

Reintroduction success of threatened Australian trout cod (*Maccullochella macquariensis*) based on growth and reproduction

Jarod P. Lyon^{A,E,G}, Charles Todd^A, Simon J. Nicol^B, Alasdair MacDonald^A, Daniel Stoessel^A, Brett A. Ingram^C, Richard J. Barker^D and Corey J. A. Bradshaw^{E,F}

^ADepartment of Sustainability and Environment, Arthur Rylah Institute for Environmental Research, 123 Brown Street, Heidelberg, Vic. 3084, Australia.

^BOceanic Fisheries Programme, Secretariat of the Pacific Community, BP D5, 98848 Noumea, CEDEX New Caledonia.

^CDepartment of Primary Industries, Private Bag 20, Alexandra, Vic. 3714, Australia.

^DDepartment of Mathematics and Statistics, University of Otago, PO Box 56, Dunedin, New Zealand.

^EThe Environment Institute and School of Earth and Environmental Science, The University of Adelaide, Adelaide, SA 5005, Australia.

^FSouth Australian Research and Development Institute, PO Box 120, Henley Beach, SA 5022, Australia.

^GCorresponding author. Email: jarod.lyon@dse.vic.gov.au

Abstract. Internationally, re-introductions of endangered species into their former ranges have largely failed. Here we assess a successful reintroduction program of the endangered trout cod (*Maccullochella macquariensis*) and examine factors contributing to this success. Stocking of marked fish (all stocked fish were marked) occurred between 1997 and 2006 in the Ovens River, south-eastern Australia, where trout cod were historically abundant but locally extinct by the 1980s. We found no natural recruits (i.e. from spawnings of stocked fish in the wild) over the age of six, indicating that natural recruitment started at most five years after stocking began. Of the 83 fish we examined for sexual maturity, 12 were immature, 20 were male, and 51 were female. The body length at which 50% of the population can be considered mature was 325 and 250 mm for females and males, respectively. The length at which 90% of the population was mature was 394 and 318 mm for females and males, respectively. The smallest mature female was 245 mm. Average relative fertility was 9 eggs g⁻¹ fish weight. The results we obtained provide valuable insights into the aspects contributing to the success of reintroduction programs for endangered freshwater species.

Additional keywords: fish, *Maccullochella macquariensis*, recruitment, reintroduction, stocking, threatened.

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Introduction

Reintroduction of native fishes into areas from which they have been extirpated is being embraced globally as a means to reduce the probability of species-wide extinctions. There are two main reasons why fish are reintroduced to the wild: (i) for fisheries management (i.e. restocking to replace fish removed) and (ii) conservation (i.e. to prevent extinction) (Brown and Day 2002). For native fish conservation, a basic premise needs to be considered when reintroducing individuals: the threats suspected or known to have caused extirpation need to be managed and attenuated before, during and after the reintroduction itself (Reading *et al.* 1991; Brown and Day 2002). However, in some cases, the resources enabling successful

management are not available even though the threats are known. As such, reintroductions of animals into their former range have largely failed (Reading *et al.* 1991).

Many fish species have a long history of captive breeding and subsequent release, both for fisheries and conservation objectives. The general life history of most fishes, which allows large numbers of offspring to be produced for a relatively small investment (compared to other taxa), makes this possible (Rakes *et al.* 1999; Brown and Day 2002). Reading *et al.* (1991) discussed a holistic model for reintroduction which takes into account not only biology, but also socioeconomic, organisational and authoritative aspects in considering why a species has become extinct in parts of its natural range. From a global

perspective, reintroductions of threatened fishes are only occasionally successful. In the Colorado River Basin, USA, stockings of the endangered razorback sucker *Xyrauchen texanus* have been occurring for more than thirty years, with limited success (Schooley and Marsh 2007), and while small-scale captive breeding of threatened fishes in the south-eastern USA has been done, the success of re-introductions could be considered mediocre (Rakes *et al.* 1999).

The Murray–Darling Basin in south-eastern Australia has experienced considerable land-use change since European settlement in 1788 (Bradshaw 2012). As such, fish populations there have declined because of habitat loss, altered flow and temperature patterns, in-stream sedimentation, introduced (alien) fishes and population fragmentation caused by in-stream barriers and over fishing – issues that have been consistent with reduction in freshwater fish numbers in many river systems globally (Barrett 2004; Kondolf *et al.* 2008; Honea *et al.* 2009; Winter *et al.* 2009). The Australian trout cod (*Maccullochella macquariensis* Cuvier) was once considered widespread in the southern tributaries of the Murray–Darling Basin (Berra 1974; Harris and Rowland 1996). However, over the past fifty years, its distribution and abundance have declined. Trout cod are a long-lived (>20 years), large-bodied species, with a maximum size of 16 kg and 850 mm total length (Harris and Rowland 1996). They occupy a range of habitats, but are strongly associated with large woody instream habitats (Nicol *et al.* 2007). Trout cod are now listed nationally as *Endangered* under the Australian Environment Protection and Biodiversity Conservation Act (*EPBC Act 1999*) and are listed as *Endangered* by the IUCN Red List (www.iucnredlist.org, accessed 10 November 2011).

In the Ovens River system located in the southern Murray–Darling Basin, trout cod were locally extinct by the 1980s (Cadwallader and Gooley 1984). In an attempt to re-establish viable populations, captive breeding programs were established in the 1980s to produce fingerlings for recovering populations (Ingram *et al.* 1990). Hatchery-reared juveniles bred at the Department of Primary Industry (DPI) Snobs Creek Hatchery were stocked into the Ovens River for 10 consecutive years starting in 1997. In this paper, we assess the success of this stocking regime to test the following hypotheses: (1) the duration of this stocking program (10 years) was an essential element for its ultimate success; (2) hatchery fish do not differ from wild-bred individuals (measured by chemical marking of hatchery fish before release) in terms of growth and reproduction; (3) the release of yearling fish improves the viability of such stockings; and (4) genetic diversity (as measured by the number of alleles) in a stocked population is less than in a natural population because of inbreeding and the founder effect (Frankel and Soulé 1981). The results have implications for other conservation programmes for endangered species that use re-introduction as a tool for recovery.

Methods

Fish were stocked in the region of the lower Ovens River system downstream from Wangaratta and upstream of the influence of Lake Mulwala (Fig. 1). The lower Ovens River in this area is a typical lowland river characterised by deep pools with shallower

connections, and a braided floodplain with numerous anabranches and backwaters. Long-term average discharge (1891–2000) for the river is 1640 giganlitres year⁻¹ (MDBA 2003).

Fish were stocked as both fingerlings (0+) and one-year olds (1+) to test the assumption that yearling fish had a higher probability of survival to adult size than fingerling fish (Table 1). We applied chemical marks to otoliths of hatchery-reared fish, by immersing fish for 24 h in a solution of oxytetracycline (a chemical which permanently marks the otolith). In 2007, twelve months after the cessation of the 10-year stocking program, we collected a sample of the population using electrofishing and angling: 47 trout cod in February and 84 fish in the August to November spawning period (when sexual maturity was more easily determined). We killed captured fish and returned them to the laboratory for further processing.

Otoliths

We examined otoliths to assess growth, age structure, and to determine whether or not sampled fish were hatchery-reared or wild-bred. From each of the 131 fish sampled, we removed sagittal otoliths, then washed and stored them dry before sectioning. We first examined sections using fluorescence microscopy for specifically marked growth rings to determine whether each sampled fish was originally stocked, and also used otoliths to determine the age and growth of each individual fish. Sections were viewed with transmitted light at 25× magnification. Ages were estimated by counting the completed zones (translucent – opaque sequence). A customized image analysis system (Morison *et al.* 1998) was used to mark and count increments along a transect between the primordium and the proximal edge of the section, adjacent to the dorsal side of the sulcus.

Gonads

From the sample of 131 fish, we examined the gonads of 84 trout cod to determine sex and sexual development. Our protocol ensured collection over the trout cod spawning period to provide information on spawning times and condition throughout the season. We weighed all gonads to the nearest gram. For females, we preserved gonads in a solution of 85% formalin and counted a 1-g subsample of oocytes from each gonad to estimate fertility, determining reproductive maturity of individuals using an adaptation of a macroscopic, eight-stage descriptor (FI–VIII) developed for Murray cod *Maccullochella peelii*, a closely related species (Gooley *et al.* 1995). We did histology on a portion of the fish sampled to confirm macroscopic estimates of development by embedding a transverse medial sub-sample of each individually preserved gonad into a paraffin wax block, sectioning the block (6 µm), and staining it with Harris' haematoxylin and eosin (Luna 1968). We then mounted the section on a slide under a coverslip, numbered it, and staged it as per Gooley *et al.* (1995).

To estimate size at sexual maturity (L_{m50}), we categorised female and male trout cod as mature when they were macroscopically within stages IV–VIII (see Gooley *et al.* 1995 for stage description). For each sex, we estimated size (total length) at maturity by determining the proportion of mature and

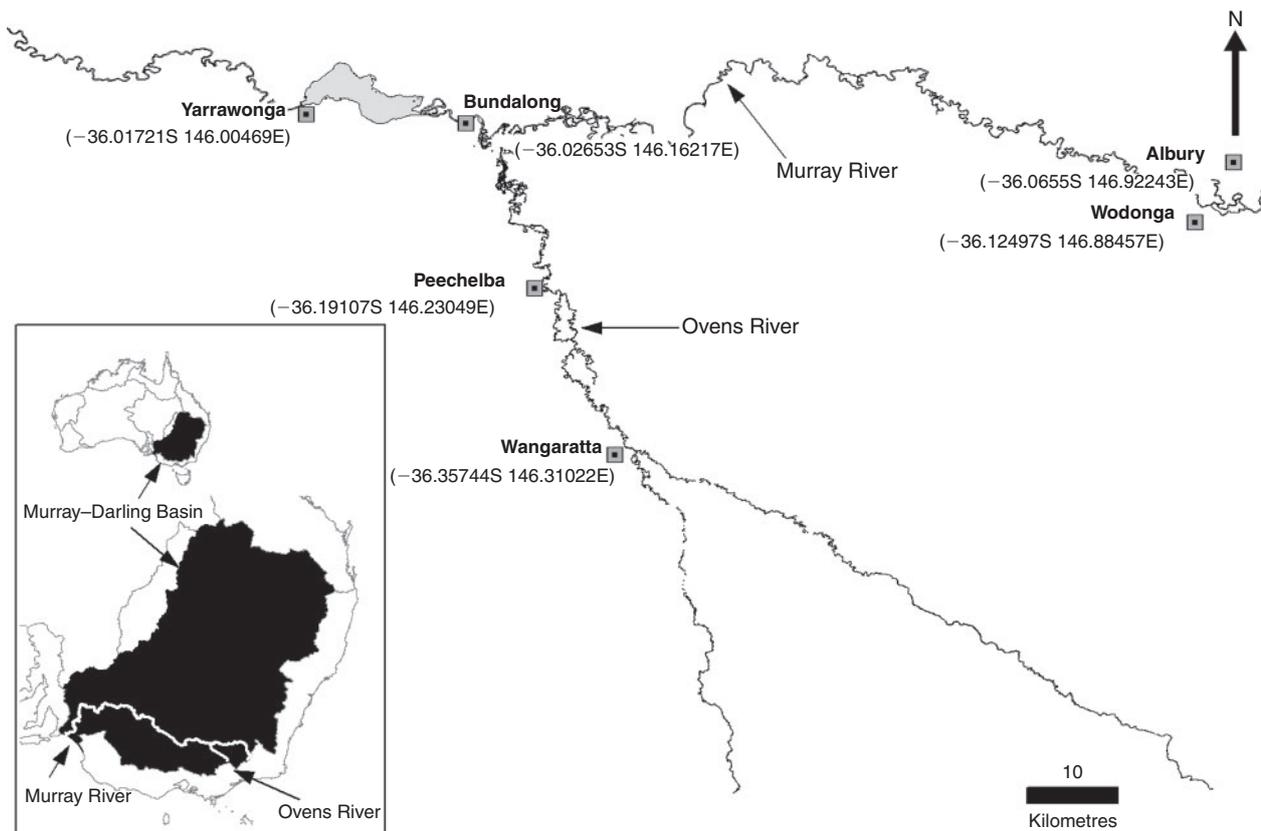


Fig. 1. Map of study area.

immature fish in each 10-mm length class, and then fitted a logistic function to the data using a non-linear, least-squares procedure weighted by sample size on each length class (see Brown *et al.* 2005).

Genetics

We extracted and purified DNA from finclip tissue collected from 50 trout cod caught from the Ovens River in the area where fish were originally released, and 50 fish from the Murray River between Yarrowonga Weir and Cobram (an area from which trout cod have not been stocked). The latter population was the source of broodstock used in the captive breeding program at DPI, Snobs Creek. We extracted total genomic DNA using the QIAGEN DNeasy tissue kit (QIAGEN Pty Ltd Doncaster, Melbourne). We genotyped individuals using nine microsatellite loci (Rourke *et al.* 2007), and analysed these data using GENEPOP version 3.3 (<http://wbiomed.curtin.edu.au/genepop>, accessed 2 July 2011) and ARLEQUIN 3.01 (Excoffier *et al.* 2005). We assessed whether the data conformed to Hardy–Weinberg equilibrium genotype frequency proportions (expected with random mating within populations), if F_{ST} deviated from zero (expected if there is genetic structure between Ovens and Murray populations), differences in number of alleles between populations and probability of assignment (showing how individuals can be best allocated to the alternative populations).

Table 1. Numbers of hatchery-reared trout cod released into the Ovens River between 1997 and 2006

Year	<i>n</i> fingerlings (~35 mm TL)	<i>n</i> yearlings (~180 mm TL)
1997	13 100	0
1998	24 000	653
1999	30 000	2430
2000	20 000	0
2001	51 700	0
2002	30 000	0
2003	44 260	0
2004	30 000	900
2005	24 000	0
2006	10 400	0
Total	277 460	3983

Growth model

To test whether growth in hatchery fish differed from wild-bred individuals, we constructed a standard nonlinear regression model of age against length using two common growth curves, 1. von Bertalanffy (1938) and 2. Gompertz (1825) respectively:

$$\text{Length} = L_a \times (1 - \exp(-k \times (\text{Age} - t_0))) \quad (1)$$

$$\text{Length} = L_a \times \exp(\exp(-k \times (\text{Age} - t_0))) \quad (2)$$

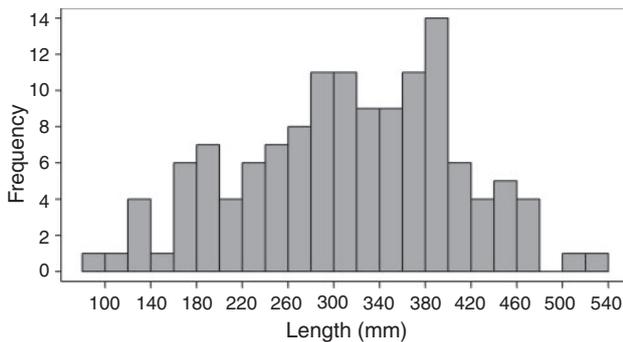


Fig. 2. Length–frequency of trout cod captured in the Ovens River, 2007 ($n = 131$).

where L_a is the asymptotic length, k the annual growth rate, and t_0 the theoretical age at zero length. We applied nonlinear least-squares estimation of the parameters for both growth models using the R package (R Development Core Team 2011) to examine differences between growth in wild and stocked fish.

Sexual maturity

To test whether maturity (as a function of length) differed between hatchery fish and wild-bred individuals, we fitted a multi-level logistic regression model treating the response variable stage (mature or immature) as exchangeable observations of a Bernoulli random variable, with the logit of the probability of being mature expressed as a linear function of effects according to sex, age, length and origin (hatchery-reared or wild-reared). The model included an interaction between age and length. We also modelled sex as exchangeable observations of a Bernoulli random variable with the logit of the probability of being male expressed as a linear function of effects according to length and origin.

We used Bayesian inference implemented through the program WinBUGS (Lunn *et al.* 2000) implemented in the R package (R Development Core Team 2011). Included in the data were fish that were of unknown sex and stage of maturation. In both cases we predicted missing values as part of model fitting; this technique allows for efficient use of all available data.

Results

We captured 131 trout cod in the lower Ovens River ranging in size from 88 to 522 mm total length (Fig. 2). Of the 131 tested fish 96 (72%) had a definite oxytetracycline mark (indicating that they were stocked), 35 had no mark. Wild-spawned and hatchery trout cod were present up to 9 years of age (Fig. 3).

Growth model

Age-length analysis indicated differences in growth between wild-spawned and hatchery-sourced trout cod (Table 2, Fig. 4). There were only marginal differences between both growth models explored (Table 2, Fig. 4).

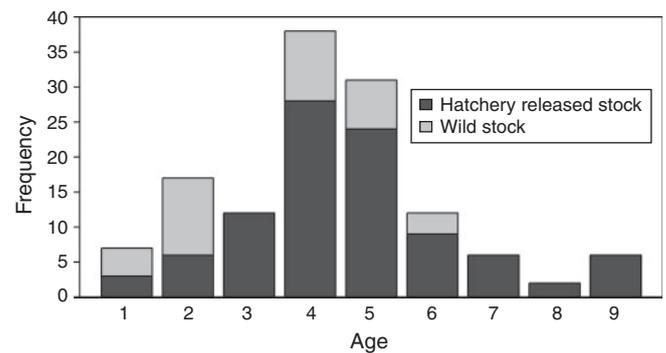


Fig. 3. Age (years) frequency distribution.

Sexual maturity

Of the 83 fish used to determine sexual maturity, there were 51 females, 20 males and 12 indeterminate-sex juveniles. We observed the earliest indication of spawning in a spent (F7) 385-mm female fish captured on 10 October 2007. By 12 November 2007 (our last sampling event), ~65% of mature fish had spawned. The smallest mature female (i.e. $\geq F4$) recorded on 30 Aug 2001 was 245 mm and four years old. The smallest female we confirmed to have spawned (i.e. F7) was caught on 12 November 2007 and was 283 mm and five years old. A six-year-old (373 mm and 688 g), unmarked fish (i.e. progeny of stocked fish) was confirmed as F7.

For females, Lm_{50} (i.e. length at which 50% of the population is mature) was 325 mm (95% credible interval: 289 to 381 mm), and Lm_{90} (i.e. length at which 90% of the population is mature) was 394 mm (347 to 486 mm) (Fig. 5). Eighty-three percent of the trout cod captured were females. For males, Lm_{50} was 250 mm (220 to 275 mm), and Lm_{90} was 318 mm (287 to 368 mm) (Fig. 6). For females, Am_{50} (i.e. age at which 50% of the population is mature) was 5.4 years (4.8 to 6.3 years), and Am_{90} was 6.6 years (5.8 to 8.1 years). For males, Am_{50} was 4.2 years (3.7 to 4.6 years), and Am_{90} was 5.3 years (4.8 to 6.1 years).

Gonads

Fertility ranged from 2027 oocytes in a 291 mm fish, to 14000 oocytes in a 450 mm fish, and average fertility increased with both length and weight. Average relative fertility was 9 eggs g^{-1} fish weight (range: 7 to 14 eggs g^{-1}).

Genetics

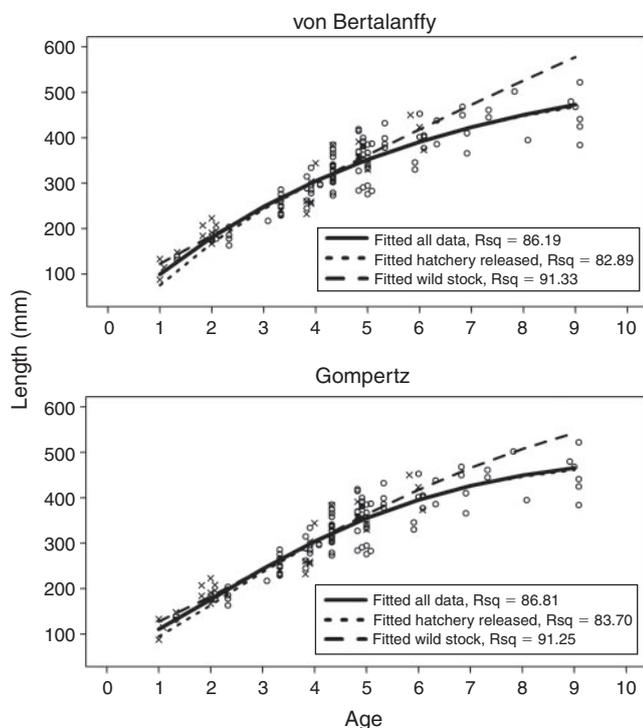
The proportions of homozygous and heterozygous genotypes in the Ovens River population differed from that expected under Hardy–Weinberg equilibrium. We detected no genetic structure between populations in the Murray and Ovens Rivers, and both populations appear to be homogeneous. The total number of alleles we detected in the Murray River population (49) was consistently lower than the number found in the Ovens River population (55).

Discussion

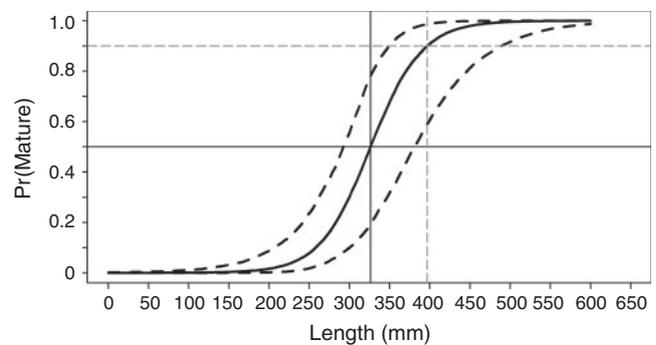
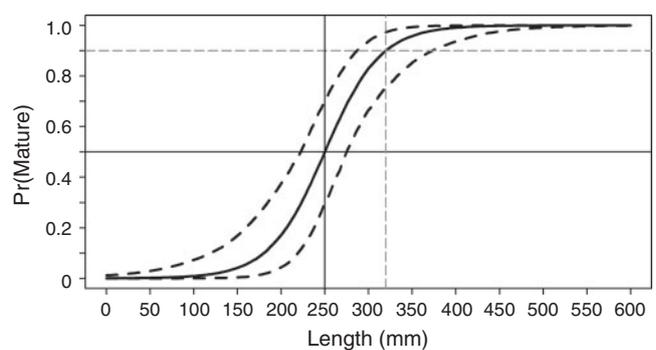
We have assessed the success of reintroduction of trout cod into the Ovens River by determining whether stocked trout cod

Table 2. Results from the age-length analysis of trout cod data

Model	All data		Hatchery-released		Wild-bred	
	$\hat{\beta}$ estimate	SE	$\hat{\beta}$	SE	$\hat{\beta}$	SE
<i>von Bertalanffy</i>						
L_a	582.53	44.66	552.16	40.24	2450.83	7014.30
k	0.19	0.03	0.22	0.04	0.03	0.09
x_0	-0.01	0.2	0.32	0.26	-0.88	0.70
		$r^2 = 86.2$		$r^2 = 82.9$		$r^2 = 91.3$
<i>Gompertz</i>						
L_a	505.70	21.16	491.83	20.65	697.05	242.80
k	2.19	0.14	2.48	0.28	2.16	0.21
t_0	-0.36	0.03	-0.41	0.05	-0.24	0.09
		$r^2 = 86.81$		$r^2 = 83.70$		$r^2 = 91.25$

**Fig. 4.** Estimated age (years)-length relationship of trout cod for both von Bertalanffy and Gompertz growth curves: circles – hatchery-released, crosses – wild-bred.

survive in the Ovens River, whether they produced viable offspring, and their growth, fertility and genetic composition are comparable to wild-bred fish. According to these criteria, we can conclude the programme was successful. Since commencement of stocking, trout cod have been reintroduced into 32 sites in eight river catchments across the Murray-Darling Basin. Of these areas, natural recruitment has been confirmed in four rivers, and currently these stockings have resulted in what might be described as ‘self-sustaining populations’ at only three areas: the Goulburn, Ovens and Murrumbidgee Rivers (Ingram and Thurstan 2008). Other re-introduction sites, which were generally smaller, upland streams stocked over shorter time frames, have had limited success.

**Fig. 5.** Female trout cod maturity ogive. Solid line = median estimate; dashed lines represent 2.5 and 97.5% credible limits. Solid horizontal and vertical lines show $L_{m50} = 325.12$ mm; dashed horizontal and vertical lines show $L_{m90} = 394.18$ mm.**Fig. 6.** Male trout cod maturity ogive. Solid line = median estimate; dashed lines represent 2.5 and 97.5% credible limits. Solid horizontal and vertical lines show $L_{m50} = 249.54$ mm; dashed horizontal and vertical lines show $L_{m90} = 318.30$ mm.

Length of re-introduction program

Previous population modelling for this species (Bearlin *et al.* 2002; Todd *et al.* 2004) indicates that there are a variety of re-introduction strategies that can be adopted to establish viable populations. The stocking strategy applied here was a long-term (over 10 years), moderate stocking regime (as described in Douglas *et al.* 1994) of between 10 400 and 51 700 fingerlings,

including three years where more yearlings were stocked (Table 1). This strategy is conservative (as defined by Bearlin *et al.* 2002), where such stocking rates are expected to achieve a high probability of success (establishment of a self-sustaining population, Bearlin *et al.* 2002). Additionally, Bearlin *et al.* (2002) and Todd *et al.* (2004) describe other stocking strategies where more fish are stocked over a shorter period, to achieve the same result.

We postulate that an important facet of a long-term stocking program for endangered species recovery is the increased chance of a stocked cohort encountering favourable environmental conditions that promote local survival, and in particular in the period immediately following release. In the current stocking program, fish stocked in 2003 and 2004 are more highly represented in our data than other stocked years and in particular subsequent years, indicating a higher rate of survival for the fish released in these years. If this were the case, it supports the conservative approach of releasing stock over a longer period of time by increasing the likelihood of encountering favourable conditions for survival. The proportion of fish stocked in 2003 and 2004 observed in our data could be because of sampling, spatial arrangement of fish within the river, and possibly dispersal. However, without information to the contrary, we conclude that the long-term stocking approach was important for establishing a wild breeding population where it appears that stockings from years other than 2003 and 2004 contributed relatively few fish to the population (and indeed, in some years, none).

Size, age and growth

As a general rule, large fish have a higher survival probability than smaller fish of the same species (Masuda and Tsukamoto 1998; Svasand *et al.* 2000). However, our results suggest that the stocking of yearling trout cod (in conjunction with fingerlings) had no discernable impact on the final population size or structure. Ebner *et al.* (2007) found that two year-old trout cod have high mortality rates (~100%) after release into the Murrumbidgee and Cotter Rivers in south-eastern Australia; a high proportion of these mortalities were probably caused by inappropriate conditioning (i.e. fish were reared in indoor tanks on an artificial diet, and had no previous contact with predators or prey). In contrast to yearlings, fingerlings released into the Ovens River were reared in outdoor earthen ponds, only live prey was available and fish were more likely to be exposed to predation (other fish, macro-invertebrates and birds).

The age structure of this population is approximately Gaussian (Fig. 4); however, few large (>500 mm) fish were captured and this is a cause for concern. Ongoing misidentification of trout cod by anglers might explain why large fish were poorly represented. Trout cod are voracious fish and therefore easily angled, so it is plausible that large trout cod (over the legal Murray cod length of 500 mm at the time of sampling, but now 600 mm) are being misidentified as Murray cod and removed.

Growth is different between released and wild fish; however, the difference is more likely associated with the sample size and/or the age/size distribution for wild-spawned (1–6: 88–450 mm) and hatchery (1–9: 132–522 mm) than those arising from physiological or metabolic differences between released and wild fish, or any effects of the marking procedure. We used two

standard growth models to estimate growth rates in both released and wild fish. The Gompertz model estimated lower parameter errors (Table 2); however, there were only marginal differences between the models explored (Table 2, Fig. 4). The rate of growth begins to slow between ages 4 and 5 most likely because of the onset of sexual maturity.

It is unlikely that the Ovens river population is receiving recruits from other populations (either stocked or natural) because of the absence of fish upstream before stocking, and the barrier formed by Lake Mulwala downstream. In addition, the fact that the species was considered functionally extinct in the Ovens river before the stocking event means that immigration into the area was absent or at least infrequent. As such, we are confident that the success was a result of the stocking program, rather than an artefact of immigration. It is noteworthy that the range of trout cod in the Ovens river has now expanded approximately 50 km both upstream and downstream from the original stocking sites (J. Lyon DSE, unpubl. data).

Genetics

Fish-stocking programs have received considerable criticism because of perceived impacts of hatchery-bred fish breeding with wild populations resulting in loss of genetic diversity or reduced viability (Allendorf 1991; Nock *et al.* 2011). Stocking Murray cod into the Murray–Darling Basin has resulted in a range of genetic effects from non-detectable change to substantial change in wild populations (Rourke *et al.* 2010, 2011). We showed that the stocked population in the Ovens River and the Murray River population, where parent fish for the captive breeding program were captured, are genetically homogeneous. The number of alleles detected in the Murray River (49 total) was consistently lower than the number of alleles found in the Ovens River population (55 total). These results might reflect the successful application of the breeding program protocol that aimed to maximise the genetic diversity of the fish produced. The breeding program followed genetic guidelines described by Douglas *et al.* (1994) which included regular replacement of captive broodstock with new broodstock caught from the Murray River population, maintaining a sex ratio of 1:1 in spawned broodstock, undertaking single-pair matings, avoiding repeat matings of the same pairs of fish, and mixing progeny from all matings together before re-stocking.

Wild-spawned fish and sexual maturity

Our data show that the first natural recruits into the system (i.e. spawned by hatchery-released fish) occurred three years after the beginning of the stocking program – a result that concurs with our data describing age at maturity where some Ovens River trout cod become sexually mature at 3 to 4 years. However, we only captured a few natural recruits until years 6 and 7 (when there was also a high survival of stocked fish) of the stocking program, again indicating the importance of sustained introductions to account for changes in yearly survival rates. Our smallest mature female fish, having spawned at 283 mm, 299 g and five years of age, is also smaller than expected, with the spawning weight range previously reported at 750–1500 g (Douglas *et al.* 1994; Koehn *et al.* 2008). Sarrazin and Barbault (1996) indicate that successful breeding of the first-born generation can be used as an indication of re-introduction success,

and here we recorded many stocked fish and one wild spawned female as having spawned (F7).

We found evidence for deviation from the expected 1 : 1 sex ratio; females dominated 2.5 : 1. Sex determination in many fish species can be influenced by physical conditions such as temperature (Devlin and Nagahama 2002; Penman and Piferrer 2008). A highly skewed sex ratio was observed in small population of hatchery-reared trout cod from the Snobs Creek Hatchery where there were 9 males to 1 female (B. Ingram DPI, unpubl. data). There might have been a sex ratio bias in the production of hatchery fingerlings, or that male survival in the wild after release is lower than that of females.

Lessons learnt

Our data clearly show the dynamics of a successful reintroduction of a threatened species, by applying a long-term stocking program to overcome environmental variability, which can result in failure of any particular stocked cohort. What is more difficult to explain is why the original extinction occurred, and why these threats did not prevent restocking success. The reasons for human-induced species decline are usually a synergistic combination of several processes (Brook *et al.* 2008), and here we assume that the processes that led to the decline included competitive exclusion and/or predation by introduced species such as carp (*Cyprinus carpio*) and redfin (*Perca fluviatilis*), barriers to migration, land-use change and overfishing (Cadwallader 1978; Ingram *et al.* 1990; Douglas *et al.* 1994). Although these threats likely have not totally abated in the Ovens River, the scale of their impact is greatly reduced. For example, the period of intensification of invasive species spread occurred between the 1950s and 1990s, after which their populations stabilised. There is also an increasing global emphasis on more sustainable recreational fishing practices (Granek *et al.* 2008; Douglas *et al.* 2010). As such, we suggest that species reintroductions can be done provided threat reduction occurs prior or in concert with restocking.

Ongoing threats to freshwater fish populations mean that re-introduction programs will need to continue if threatened populations are to be brought back from the brink of extinction, because river restoration alone is unlikely to provide enough impetus for fish to recolonise previous habitats on their own. The example presented here shows that large-bodied native fish reintroductions can be successful in lowland rivers. However, such re-introductions should be sustained and of sufficient magnitude to maximise establishment.

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