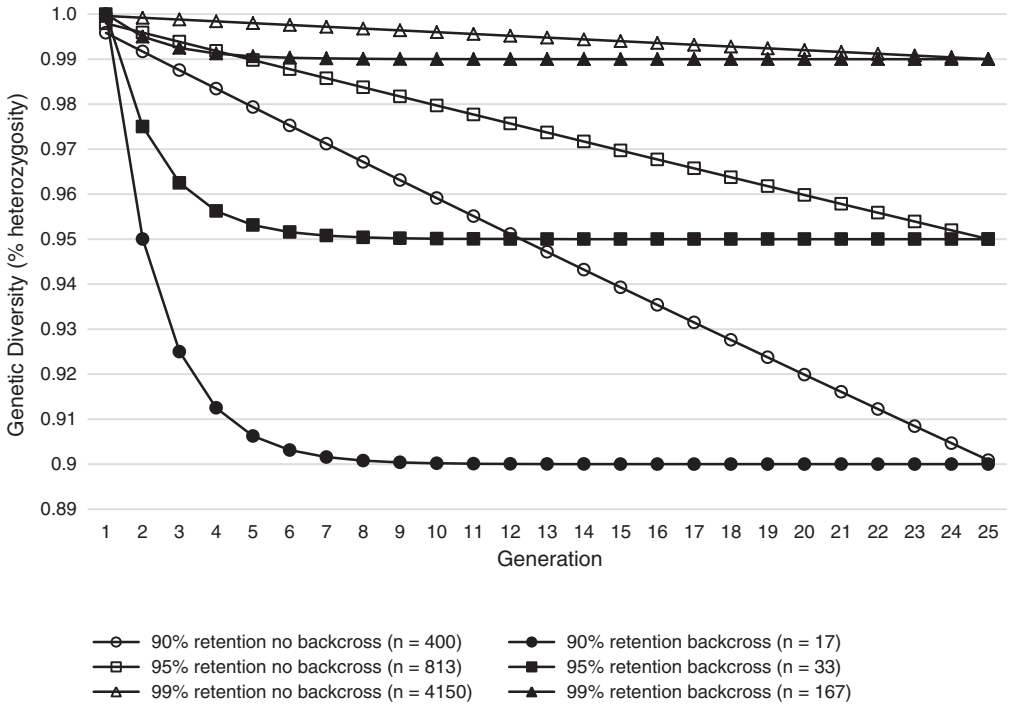

Fig. 1. Genetic retention (per cent initial heterozygosity, converted to decimal value) across 100 years (25 generations) in hypothetical captive populations of orange-bellied frogs *Geocrinia vitellina* and white-bellied frogs *Geocrinia alba* designed to meet different genetic retention goals (90%, 95% and 99% of source population heterozygosity for 100 years) without back-crossing or with back-crossing live females to founder males using cryopreserved *G. vitellina* and *G. alba* founder spermatozoa each generation (4-year intervals).

We present estimates of costing scenarios (comprised of additional infrastructure and variable labour costs) for incorporating biobanking technology into the modelled amphibian captive breeding program if it were to be conducted at husbandry costs currently encountered in Perth Zoo's Native Species Breeding Program (Table 3).

In *G. vitellina* and *G. alba* populations designed to retain 90%, 95% and 99% heterozygosity, the rate of inbreeding (F_t) is decreased considerably when back-crossing every generation (Table 3; Fig. 1). Biobanked *G. vitellina* and *G. alba* populations provide substantial cost reductions against the non-biobanked colony sizes required to achieve equivalent genetic targets (Table 3) and are even less expensive than estimates of the cost of the current head-starting program (Figs 2, 3) for 90% and 95% targets. For biobanked populations designed to meet the more ambitious genetic retention target of 99% H_t/H_o, the costs are considerably higher in *G. vitellina* and *G. alba* (Table 3; Figs 2, 3), but not excessively larger than maintaining the current head-start wild population supplementation program. Reductions in live captive colony numbers significantly increase rates of inbreeding in non-back-crossed populations (Fig. 1), leading to very large increases in costs required to achieve genetic targets (Table 3).

Discussion

Based on genetic and cost modelling scenarios generated in this study, we propose biobanking technology could be incorporated as a lower-cost approach to the operation of long-term captive breeding colonies of *G. vitellina* and *G. alba*, where the aim is to maintain a high level of wild-type, source population genetic diversity in a colony established from a single group of founders. The implementation of this approach would be subject to the development and optimisation of biobanking protocols for these species. Our modelling suggests significant cost savings, greatly reduced inbreeding and reduced requirement for large live colony sizes from combining conventional colony maintenance approaches with biobanking and ARTs. Extending this approach to other species, biobanking could allow heterozygosity retention targets of 90% of source populations (Soulé *et al.* 1986) to be achieved across a range of Australian amphibian captive breeding programs, and could be extended to more ambitious targets of 95–99% heterozygosity (Howell *et al.* 2020) within realistic cost frameworks.

We postulate a broad transitional pathway of actions to incorporate biobanking technology into Australia's *ex situ* management of amphibians (Table 4). This would require species-specific protocol development delivered by well-funded applied research programs designed to close knowledge gaps in the reproductive sciences for candidate amphibian species (Table 5). Recognising the potential merits of biobanking technology could provide a new direction for Australian amphibian *ex situ* management that has the potential to deliver substantial genetic and economic outcomes, and ultimately free economic resources to support captive programs for a greater quantum of at-risk Australian amphibian species.

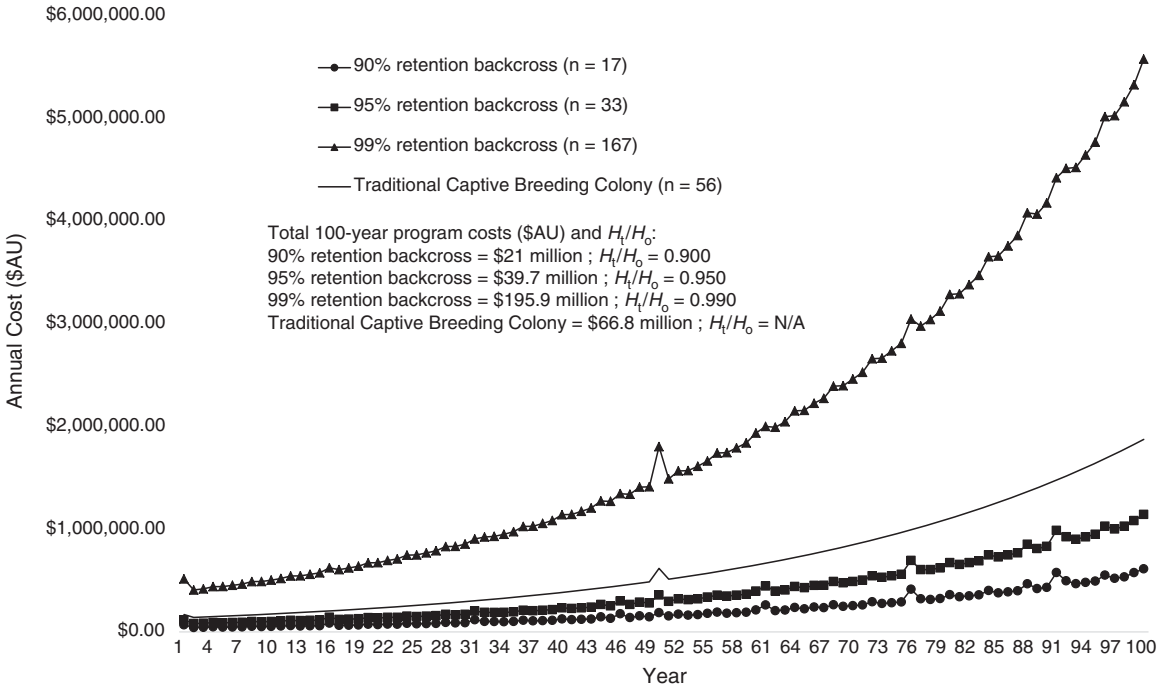

Fig. 2. Projections of 100-year annual costs for hypothetical captive orange-bellied frog *Geocrinia vitellina* populations designed to meet different genetic retention targets by incorporating biobanking technology. Projected 100-year total program costs are shown for modelled populations of *G. vitellina* designed to retain 90%, 95% and 99% of source population heterozygosity by recurrent genetic back-cross with frozen founder spermatozoa (4-year intervals). Projected costs for back-crossed populations represent colonies under an assumed mean N_e/N ratio of 0.3. Projected 100-year annual program costs are also shown for the actual *G. vitellina* captive population size held at Perth Zoo as at 2017–18 for comparison. All dollar amounts are shown in Australian dollars (A$), starting in 2019. *n*, colony size for all modelled populations and the current captive holdings held for wild population head-starting.

In the genetic modelling of Howell *et al.* (2020), the three drivers of census population size in captive colonies are the N_e/N ratio, the generation interval and the proportion of initial heterozygosity retained at the end of 100 years. Of these, N_e/N and generation interval vary as a function of life history traits and will vary from species to species. Hence, in the present study, both *G. vitellina* and *alba* achieve 90% heterozygosity retention in non-back-crossed colonies over 100 years with $N > 400$, whereas the equivalent for *R. pretiosa* is $N > 1800$ live animals (Howell *et al.* 2020); these differences are driven by the generation interval (3 years for *R. pretiosa*; 4 years for *G. vitellina* and *G. alba*) and the N_e/N (0.086 for *R pretiosa*; 0.3 for *G. vitellina* and *G. alba*; Mace 1986; Phillipsen *et al.* 2010). Inspection of model outputs from Howell *et al.* (2020) and the present study shows that the *N* required to meet 90% heterozygosity retention with back-crossing for *R. pretiosa* is 58 (97% decline from 1826), compared with 17 (96% decline from 400) for *G. vitellina* and *G. alba*. There is a much greater reduction in census *N* in absolute terms for species with a lower N_e/N and longer generation interval. This means that species with shorter generation intervals and lower N_e/N ratios will benefit most in terms of cost benefits and a reduced number of live animals required in the colony if biobanking is used.

Economic costs from modelling captive breeding will also vary with life history traits, for example aquatic breeding species such as *R. pretiosa* (Pearl *et al.* 2009) versus terrestrial breeding species without an aquatic tadpole stage such as *G. vitellina* and *G. alba* (Clulow and Swan 2018), with differing costs of biobanking and ART in the back-crossed colonies. Taking genetic and costing models together, the outcomes of using biobanking may therefore differ between species, in addition to the economic benefits. Nevertheless, absolute numbers that must be kept alive in colonies for equivalent genetic heterozygosity targets are reduced in all cases when back-crossing with biobanked genomes is used. In addition, for a given census population size, there is always a genetic benefit in using biobanking. In practice, benefits to captive breeding institutions will likely vary across species with clutch size (Morrison and Hero 2003). Species with larger clutch sizes are expected to generate a higher output of animals optimal for reintroductions, but also raise overall holding costs if animals are not removed from the colony; here the key benefit is the quantum of genetically fit individuals for release to the wild. Although the overall output benefits of incorporating the biobanking approach into the management of species with small clutch sizes is expected to be lower, the clear potential benefit in this case is potentially maximising the contribution of all females in the colony through assisted reproduction, as well as the genetic fitness of output animals. This approach may be particularly powerful for species with small clutch sizes with captive

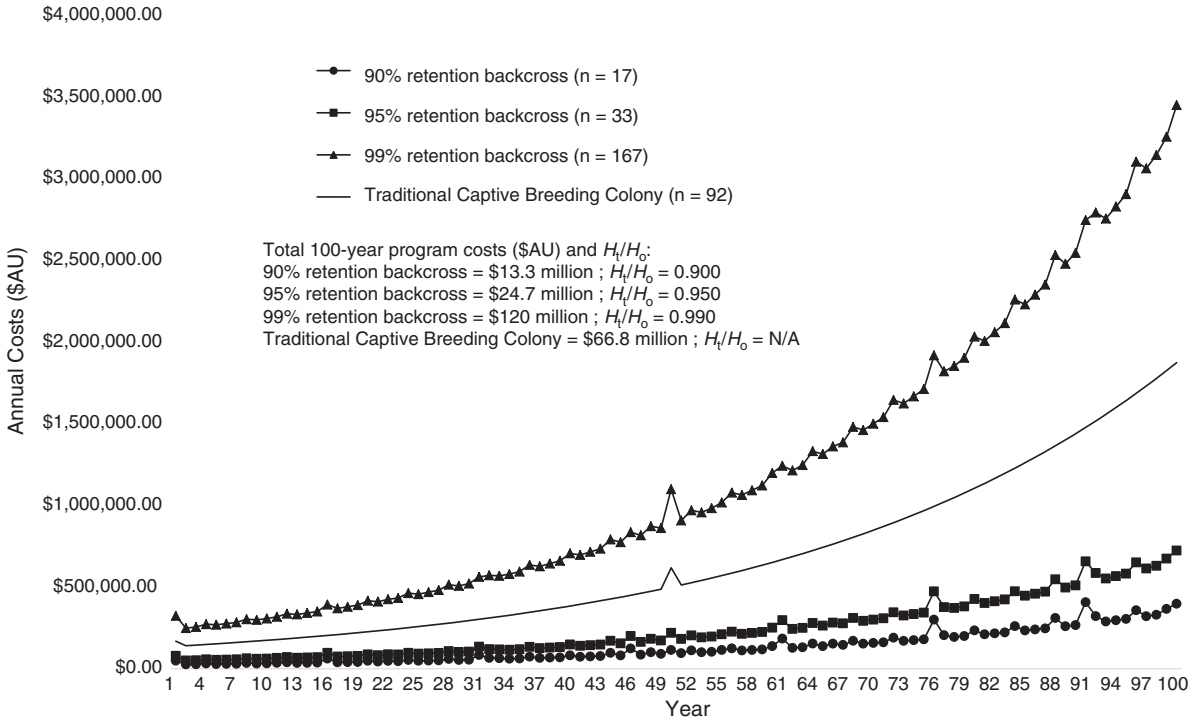

Fig. 3. Projections of 100-year annual costs for hypothetical captive white-bellied frogs *Geocrinia alba* populations designed to meet different genetic retention targets by incorporating biobanking technology. Projected 100-year total program costs are shown for modelled populations of *G. alba* designed to retain 90%, 95% and 99% of source population heterozygosity by recurrent genetic back-cross with frozen founder spermatozoa (4-year intervals). Projected costs for back-crossed populations represent colonies under an assumed mean N_e/N ratio of 0.3. Projected 100-year annual program costs are also shown for the actual *G. alba* captive population size held at Perth Zoo as at 2017–18 for comparison. All dollar amounts are shown in Australian dollars (A$), starting in 2019. *n*, colony size for all modelled populations and the current captive holdings held for wild population head-starting.

husbandry issues, which could otherwise be a detrimental combination in breed-for-release programs.

We focused on modelling the minimum numbers of animals needed to be maintained alive in colonies and the minimalist biobanking scenario required to achieve the heterozygosity targets. In practice, larger numbers and variations in biobanking protocols may be pursued to achieve additional outcomes, at relatively little additional expense. For high-value banked genetic resources, some risk management strategies would be recommended, such as storage of replicated samples in multiple locations (Mintzer *et al.* 2013; Morrin and Robinson 2013). This would increase the costs of biobanking, but not substantially (Della Togna *et al.* 2020). In addition, some captive-assurance populations may hold larger numbers of live animals to deal with catastrophic demographic events in wild populations, and perhaps an N of around 100 may be closer to a realistic minimum. For example, Species Survival Plan Programs had an average N of 137 (Hodskins 1997). Adjusting live numbers to accommodate these scenarios falls within the range of modelling scenarios in this paper (Table 3; Figs 2, 3), and would not add substantially to costs compared with non-banked scenarios.

The approach suggested here for the *Geocrinia* species may also deliver major genetic and cost efficiencies to captive breeding programs for other Australian amphibians. For example, consider the maximum numbers of other amphibians kept in captive programs for various Australian amphibians provided in Harley *et al.* (2018): 410 southern corroboree frogs *Pseudophryne corroboree*, 465 northern corroboree frogs *Pseudophryne pengilleyi*, 1000 Baw Baw frogs *Philoria frosti*, >1000 spotted tree frogs *Litoria spenceri* and 1340 Alpine tree frogs *Litoria verreauxii alpina*. It is uncertain what the intrinsic rate of inbreeding in these captive breeding programs is because these captive breeding programs do not yet have any DNA profiling or genetic studbooks. It is also uncertain what the actual N_e/N ratios are (as for the *Geocrinia* complex in captivity), but these may vary considerably. In modelling for the present paper, we opted for a conservative approach by using the mean captive N_e/N ratio of 0.3 for vertebrate species (Mace 1986) in the absence of more definitive values. Although species-specific values do exist for *G. vitellina* and *G. alba* (Table 1; Driscoll 1999), these are much higher than other estimates for amphibians that account for the same variables (Table 1). Nevertheless, we have also run the models with other N_e/N values, resulting in low (Table 1; Supplementary Table S4) and high (Table 1; Supplementary Table S5) extremes of possible colony size under alternative N_e/N ratios (the species-specific wild-derived values as well as the median for various temporal estimates for amphibians) and the relative outcomes

Table 4. Pathway of broad interinstitutional actions required to transition biobanking technology into Australian amphibian captive breeding across the existing network of *ex situ* institutions

Pathway to transition biobanking technology into amphibian captive breeding programs
1 Build the case for amphibian biobanking using economic and genetic arguments: desktop feasibility studies for candidate amphibian species including the economic and genetic modelling presented here and in Howell *et al.* (2020)
Develop research partnerships and/or encourage transparency and data sharing regarding program costs, as well as pedigree and studbook data (if this information is available or exists), to facilitate modelling and recognition of the potential genetic and economic merits of biobanking technology across the *ex situ* network: this will give a clear sense of the long-term costs and the number of animals required to be banked in Year 1 to meet genetic targets, and will provide leverage for funding (Della Togna *et al.* 2020)
2 Secure target species captive colony for research and program development: this may require establishment of new captive colonies or require interinstitutional partnerships. Some candidate species include the orange-bellied frog *Geocrinia vitellina*, white-bellied frog *Geocrinia alba*, the Baw Baw frog *Philoria frosti*, giant burrowing frog *Heleioporus australiacus*, Littlejohn's tree frog *Litoria littlejohni*, northern corroboree frog *Pseudophryne pengilleyi*, southern corroboree frog *Pseudophryne corroboree* and the spotted tree frog *Litoria spenceri*
Where partnership is not possible, or for novel candidate species, develop *ex situ* programs ideally within an existing *ex situ* institution (this will lower overall costs; see comparisons in Table 5); for novel candidate species, select founders from discrete *ex situ* or *in situ* populations where possible
3 Financial planning and funding mechanism development (as presented in Della Togna *et al.* 2020), with an emphasis on long-term biobank sustainability and efficiency: focus on a clear understanding of the desired long-term economic and genetic outcomes and the associated recurring input costs required across the life of the infrastructure to achieve these outcomes (Step 1). Developing a funding mechanism for the long-term biobanking of amphibians requires a clear understanding of the associated costs, which are species specific. These data are lacking for most species, but an approach to model these costs is presented in Howell *et al.* (2020) and in the present study. Long-term biobanking programs will demand dedicated funding commitment in line with genetic targets (minimum 100-year durations) and it is unclear whether this funding security exists. Various funding mechanisms exist across biomedical biobanks (e.g. cost recovery, public perception and acceptance, analysis of impact and value studies, online economic planning tools, user pays systems and commercialisation; Della Togna *et al.* 2020); however, their applicability for wildlife-based biobanking is unexplored and, until these models are better understood, long-term government or institutional funding will be required (Della Togna *et al.* 2020)
4 Assess existing infrastructure capacity and requirements across the *ex situ* institution networks (e.g. zoo associations) and infrastructure across stakeholder and partnership networks (e.g. government, philanthropic, museums)
Ensure interinstitutional partnerships allow necessary access to facilities and infrastructure (including access to captive colonies)
Determine any additional biobanking-specific infrastructure required: freezing infrastructure, including programmable freezers, and liquid nitrogen dewars
5 Assess capacity to fill knowledge gaps and research and development capacity across stakeholder and partnership networks, including the availability of research funding, and the availability of skilled personnel and expertise across the *ex situ* network
Encourage interinstitutional knowledge and skills transfer to build and/or spread capacity across the *ex situ* network
6 Identify knowledge gaps in underlying reproductive sciences precluding the biobanking approach for candidate amphibian species via communication and interinstitutional knowledge sharing, training and collaborative research. Species-specific knowledge gaps are likely to differ considerably across species with different life histories and reproductive strategies. Research will be required to close knowledge gaps in the broad areas of hormone induction of ovulation and spermiation, cryopreservation and thawing of spermatozoa and successful IVF
7 Capacity build for research and additional infrastructure requirements: aim to leverage multisectoral and collaborative research funding (philanthropic, industry and/or all levels of government) using the end-stage potential species-specific genetic and economic outcomes generated by modelling (Step 1)
8 Conduct applied research programs to close knowledge gaps in the underlying reproductive sciences for target candidate amphibian species. This will require well-funded research programs using colony animals in areas precluding biobanking protocols (Step 6; we provide per-species budget estimates for this step in Table 5)
9 Use knowledge and research outcomes to develop and optimise species-specific biobanking protocols for candidate species, including a planned backcross schedule (with strict resourcing and financial planning; see Steps 1 and 2) based on species reproductive system and life history (e.g. generation length). Detailed protocols based on the species-specific backcross requirements should be imbedded within the program and institutional policy and be optimised and repeatable (to pass down across long time periods with inevitable staff turnover). This information should be captured in the same platforms available to conventional captive breeding programs (i.e. Species360 (https://www.species360.org) or annual report and recommendation documents associated with Zoo and Aquarium Association of Australasia studbooks)
10 Design and implement Year 1 of the *ex situ* program incorporating biobanking technology, followed by long-term monitoring of progress towards genetic and economic outcomes
Perform DNA profiling of all founders before Year 1 and maintain documented genetic pairings and studbook data across the life of the program

are the same. These scenarios still result in similar proportionate benefits genetically (Supplementary Tables S4, S5; Supplementary Figs S1–S4) and from a cost perspective when backcrossing with biobanked founder spermatozoa is undertaken. Because we do not know N_e/N for these other Australian species or the generation intervals in practice in those captive breeding programs, we cannot be certain of the potential reduction in live colony size that could be achieved while achieving 90% (or other) heterozygosity retention targets. Nevertheless, for each of these species, it is likely to be substantial, as for *Geocrinia* and *R. pretiosa* mentioned above, with a well over 90% reduction in the required number of live animals in captive colonies.

There is some uncertainty as to the actual costs associated with incorporating biobanking technology into the captive

Table 5. Budget and time frame estimates for closing knowledge gaps in candidate amphibian species to incorporate biobanking into captive management

Estimates presented are per candidate species and a general estimate based on author experience. We assume several species of similar life histories could be covered under the presented budgets for labour; however, we provide a general per-species estimate. All dollar amounts are in Australian dollars (A$, 2020). For the assumed timeline of 2–5 years, we provide the minimum 2-year budget and maximum 5-year budget

	Cost (A$)	Total cost (A$)
Costs assuming research is conducted within existing *ex situ* network (zoo and/or university partnership) where infrastructure and overhead costs are covered		
Labour and research personnel[A] (2–5 years)		
Experienced postdoctoral researcher	150 000 p.a.	500 000–1 250 000
Research technician support	100 000 p.a.	
Minor equipment and consumables[B] (2–5 years)	30 000 p.a.	60 000–150 000
Additional low-cost freezing infrastructure[C]	14 000	14 000
Minimum subtotal		574 000–1 470 000
Costs for a novel research program without existing infrastructure and systems to absorb infrastructure and overhead costs		
Labour and research personnel[A] (2–5 years)		
Husbandry staff	100 000 p.a.	700 000–1 750 000
Experienced postdoctoral researcher	150 000 p.a.	
Research technician support	100 000 p.a.	
Operating costs[D] (2–5 years)	50 000 p.a.	100 000–250 000
Minor equipment and consumables[B] (2–5 years).	30 000 p.a.	60 000–150 000
Conventional captive colony costs[E] (per species)	200 000 p.a.	500 000–1 100 000 (per species)
Additional captive colony infrastructure costs, including low-cost freezing infrastructure[C]	100 000	
Minimum subtotal		1 260 000–3 250 000

[A]Could be spread across related species.
[B]For example, freezing straws, hormones, syringes, liquid nitrogen, microscopes, slides, pipettes etc.
[C]For example, liquid nitrogen dewars and programmable freezer, as proposed in the present study and by Della Togna *et al.* (2020) and Howell *et al.* (2020).
[D]Management, utilities etc.
[E]For example, food, utilities, facilities etc.

management of amphibian species, regardless of species, because there are no captive programs where such procedures are routinely incorporated into the breeding programs. Our modelling suggests that when these procedures are developed and optimised, these would be minimal compared with total program costs (Table 3). In this respect, our modelling for *Geocrinia* is consistent with modelling for *R. pretiosa* (Howell *et al.* 2020), and likely to hold true for most amphibian species in captive programs. Costs associated with cryostoring *G. vitellina* and *G. alba* spermatozoa (A$14 000 in set-up costs and more than A$700 in Year 1 labour costs) and using frozen samples through IVF to generate live offspring (more than A$460 000 in potential accumulated 100-year labour costs) represent 4.5% and 7% of total captive program costs for *G. vitellina* and *G. alba* respectively in populations designed to retain 90% heterozygosity (Table 3). Costs associated with performing recurring back-crosses within captive colonies (using ARTs such as IVF) will vary across species and taxa due to differences in life history traits (particularly generation length), as well as in reproductive strategies, which will determine the applicability of available ARTs (e.g. aquatic breeding species vs terrestrial breeders, as mentioned above). *R. pretiosa* populations designed to retain 90% heterozygosity required accumulated labour costs of C$67 000 (A$69 000) across 100-years for recurrent backcrosses at 3-year intervals (Howell *et al.* 2020), compared with A$460 000 proposed here for *G. vitellina* and *G. alba* for recurrent back-crosses occurring every 4 years (Table 3).

The parameters and assumptions of the genetic modelling (Table 2) ensure that biobanked populations retain the benefits of traditional captive breeding programs and would allow the continued contribution of captive breeding institutions to *in situ* and *ex situ* conservation outcomes. A biobanking program for *Geocrinia* and other species would still provide an output of individuals for research, translocation and disease mitigation, as well as individuals for public displays (providing public engagement and education), transfer of individuals (also applies to frozen material) and the subdivision of populations between institutions to bolster genetics and establish new programs. Howell *et al.* (2020) noted that reintroducing founder genomes reflecting wild-type, source population genotypes each generation to the captive population would have a range of fitness benefits, including reduced selection for domestication alleles that would benefit translocated animals (Frankham 2008; Allentoft and O'Brien 2010). Output animals from biobanked populations would likely be better suited for successful translocation (Griffiths and Pavajeau 2008; Robert 2009; Skerratt *et al.* 2016), due to genetic fitness, with the additional benefit of lower costs of colony maintenance before release (Table 3; Figs 2, 3).

There is no doubt that there are various successful captive breeding programs for Australian amphibians mentioned above, and presented in Table 4 and Harley *et al.* (2018). The *Geocrinia*

species program represents a good example, with high survivorship and regular reintroductions to the wild to bolster *in situ* populations. Despite documented success, it is unclear whether the financial sustainability exists for this success in Australia's captive breeding efforts to be maintained long enough to continue bolstering wild populations under persistent threats or to meet the time frames demanded by current accepted global targets for genetic diversity (90% genetic retention for 100 years; Soulé *et al.* 1986; Harley *et al.* 2018; Howell *et al.* 2020). The *Geocrinia* program would also have the added genetic benefit of regular sourcing of new animals from the wild (therefore likely being subject to minimal domestication and inbreeding, although this cannot be quantified in the present study) and it would be expected that this program would have higher genetic diversity than closed captive breeding programs from the same group of founders. However, programs that rely heavily on collection from *in situ* environments (particularly for highly threatened species) are highly vulnerable to stochastic events (e.g. bushfire and drought; Hoffmann *et al.* 2021) as well as the persistent threat of disease (Bower *et al.* 2017; Scheele *et al.* 2017). Mass mortality and emerging threats can wipe out significant genetic diversity from *in situ* populations permanently without added frozen insurance (e.g. the mass amphibian mortality seen in the 2019–20 Black Summer Bushfires; WWF 2020) and the persistence of disease in the landscape usually means this driver of amphibian decline cannot yet be properly mitigated before the release of captive-bred animals back into the wild (Skerratt *et al.* 2016; Scheele *et al.* 2017), although see Clulow *et al.* (2018*a*) for some emerging hope. Species in other conventional captive breeding programs would be equally vulnerable to this phenomenon without the biobanking approach, and species under no captive management are especially vulnerable. The closed system we advocate in the present study would maintain high levels of source population heterozygosity from single founder groups at lower long-term costs (Table 3; Figs 1–3) and would therefore be largely immune from these challenges and could allow long-term insurance while *in situ* threats persist.

Della Togna *et al.* (2020) and Howell *et al.* (2020) argued that the most promising model for incorporating biobanking and ARTs into the captive management of amphibians to deliver outcomes and make practical use of frozen samples is additional infrastructure within established captive breeding institutions and frameworks. We argue here that many of the strategic partnerships, infrastructure and skills already exist across Australia's *ex situ* network that could produce practical biobanking examples with a range of potential candidate species in well-resourced captive breeding and research programs across the network (Harley *et al.* 2018). An important transitional step towards practical biobanking in captive breeding will be the closing of species-specific knowledge gaps in the underlying reproductive sciences through applied research programs to develop biobanking protocols for candidate species (Table 4). Recent advances in the reproductive sciences for amphibians (Clulow *et al.* 2014; Clulow and Clulow 2016, 2018), could allow knowledge gaps to be closed in 2–5 years for individual species candidates with targeted applied research programs on the key knowledge gaps precluding the biobanking approach (Table 4). This includes the technological tools of hormone induction and the freezing, cryostorage and thawing of spermatozoa for use in optimised IVF protocols. This will require funding support, leveraged by the argument for the potential genetic and economic benefits of the technology. Funding support will require a focus on ARTs for amphibian conservation, both internally by captive breeding institutions and externally by policy makers, research funding bodies and the non-profit sector. We have provided general per-species budget estimates as an indicator of potential funding requirements and time frames (Table 5).

The actions proposed here could provide an efficient pathway to transition biobanking technology into the *ex situ* management of Australian amphibians by recognising and quantifying the potential cost and genetic benefits in order to leverage research funding and in-kind support, and facilitate a focused and rapid approach to the required applied research (Table 4). Once a practical reality, biobanking technology in captive breeding programs could generate considerable institutional cost savings, freeing economic resources to allow captive programs with high genetic retention rates for a greater number of at-risk Australian amphibians.

Conflicts of interest

John Clulow, Simon Clulow and Natalie Calatayud are guest Associate Editors. Despite this relationship, they did not at any stage have editor-level access to this manuscript while in peer review, as is the standard practice when handling manuscripts submitted by an editor of this Journal. The authors have no further conflicts of interest to declare.

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