RESEARCH **3**

2017-148

WILL CARP VIRUS BIOCONTROL BE EFFECTIVE?

NATIONAL CARP CONTROL PLAN

Exploring genetic biocontrol options that could work synergistically with the carp virus

This suite of documents contains those listed below.

NCCP TECHNICAL PAPERS

1. Carp biocontrol background
2. Epidemiology and release strategies
3. Carp biocontrol and water quality
4. Carp virus species specificity
5. Potential socio-economic impacts of carp biocontrol
6. NCCP implementation
7. NCCP engagement report
8. NCCP Murray and Murrumbidgee case study
9. NCCP Lachlan case study

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Will carp virus biocontrol be effective?

1. 2016-153: Preparing for Cyprinid herpesvirus 3: A carp biomass estimate for eastern Australia
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3. 2017-148: Exploring genetic biocontrol options that could work synergistically with the carp virus
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12. 2019-176: Determination of the susceptibility of Silver Perch, Murray Cod and Rainbow Trout to infection with CyHV-3
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Synergistic genetic biocontrol options for common carp (*Cyprinus carpio*)

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Executive Summary

The common carp is a long-lived and prolific species that has invaded the Murray-Darling basin in Australia and has become a threat to this ecosystem. Here, genetic biocontrol technologies are reviewed to help the Science Advisory Board of the National Carp Control Plan (NCCP) identifying suitable technologies that could be combined to control carp populations.

Potentially synergistic genetic biocontrol technologies can be grouped into those that do not involve engineered DNA sequences and those that do. The former include the “sterile male” and the “Trojan Y chromosome” technologies. The latter include genetic constructs that lead males to produce only male fertile offspring while daughters are either sterile or non-viable (“daughterless carp”), or to various types of engineered gene-drive technologies that would be sexually propagated but could still reach 100% inheritance. The high inheritance would allow them to spread even if the introduced engineered DNA sequence reduced the fitness of the host, for example by killing female offspring or rendering them infertile. Some gene-drive technologies could therefore lead to the extinction of a problem population. However, unintended spread of the engineered drive sequence beyond the target population is possible. Worst-case scenarios of applying gene-drive technologies may therefore include the extinction of an entire species, and, if gene flow is possible as is the case in many cyprinid fishes, of related species.

The idea of the “sterile male technique” is to introduce large numbers of sterile males into a problem population and to let them compete with feral males over access to females. The technique has been successfully used for controlling some insect populations. In fish, arguably the most promising method to produce large numbers of sterile males would be to breed males to sterile triploids. However, the large amount of fish that would have to be stocked makes this technique not very promising for controlling carp in Australia.

The “Trojan Y chromosome” technology exploits the fact that, in many fish with XX/XY sex determination, sex differentiation is labile and Y chromosomes are barely decayed. In the case of the carp, sex determination in carp is male dominant (XX/XY), and genetic sex determination can be overruled, for example, by exposing juveniles to exogenous hormones during a critical period during early sex differentiation. The resulting genotype-phenotype mismatch can then affect population demography over the next generations, i.e. they can be used to influence population sex ratio and thereby population growth. The three types of carriers of Trojan Y chromosomes are XY females (i.e. sex-reversed XY individuals), YY males (e.g. offspring of XY females), and YY females (i.e. sex-reversed YY individuals). If released into the wild, YY individuals would only produce sons, and half of the sons of YY females would be expected to be YY males that would also bias the sex ratio of the F2 generation. All else being equal, the release of large numbers of YY females would therefore have the strongest effect on population sex ratio. However, the efficiency of the Trojan Y chromosome technology depends crucially on the viability and fertility of Trojan Y carriers. Current meta-analyses suggest that the sex-reversal itself produces no significant long-term effects. However, the aberrant YY genotype is normally

expected to suffer from reduced viability and fertility as compared to natural XY and XX genotypes.

Several measures can be implemented to increase the viability and fertility of Trojan Y chromosome carriers to the level of the natural genotypes or even beyond: (i) Avoiding inbreeding depression Trojan Y carriers that are used for stocked, e.g. by actively outbreeding during the final breeding step in the production of Trojan Y carriers. (ii) Respecting local adaptation that is likely in carp of the Murray-Darling basin: Trojan Y carriers should therefore ideally be produced from samples of local populations. (iii) Improving the quality of Trojan Y chromosomes by purging them from deleterious mutations: such purging could be induced by promoting recombination between sex chromosomes, followed by selection. Because recombination between sex chromosomes is more likely in the female than in the male phenotype, selection on offspring of sex-reversed XY females or YY females is expected to purge deleterious mutations from Y chromosomes. (iv) Improving survival of juvenile Trojan Y carriers that are used for stocking: survival of stocked juvenile relative to naturally born juvenile will depend on various factors, and many of them can be actively managed. These factors include the timing of stocking, the locations, the size and condition of stocked fish relative to naturally born ones, whether or not naturally born fish were stressed and/or their numbers reduced before the stocking of Trojan Y carriers, and whether or not Trojan Y carriers have been immunized against pathogens that are relevant at a given location. (v) Improving survival and fecundity of adult Trojan Y carriers by sparing them from angling and fishing: Trojan Y carriers would then not only profit from reduced mortality but grow on average larger and therefore produce larger amounts of eggs than wild types. Moreover, their eggs would on average be of larger size and hence give rise to larger and more viable hatchlings than the eggs of wild-types females. Trojan Y carriers would therefore have to be phenotypically marked.

There are several potential marking techniques that could be used to mark carp. Arguably the most promising one is the “mirror” phenotype that has irregular and patchy scaling caused by a mutation on the paralog of a fibroblast growth factor. The mutation does not significantly reduce growth, survival, and fertility. Existing mirror phenotypes could be used to produce Trojan Y carriers. Alternatively, CRISPR/Cas9 technology could be used on local carp strains to directly edit the paralog of the fibroblast growth factor in order to promote local adaptation of mirror-type Trojan Y carriers. If mirror-type YY females are then released into the wild, the mutation would be inherited into the next generation, but because the phenotype requires homozygosity of the mutation, mirror-type carp that are not Trojan Y carriers would be expected at very low frequency only from the F2 generation on.

A simple population model illustrates the importance of increasing the viability and fertility of Trojan Y carriers. Continuous stocking of YY females into a population at carrying capacity K, for example at a rate of 1% of K, can lead to the extinction of XX females within 30 years if their average age can be kept below 7 years and the annual mortality of adult YY females below 4%, or if their average age can be kept below 5 years and the mortality of YY females below 7%. All else being equal, reproduction in the wild would then stop after the last YY female has died.

The fact that carps are potentially long-lived (over 30 years) and very prolific (hundreds of thousands of eggs per female and spawning season) is a challenge for most biocontrol measures. However, these characteristics offer interesting opportunities in the context of the Trojan Y chromosome technology, because they allow to increase the survival and fecundity of YY females to such a degree that extinction of XX females after few generations seems possible. This extinction process could happen while the population size remains at carrying capacity, i.e. compensatory population growth in reaction to changes in population density could be avoided.

There are several protocols that could be followed to produce Trojan Y carriers. Sex-specific markers that are still to be developed would be required for an efficient production of such carriers. Androgenesis could be used to shorten the time required to produce YY individuals. The first Trojan Y carriers for release into the wild could then be produced within 3-4 years. Androgenesis and especially gynogenesis could also be used to foster purging of Y chromosomes from deleterious mutations and thereby strengthen the fitness of YY females used for stocking.

In the case of common carp in the Murray-Darling basin, the effectiveness of a technique will eventually depend on its potential for deployment or self-propagation across very large (i.e. continental-scale) spatial extents with variable target species population densities, its potential for deployment in remote or inaccessible locations, and its potential for deployment across all Australian environmental conditions. Gene drive technologies that are lethal to female offspring or render them infertile have the potential to largely fulfil these criteria, but they would still have to be developed and tested. Also, these technologies may be difficult to control and can potentially spread to populations outside the Murray-Darling basin.

The social and legal acceptability of the various genetic biocontrol technologies will depend on various factors. Among the most important questions will be how well gene drive technologies can be controlled, and whether the release of engineered DNA sequences into wild population can at all be accepted. If the use of engineered DNA sequences is to be avoided, the release of hormone-treated YY females into the wild would have to be discussed, even if the hormone treatment would be confined to a limited period at fry stages. The marking of hormone-treated Trojan Y carriers (the mirror type) combined with an information campaign could potentially make the technology more acceptable for the public and for legislation.

Considering the biological effectiveness, the relevant logistical factors of each techniques, the risks involved, and their likely public acceptability, the potentially most appropriate technique for deployment against carp in Australia is the Trojan Y chromosome technology, specifically the production and release of mirror-type sex-reversed YY individuals, combined with all measures that increase the survival and fecundity of these stocked animals.

Keywords: Biocontrol, carp, *Cyprinus carpio*, daughterless carp, gene drive, sterile male, Trojan Y chromosome, sex reversal, mirror carp, marking, population sex ratio

1. Introduction and objectives

The common carp (*Cyprinus carpio*) is one of the most important aquaculture fish worldwide, with a global annual aquaculture production of over 4 million tonnes (www.fao.org). It is also a very successful invasive species in various parts of the world and is even listed among the 100 worst invasive alien species of the world (Lowe et al. 2004). The species has been introduced into Australia where it is now widespread and considered a pest that needs to be controlled. In the Murray-Darling basin, Australia's largest river system, carp seem to have reached up to about 90% of the fish populations in some parts of the system. Biomasses have reached up to 3,144 kg/ha (Harris and Gehrke 1997), and commercial fisheries in this region have reported large annual catches over the last decades, especially in the late 1970s and early 1980s (Forsyth et al. 2013). Invasive carp can have a significant impact on their environment, especially at the high population densities recorded in the Murray-Darling basin. Feeding carp, for example, stir up mud and can thereby increase turbidity (Semenchenko et al. 2017).

Carp are long-lived and prolific breeders that, under optimal conditions, grow fast and quickly reach several kilograms body weight. Males can reach sexual maturity during their third growing season, i.e. at 2+ years. They can then spawn the first time as 3-year old (Fernandez-Delgado 1990). Females often reach sexual maturity one year later, i.e. as 3+ or even 4+ year old individuals. In previous population models, the onset of sexual maturity was therefore often set at 5 years (Bax and Thresher 2009; Thresher et al. 2014b). An adult female can lay several hundred thousand eggs per spawning season. Fecundity is dependent on size and condition: larger females and females in good conditions produce more and larger eggs (Weber and Brown 2012). Carp typically spawn in spring in response to rising water temperature and rainfall (Geldhauser and Gerstner 2002), but successful recruitment can be sporadic, i.e. mortality of eggs and fry can be close to 100% in some years (Thresher et al. 2014b). Population growth is often density-dependent (Koehn et al. 2018). i.e. compensatory responses to harvest or other induced changes in population density are likely and can keep population densities high (Weber et al. 2011; Weber et al. 2016).

Controlling population growth of such a long-lived and prolific fish species is obviously challenging and is not without risks, especially if based on the release of viruses as planned in the case of the carp populations of the Murray-Darling basin (Kopf et al. 2017; Lighten and van Oosterhout 2017; McColl et al. 2017; Marshall et al. 2018). It seems important to integrate various biocontrol mechanisms in order to profit from the possible synergistic effects that could be demonstrated in population models (Thresher et al. 2014a; Thresher et al. 2014b).

Potential synergistic genetic biocontrol technologies can be grouped into two categories: technologies that are based on introducing engineered DNA sequences into the carp's genome and other technologies. Some of the latter technologies profit from protocols that

have been developed and often extensively tested in aquaculture in order to improve desirable traits of fish. Technologies that are based on engineered DNA sequences are more theoretical and largely untested in fish, with the notable exception of sex-ratio-biasing constructs that were successfully tested in laboratory populations of zebrafish (*Danio rerio*) (Thresher et al. 2014a).

The present review identifies and compares genetic biocontrol options that could potentially be used to control fish populations and especially common carp in Australia. It will also discuss whether a given technology is in a sufficiently advanced state of development to enable the possibility of real-world deployment within the next 5-10 years. The aim of this review is to help the Science Advisory Group of the National Carp Control Plan (NCCP) and other decision-makers to identify suitable technologies or approaches for potential inclusion in the NCCP.

2. Genetic biocontrol technologies not based on engineered DNA sequences

2.1 Introduction

Various genetic biocontrol technologies that do not rely on engineered DNA sequences are possible especially in fishes or amphibians, including the carp. These technologies have a number of important advantages over technologies that are based on genetic modifications. Among these advantages are that they can mostly be based on existing and proven technologies, they are species-specific and often more likely to be reversible than technologies based on engineered DNA sequences, and they are arguably more likely to obtain public acceptance. Major disadvantages are that they require high stocking rates and that important parameters like, for example, the viability, fertility, and competitiveness of males and females with chromosomal constructs or with phenotype-genotype mismatches need to be separately determined for each species or population. As with technologies based on engineered DNA sequences, technologies that avoid them have not been much tested in fish.

In the following I outline and discuss the sterile male technique and the Trojan Y chromosome technology. In the case of the carp, the latter technology may be more promising than the former. I will therefore present and discuss different protocols that could be used to produce the various types of Trojan Y carriers, and I will outline how potential genetic problems that may, in the past, have reduced the viability of Trojan Y carriers, can be avoided. I will discuss androgenesis (induced all male inheritance) as a potential way to significantly speed up the production of Trojan Y carriers, and both androgenesis and gynogenesis (induced all-female inheritance) as a possibility to reduce genetic load and hence increase viability of Trojan Y carriers. I will also discuss phenotypic markers that can potentially be used to monitor the progress of measures based on Trojan Y carriers. I will argue that such phenotypic markers can be crucial for synergistically combining the Trojan Y chromosome technology with other biocontrol mechanisms.

2.2 The sterile male technique

The objective of the sterile male technique is to produce and release large numbers of sterile males into a problem population, hoping that these males then compete with feral males over access to females. The higher the mating success of these sterile males, the more eggs are left unfertilized. Large numbers of unfertilized eggs may then lead to a reduction of population growth. The technique has repeatedly been used to control insect populations, with much success in some cases (for example, the eradication of the tsetse fly from

Zanzibar) and less success in others (Dyke et al. 2005). Although mainly used in insects, the idea of applying this concept to controlling unwanted fish population has been discussed over decades (Kapuscinski and Patronski 2005). The principle could potentially work for fish, especially for small unwanted populations, but that there are some risks involved (Thresher et al. 2014b).

Insect males are often sterilized by exposure to low doses of radiation (Dyke et al. 2005). In fish, sterility is usually induced by procedures that lead to triploids, i.e. individuals whose normal diploid set of chromosomes is augmented with a further set of unpaired chromosomes. Production of triploids has become a routine in aquaculture of some fishes, on the one hand, to minimize the risks of hybridization between escapes from aquaculture and feral individuals, and, on the other hand, because triploid females usually invest less energy into gonadal tissue than diploid females and may therefore, in some cases, grow faster (Piferrer et al. 2009; Arai and Fujimoto 2019). Triploidy can be induced by crossing tetraploids with diploids or by manipulating meiosis, i.e. by physically (heat- or cold-shock treatment) or chemically (for example, application of cytochalasin B) inhibiting the separation of chromosomes to polar bodies and daughter cells (Tiwary et al. 2005; Piferrer et al. 2009; Arai and Fujimoto 2019). Triploid males usually produce functional spermatozoa and are expected to compete with diploid males over fertilization of eggs.

Among the advantages of this technology are that (i) the production of triploid fish can be comparatively simple and inexpensive, (ii) the technology is species-specific, (iii) the release of sterile males can be stopped at any time, and (iv) there might be little public concern about releasing triploids into the wild because triploids are already widely used in aquaculture and because they are not considered to be genetically modified (Thresher et al. 2014b). However, high stocking rates would be required, which by itself could lead to undesirable ecological effects. Moreover, stocked males would have to achieve high success rates in competition with feral males over fertilization of eggs, while, in some species, triploid fish show abnormal behaviour. Among these abnormal behaviours are lower responsiveness to different types of environmental stimuli, and lower plasma levels of various sex hormones than diploid fish (Tiwary et al. 2005). For a given species, the competitiveness of triploid over diploid males would have to be established in order to estimate the potential of the sterile male technique to control population growth.

Bergstedt et al. (2003) used the sterile male technique to control population growth of sea lamprey (*Petromyzon marinus*). They caught wild males, sterilized them by injection of P,P-bis(1-aziridinyl)-N-methylphosphinothioic amide (bisazir; 100 mg/kg), and released them into the wild again. These sterilized males turned out to compete for mating with non-sterilized males. They produced non-fertilized eggs in a proportion that was close to the one expected from the frequency of sterilized males. Bergstedt et al.'s (2003) observations suggested that the sterile male technique, together with removal of females, led to significant reductions of population sizes over several years.

Because of the potential human health hazards of bisazir (Rudrama and Reddy 1985) (www.pesticideinfo.org assessed on Dec. 23, 2018), Bergstedt et al. (2003) tested other methods of sterilizing males caught from the wild, including other chemicals, hormones, or ionizing radiation. They concluded that radiation was the most effective alternative to bisazir. However, radiation caused damage to lampreys' immune system that would significantly reduce the competitive success of released males. Therefore, neither bisazir nor radiation may be an option for sterilizing carp in the Murray-Darling basin.

Wagner et al. (2006) compared diploid and triploid rainbow trout (*Oncorhynchus mykiss*) in hatchery strains that originated from three different populations and found in only one strain a slightly higher early rearing mortality of triploids than diploids but no difference in growth, their aggressive behaviour (attacks, retreats, counterattacks), nor their stress tolerance (transport, change in temperature, change in pH). Scott et al. (2015) confirmed the lack of significant effects of triploidy on other potentially fitness-related traits, with the exception that triploids were less hypoxia tolerant than diploids. Nevertheless, the rainbow trout may be an example of a species where triploid production is an attractive option in population management, for example, to avoid hybridization with native populations (Kozfkay et al. 2006) at low costs to aquaculture. Triploidy in Atlantic salmon (*Salmo salar*) is also used to reduce the ecological impact of escaped farm fish (Benfey 2016; Murray et al. 2018). Triploid males often produce functional spermatozoa, but both triploid males and females are sterile (Benfey 2016; Murray et al. 2018). Moreover, triploid males differ in various other fitness-relevant traits from diploid ones. These differences may reduce their competitiveness in intra-sexual fights for access to mating partners. Triploid males produced in hatcheries are, for example, less likely to migrate from the ocean to natal freshwater spawning places than diploid ones from hatcheries (Cotter et al. 2000; Glover et al. 2016), and they often perform less well and are less likely to outcompete natives than these diploids hatchery-born fish (Benfey 2016). Significant reduction of fitness-relevant traits were also observed in triploid pink salmon (*Oncorhynchus gorbuscha*) (Artamonova et al. 2018). It seems that the effects of triploidy are species-specific and therefore need to be studied for each problem population to test whether triploidy can be used to sterilize males in population management (Arai and Fujimoto 2019).

Basavaraju et al. (2002) induced triploidy in common carp by heat shock and found gonadal development of triploids to be more reduced in females than in males, as expected from previous observations in other fishes. At the end of their study period, diploids were heavier than triploids while their gutted weights were similar, i.e. there was no significant growth differences between triploids and diploids. In order to model the efficiency of triploids to control carp populations via competition between introduced sterile and feral fertile males, it remains to be determined whether and to what degree triploidy reduces male survival and their competitiveness in intra-sexual selection. Among the other questions that may need to be studied in this context are: (i) what are the effects of the introduced males, i.e. of male-biased sex ratios and the artificially increased population density, on female growth, survival,

and fertility (Le Galliard et al. 2005), (ii) can the survival and hence the frequency of triploid males in the wild be enhanced by synergistic measures, e.g. immunization against predominant pathogens, and (iii) how is recruitment rate dependent in the density of larvae that hatched from fertilized eggs, and is it possible that negative density effects at larval or juvenile stages cancel or even reverse the effects of the sterile male technology on population growth? In conclusion, it is currently not clear yet whether the sterile male technology can be useful for controlling carp population (Thresher et al. 2014a).

2.3 Trojan Y chromosomes

The Trojan Y chromosomes technology is based on a particularity of sex determination in many lower vertebrates and is therefore probably no option for mammals or birds. In fish, the mechanisms of sex determination are very diverse, ranging from purely genetic to purely environmental sex determination (Devlin and Nagahama 2002; Penman and Piferrer 2008; Bachtrog et al. 2014). This range can be seen as a continuum (Beukeboom and Perrin 2014). Phenotypic sex is then a threshold trait that depends on the interplay of genetic and environmental factors during sex differentiation. As a consequence, environmental factors such as extreme temperatures (Ospina-Alvarez and Piferrer 2008) or exogenous hormones (Mizoguchi and Valenzuela 2016) can overrule genetic factors of sex determination in some species. The resulting genotype-phenotype mismatches can then affect population demography and genetics over the next generations (Wedekind 2017).

Gutierrez and Teem (2006) suggested that artificially induced genotype-phenotype mismatches could be used to change the frequencies of sex chromosomes in order to control problem populations (Cotton and Wedekind 2007a). They used the term “Trojan Y chromosomes” for Y chromosomes in phenotypic females or in males with the YY genotype, because these chromosomes then have the potential to change population sex ratio, i.e. to create a male bias that may reduce population growth over the following generations or even drive a population to extinction (in some specific cases, Trojan chromosomes can even be used to boost population growth (Cotton and Wedekind 2007b)). Figure 1 illustrates how this male bias can result from the release of Trojan YY females (Figure 1a), Trojan YY males (Figure 1b), and Trojan XY females (Figure 1c).

Inducing sex reversal to produce XY females does usually not seem to create significant long-lasting negative effects on individual survival and fertility (Senior et al. 2012; Senior et al. 2016). It seems that these fish only have to recover from the acute stress imposed by the environmental conditions that induced the genotype-phenotype mismatch, for example, the exposure to an endocrine-disrupting chemical like ethinylestradiol (Brazzola et al. 2014; Marques da Cunha et al. 2019).

The YY genotype that results from mating between XY females and XY males is often viable and fertile because the Y chromosomes of many fishes are not significantly decayed (Senior et al. 2015), despite the usually suppressed recombination among sex chromosomes in the male phenotype (Bachtrog 2013; Beukeboom and Perrin 2014). Interestingly, naturally occurring genotype-phenotype mismatches may even be responsible for the maintenance of the functionality of Y chromosomes in many fishes, because recombination between sex chromosomes seem not suppressed in the female phenotype (Perrin 2009). Recombination between the X and the Y chromosomes in the female phenotype, followed by selection, is then expected to act as a "fountain-of-youth" for Y chromosomes (Perrin 2009).

It has long been known that sex determination in common carp is male-dominant (XX/XY) because family sex ratio of conventionally-bred diploid offspring is about 1:1 while gynogenetic offspring are all female (Nagy and Csanyi 1984). Male heterogamety is also found in bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*) (Liu et al. 2018), the two other intensely exploited fishes in aquaculture worldwide that have become problematic invasive species in various parts of the world. The presence of Y chromosomes in the various carp species make them potentially susceptible to the Trojan Y chromosome technology. However, sex-specific genetic markers would be required for an efficient production of Trojan Y carriers.

Liu et al. (2018) developed sex-specific markers for bighead carp and silver carp by comparing gynogenetic diploids and normal families. Chen et al. (2009; 2010) also found male-specific markers in common carp. However, Lui et al. (2018) found some of these markers again in gynogenetically produced females of other carp strains. The role that these markers play in sex determination is hence unclear and could be population specific. Rodrigues et al. (2016; 2017) found, for example, that sex determination mechanisms vary in the common frog (*Rana temporaria*), both within and between natural populations, even if this implies that "… *an apparently unstable pattern has been maintained over long evolutionary times*" (Rodrigues et al. 2017). It therefore remained to be tested whether there are different sex-determining genes in common carp. Indeed, Feng et al. (2018), who also found sex-specific linkage groups in common carp, suggested that sex determination in this species is polygenic, and that the contribution of different genes to sex determination may vary among different strains, analogously to what has been observed in tilapia (*Oreochromis niloticus*) (Eshel et al. 2011; Palaiokostas et al. 2013), zebrafish (*Danio rerio*) (Bradley et al. 2011; Anderson et al. 2012), the Atlantic salmon (*Salmo salar*) (Eisbrenner et al. 2014), and potentially in the Pacific salmon *Oncorhynchus gorbuscha* (Makhrov et al. 2018). However, Bongers et al. (1999) concluded from the unchanged sex ratio of 1:1 in repeated back-crossings that they did to produce congenic carp strain that "… *autosomal influences affecting sex differentiation are absent in this pedigree*" (p.196).

Y-chromosome-specific molecular markers would be required to efficiently produce Trojan Y carriers (Figure 2). Such markers could be developed by collecting females and males from

the target population and using a pool-and-sequence method to identify sex-linked loci (Zhang et al. 2017). Trojan Y carriers can then be feminized XY, or YY males or feminized YY individuals (Figure 2). However, producing YY individuals for release into the wild in order to control problem populations is an idea that has not been tested much yet. Arguably the best worked-out example at the moment is a program on brook trout (*Salvelinus fontinalis*) in North America.

Schill et al. (2016) developed sex-linked genetic markers for brook trout and exposed fry to 17-beta-estradiol in order to induced sex reversal, based on a protocol that has been used before in aquaculture to establish YY brood stocks. Feminizing XY individuals to produce XY females turned out very efficient (99.6% success rate) and did not seem to reduce embryo and fry survival (Schill et al. 2016). These sex-reversed XY females showed no reduced growth compared to females when raised to maturity. They were then crossed with XY males to obtain YY males (following protocol B1 in Figure 2) and YY females (following protocol B2 in Figure 2). The YY genotypes were verified by the genetic markers. The YY males and YY females were then raised to maturity and used as brood stock to produce non-hormone treated YY male Trojan carriers.

In the case of the brook trout, the production of a YY brood stock that allows to produce large numbers of male Trojan carriers could be completed in 4 years and cost less than US$ 10k (Schill et al. 2016). However, Schill et al. (2016) started their breeding program with only 4 full-sib families and did not seem to actively prevent inbreeding, i.e. the inbreeding coefficient of the YY males produced by their brood stock is expected to be high. Accordingly, the viability of their Trojan carriers is expected to be significantly reduced because of inbreeding depression, which reduces the efficiency of the Trojan carriers as means in population management. Moreover, it remains unclear whether a reduced viability of their YY Trojan carriers, and how much of such a reduced viability, is due to the potential problems linked to the homozygosity of the Y chromosome. The increased inbreeding depression could have been avoided by starting with more families and using a protocol during the production of Trojan carriers that actively avoids inbreeding (Wedekind 2019).

Schill et al.'s (2016) protocol avoids the release of hormone-treated YY females that would potentially be more effective as biocontrols. In Schill et al.'s (2016) study, the fish were only hormone-treated during a limited period and at early fry stages (with 20 mg 17beta-estradiol per kg food), i.e. the total amount of 17beta-estradiol that was used in their study "… *was 15 mg or less*" (p.81) which corresponds to about the amount of estrogens that is released by a pregnant woman in urine over a period of less than three days (Wise et al. 2011). Such arguments may be important in a discussion of the public and legal acceptability of releasing sex-reversed fish into the wild.

The viability and fertility of Trojan Y chromosome carriers that are stocked into wild populations is crucial for the success of a biocontrol program, as shown in various simulation

studies (see references in chapter 5) and in a study that specifically simulated the situation for brook trout (Schill et al. 2017). The latter study also concluded that manual suppression of a wild population as part of an integrated pest management would increase survival of the stocked carriers and hence the effectiveness of the program. To test this idea, Kennedy et al. (2018) stocked catchable-size YY male Trojan carriers (produced in the course of Schill et al.'s (2016) study) into four different streams with non-native brook trout. In two of the streams, the wild brook trout population was suppressed before stocking (removal of about 17% of the wild population by electrofishing). This suppression created vacant feeding territories and seemed to double the survival rates of the Trojan Y carriers (from on average 9% to 18%). Genetic assignment of samples of the F_1 generation caught from the wild revealed that the Trojan Y carriers successfully reproduced in all four streams. Given that these Trojan Y carriers were likely to suffer from enhanced inbreeding depression that could have been avoided (see above), the success of stocking YY male Trojan carriers into the wild could even be increased. Given also that sex reversal seems to have little effect on viability and fertility, the efficiency of such a program could be further increased by releasing YY females instead of YY males (if releasing hormone-treated individuals is legally accepted), because half of the offspring of YY females are expected to be YY males (Figure 1).

In conclusion, computer simulations and a series of first experimental tests of the Trojan Y chromosome technology to control non-native populations of brook trout in North America helped to improve the production and stocking of Trojan Y carriers and lead to very promising first results. Production costs were small, and the viability and fertility of YY individuals was good and could be further improved. The efficiency of Trojan Y carriers as biocontrol could also be improved by stocking sex reversal YY females. Combining the release of YY individuals with other measures in the course of an integrated pest management turned out to be successful. This first experimental test of the Trojan Y chromosome technology was therefore successful.

2.4 Production of Trojan chromosome carriers via conventional breeding

There are three possible types of Trojan chromosome carriers in species with male heterogamety and possible environmental overruling of genetic sex. Figure 2 outlines the main types of protocols that could be followed to obtain these three possible types of Trojan chromosome carriers via conventional breeding. The production of Trojan chromosome carriers then takes at least two generations (Figure 2). All options would start with protocol A (Figure 2) in the first breeding season. This protocol is expected to lead to 50% XY-type females that would need to be identified to be separated from their XX siblings, for example, with genetic markers.

In protocol B1 (Figure 2), XY-type females are raised to maturity and crossed with wild-type XY males to produce offspring of which 25% are expected to have the YY genotype. If untreated, they are expected to develop into the YY males that would need to be identified in order to separate them from their XX and XY siblings. In protocol B2 (Figure 2), the offspring that result from protocol B1 are ESR-treated to produce two types of Trojan chromosome carrier, namely 25% YY- and 50% XY- females that would have to be separated from the remaining 25% XX-female siblings.

When the YY-females are raised to maturity, they could be crossed with the YY males produced in protocol B1 to obtain 100% YY males (protocol C1 in Figure 2). In protocol C2, these YY individuals would be treated to obtain YY females at a rate of up to 100%, depending on the effectiveness of the ESR treatment.

The YY-females could also be raised to maturity to be crossed with wild-type XY males (protocol D1 in Figure 2). This would lead to 50% YY males and 50% XY males. Separating the YY males from the XY males before release into the wild would avoid introducing X chromosomes into the wild. Protocol D2 adds ESR to protocol D1 and would result in 50% YY- and 50% XY- female Trojan chromosome carriers. Again, YY females could be separated from the XY females to avoid introducing X chromosomes into the wild.

If XY-females from protocol B2 (Figure 2) were raised for maturity, they could be crossed with either with wild-type XY males to repeat the B1 or B2 protocol in the 3rd season (not included in Figure 2). Alternatively, these XY-females could now be crossed with the YY males that resulted from protocol B1 during the 2nd breeding season. This crossing would either lead to 50% untreated YY males (protocol E1 in Figure 2) that would have to be separated from their XY siblings to avoid release of X-chromosomes into the wild, or, if followed by ESR, to 50% YY- and 50% XY- female Trojan chromosome carriers as in protocol D2 (protocol E2 in Figure 2).

Two of the three types of Trojan chromosome carriers, namely the XY- and the YY-type females, would have experienced environmental sex reversal (ESR), for example, by hormone treatment. If the release of hormone-treated individuals were to be avoided, for example, to prevent anglers and fishermen or -women to catch, consume, or sell hormone-treated fish, only four of the nine protocols in Figure 2, namely protocol B1, C1, D1, and E1, could be used. These four protocols would lead to the YY male Trojan chromosome carriers that would not have experienced an ESR, while protocol C1 would be most efficient because it would lead to 100% YY males. However, if hormone-treated individuals were marked with a distinct phenotype, for example, the “mirror” phenotype or a distinct colour morph (see chapter 4), the release of ESR-treated individuals, together with an information campaign, may be potentially be more likely to be acceptable by the public and by legislation.

Care would have to be taken to avoid the negative effects of inbreeding depression in the production of these carriers (Wedekind 2019). The production would therefore have to be

started with several unrelated strains in parallel so that sib-mating can be avoided in the following generation.

2.5 Production of Trojan chromosome carriers via androgenesis

Androgenesis is the (usually artificially) induced development of an embryo from only the paternal pronucleus, i.e. offspring develop without any genetic contribution of the egg nucleus. There are various protocols that could be used to induce androgenesis in carp (Arai and Fujimoto 2019) in order to skip one breeding season and hence to significantly shorten the time that is required to obtain the Trojan chromosome carriers. Figure 3 outlines the main types of protocols based on androgenesis in the first round of breeding.

Androgenesis itself (protocol G1 in Figure 3) leads directly to YY males or, if followed by ESR (protocol G2 in Figure 3) to YY females, i.e. two of the three possible Trojan chromosome carriers can be produced already in the first breeding season. However, these first types of carriers suffer from increased inbreeding depression because the genetic effects of androgenesis are equivalent to several generations of close inbreeding (Bongers et al. 1997). To get rid of these negative effects on survival and fertility, the F_1 would have to be raised to sexual maturity and crossed with non-related individuals to obtain outbred Trojan chromosome carriers. This could be achieved by breeding YY males with unrelated YY females to produce non-inbred YY males (protocol C1 in Figure 3) that could be released in to wild, or, after ESR, as YY females (protocol C2 in Figure 3). These protocols are analogous to the protocols C1 and C2 in Figure 2, as are the protocols D1 and D2 of Figure 3 in which wild-types males would be crossed with YY females and the resulting YY individuals would have to be identified from the XY siblings to avoid the release of X-chromosome carrying males.

For the sake of completeness, breeding protocols E1 and E2 (Figure 3) could be done with androgenetically produced YY males, but these protocols require sexually mature XY females that would have to be produced in the previous generation by ESR of XY individuals (protocol A in Figure 3). As with conventional breeding protocols, using protocols C1 and C2 in the second breeding season would be most efficient because it would lead to 100% YY karyotypes.

Androgenesis does not have to start from XY males but could also start from YY males (Bongers et al. 1999) as soon as they are available. All offspring would then be expected to be YY individuals, i.e. identifying and separating them from the individuals would not be necessary. Apart from this, starting the production of YY individuals via androgenesis using YY individuals may be not offer any further advantages because these androgenetically produced offspring would suffer from inbreeding depression and would need to be outbred for production of viable stocking material. However, the procedure that is analogous to

androgenesis, namely gynogenesis, could significantly increase the viability and fertility of Trojan Y carriers that are to be released into the wild.

2.6 Production of Trojan chromosome carriers via gynogenesis

Gynogenesis is the induced development of an embryo from only maternal genes, i.e. offspring develop without any genetic contributions of the sperm nucleus. Gynogenesis is a natural mode of reproduction in some fish and can be artificially induced in others, including the common carp (Arai and Fujimoto 2019). Both, androgenesis and gynogenesis are forms of severe inbreeding, i.e. androgenetically or gynogenetically produced offspring are expected to express fitness effects of homozygous deleterious mutations (i.e. they suffer from increased inbreeding depression). This can be useful to purge Y chromosomes from deleterious mutations and hence make the YY karyotypes more viable.

An important difference between androgenesis and gynogenesis is the level of recombination that is expected to have happened between sex chromosomes before androgenesis or gynogenesis would induce inbreeding depression and hence purging. In the case of androgenesis, the sex chromosomes would have been in the male phenotype first and little recombination between these the sex chromosomes would be expected to have happened (Beukeboom and Perrin 2014). In the case of gynogenesis, the sex chromosomes would have been in the female phenotype first that is expected to promote recombination (Perrin 2009).

Purging Y chromosomes from deleterious mutations would therefore be most effective if the Y chromosomes are first allowed to recombine in the female phenotype, i.e. in sex-revered XY or YY females. Recombination followed by selection, i.e. using only the strongest individuals among, for example, gynogenetically produced YY offspring for a next step of outbreeding (Figure 3), is then expected to increase the viability of the resulting YY carriers. Such a protocol could start from XY females or YY females. Using XY females would allow for recombination between the X and Y, which may in some cases be more effective than recombination between Y chromosomes only, but this would lead to only 50% YY offspring that would still have to be identified as such. Starting the protocol with YY females would directly lead to 100% YY offspring.

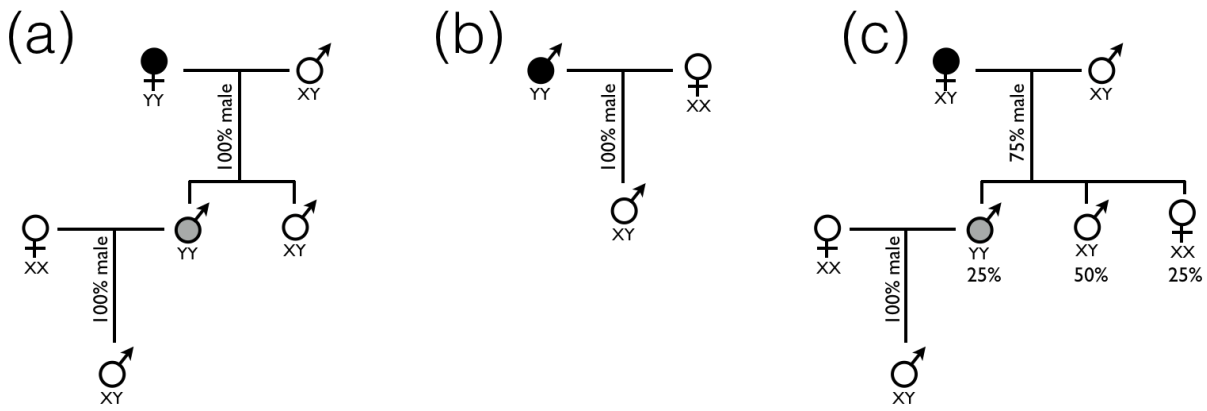

Figure 1. Introducing male bias using Trojan Y chromosome carriers
Induced male bias in generation F_1 and F_2 after the release of (a) Trojan YY females, (b) Trojan YY males, and (c) Trojan XY females into a population. Released Trojan Y carriers are marked with filled gender symbols, their Trojan Y carrier offspring are marked in grey.

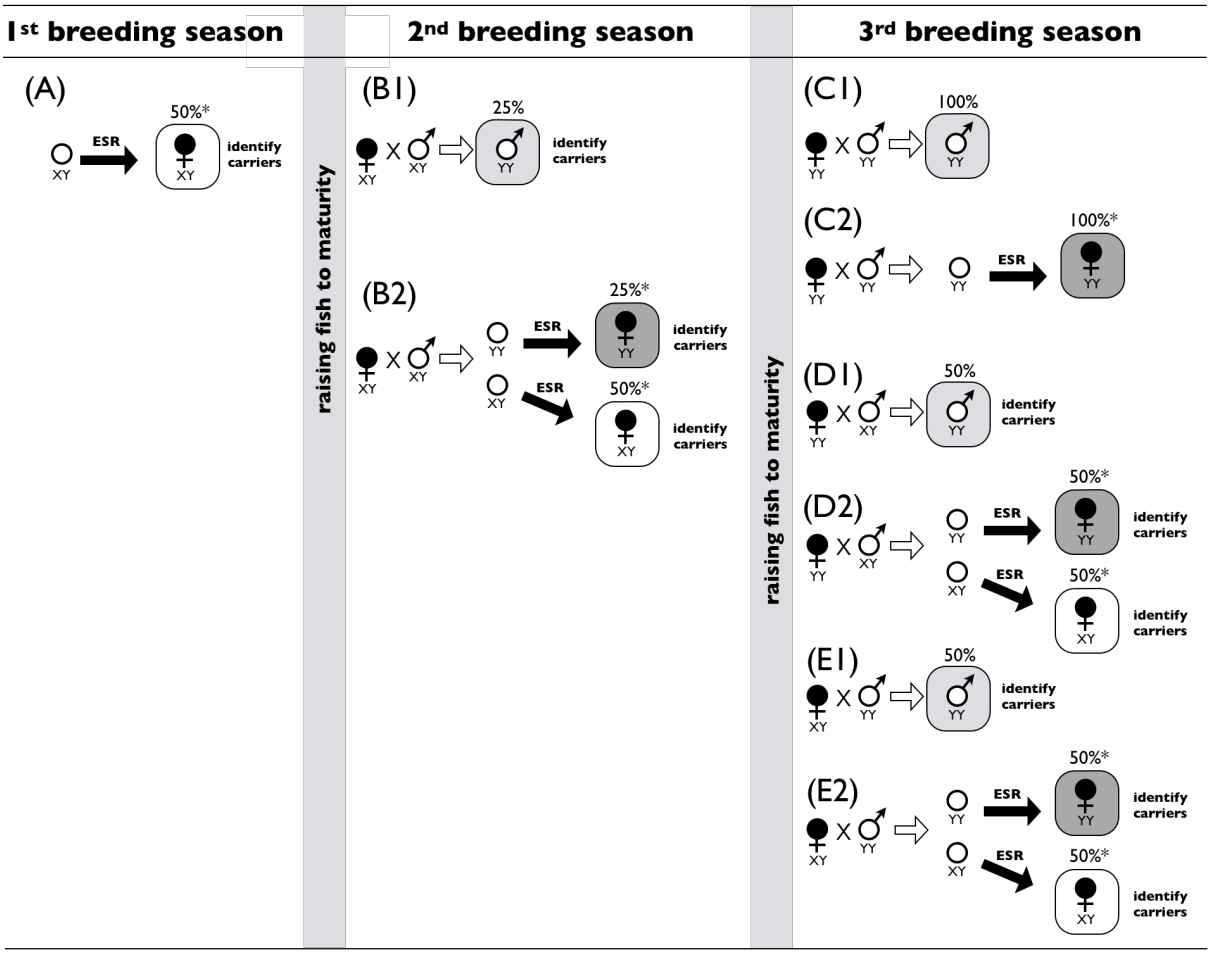

Figure 2. Production of the various types of Trojan Y chromosome carriers by conventional breeding in a species with male heterogamety

The three different types of Trojan chromosome carriers are marked in boxes. The grey value of the box indicates the expected family sex ratio of the respective type when crossed with wildtypes (the darker the shading, the more males are expected in the F_1 and F_2 progeny: empty box: 75% male offspring of which 33% are YY individuals that can only produce male offspring in the F_2, light grey boxes: 100% male offspring of the XY genotype, dark grey boxes: 100% male offspring of which 50% are YY individuals that can only produce male offspring in the F_2; see also Figure 1). Phenotype-genotype mismatches (filled gender symbols) are induced by environmental factors ("ESR" for environmental sex reversal) such as, for example, hormone treatment of sexually undifferentiated individuals at embryo and/or early larval stages. The percentages give the expected rate of the respective type of Trojan chromosome carrier within the clutches, the asterix mark the simplifying assumption that the environmental treatment (for example, the induced hormone treatment) is fully effective. See text for discussion of the various protocols.

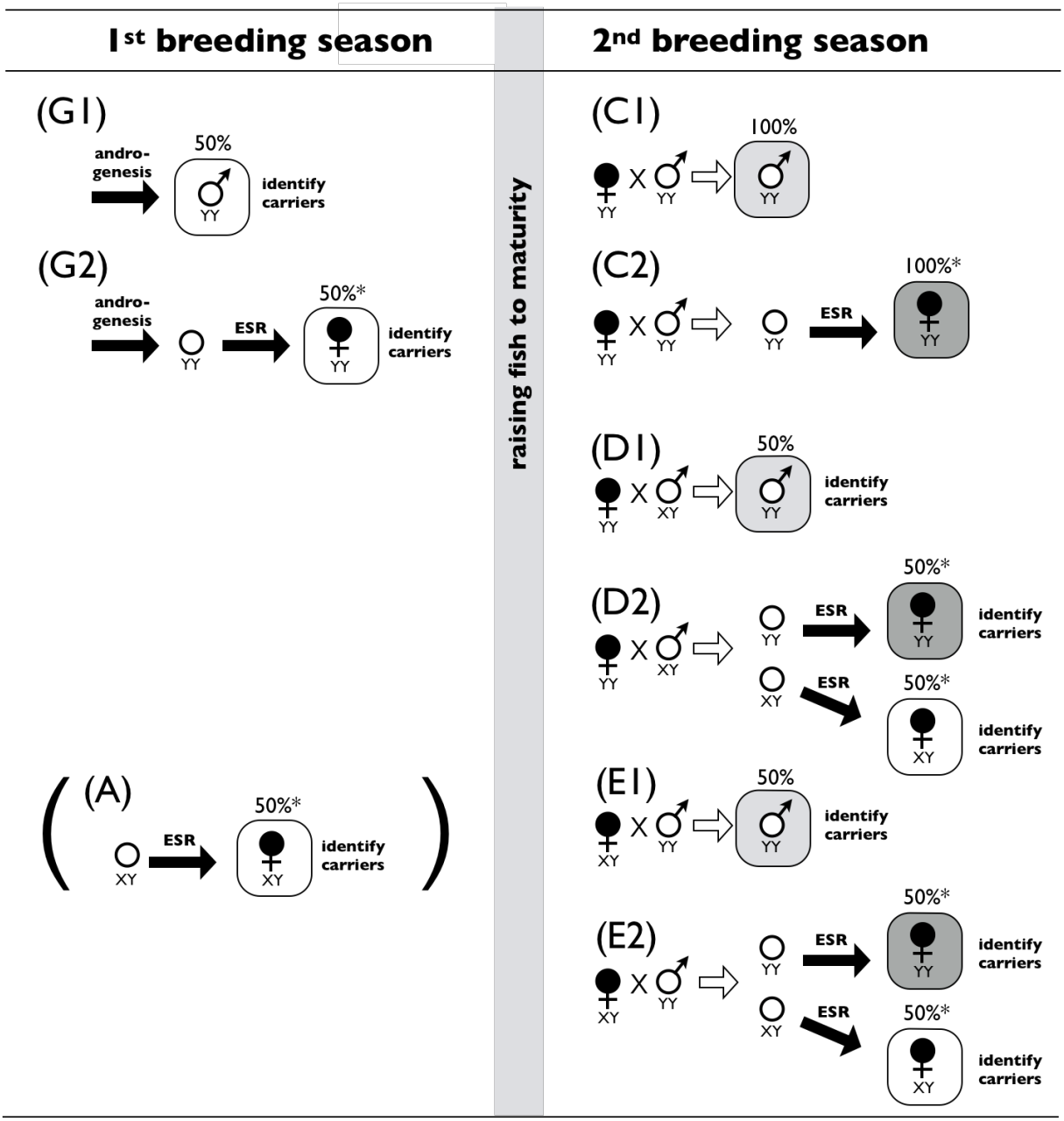

Figure 3. Production of the various types of Trojan Y chromosome carriers starting with androgenesis in the first breeding season

As in Figure 2, carrier types are marked in boxes shaded in different grey values that indicate the expected family sex ratios of the respective type when crossed with wildtypes (the darker, the more males are expected in the F_1 and F_2 progeny). Protocol A is repeated for the sake of completeness because it would be required if protocols E1 or E2 based on androgenetically produced YY males were planned for the second breeding season.

3. Genetic biocontrol technologies based on engineered DNA sequences

3.1 Introduction

There are various ideas on how to reduce fertility or even sterilize fish using engineered DNA sequences. Most of these ideas seem very effective. However, genetic modification of populations or even entire species require the development of stringent safety criteria that would then have to be verified and followed up by a well-defined safety leadership (Kapuscinski et al. 2003; Hayes et al. 2014). Genetic biocontrol technologies based on genetic constructs will require changes in legislative or policy settings before they could be tested in the wild. Some ideas are so powerful that they could, in worst-case scenarios, lead to extinction of a species and even infect species within the same family (even across genera) through hybridization and drive them to extinction, too. These potentially very powerful technologies will be discussed in chapter 3.4.

3.2 Genetic constructs that reduce male fertility

Genetic modifications can lead females to produce sterile male offspring or male offspring of low fertility while the fertility of female offspring may or may not be affected (Gemmell et al. 2013; Thresher et al. 2014b). Analogously to the sterile male technique (chapter 2.2), the objective of a recombinant technology that reduces male fertility is that males of low fertility reduce recruitment rate by competing with feral males over access to females (Gemmell et al. 2013; Robertson et al. 2017; Wolff et al. 2017). However, contrary to the sterile male technique (chapter 2.2), the recombinant technology can sometimes require only a single large release and small repeat releases to be effective (Gemmell et al. 2013).

3.3 Genetic constructs that reduce female fertility or survival ("Daughterless Carp")

Some genetic modifications can lead males to produce only male fertile offspring either by biasing sex differentiation towards the male phenotype or by rendering daughters sterile or non-viable (Bax and Thresher 2009; Thresher et al. 2013; Thresher et al. 2014b). Female-specific lethal genes have been used to control insects (Thomas et al. 2000; Fu et al. 2007). Thresher et al. (2014a) were the first to provide a successful test of this idea in a fish, using laboratory populations of zebrafish and testing genetic constructs that caused female-specific sterility or female-specific lethality. Thresher et al. (2014a) demonstrated that releasing carriers of such genetic constructs into populations can lead to significant

population declines, especially if combined with classic biological control mechanisms. Population models by Brown & Dilligan (2014) demonstrated the potential synergies effects of combining a biocontrol program based on the release of CyHV-3 and genetic constructs that bias population sex ratio. Such sex-ratio distortion genetic technologies have the potential for high species specificity (Thresher et al. 2014a).

3.4 Engineered gene drives

In sexual organisms, genes usually have a maternally and a paternally inherited copy. Following Mendelian laws, these alleles would usually have a 50% chance of passing to a descent. However, there are naturally occurring so-called “selfish” genetic elements that can cause segregation distortion by using molecular mechanisms to bias inheritance to their favor even if they provide no fitness benefits to their hosts (Hurst and Werren 2001; Werren 2011). Various types of engineered gene drive mechanisms are now being developed that would allow to also bias the inheritance of genes, for example, to increase the frequency of certain genes in wild populations (Champer et al. 2016). Endonuclease gene drives are based on the following molecular mechanisms: chromosomes are cut at a specific site (for example, by an RNA-guided endonuclease) which induces a repair mechanism that copies a drive sequence, i.e. an endonuclease-containing cassette, into the damaged chromosome via homologous recombination. The copying causes a heterozygote for the drive sequence to be converted into a homozygote. As a consequence, gene drive insertion into the genome will be sexually propagated even if they reduce fitness of their hosts (Esvelt et al. 2014). Such sexual propagation would be expected to be 100%, i.e. every offspring would eventually become homozygous for the drive sequence (Gantz and Bier 2015) (Figure 4). A homing gene drive could already be used to successfully suppress mosquito populations in cages (Kyrou et al. 2018).

The gene drive technology is currently being further developed to possibly control, for example, vector-borne diseases by adding or deleting genes (Champer et al. 2018). The technology could also be used to control, in principle, all problem populations that reproduce sexually (KaramiNejadRanjbar et al. 2018; McFarlane et al. 2018; Moro et al. 2018). A CRISPR homing drive directed to recessive female sterile gene in the carp genome by a guide RNA could, for example, be developed to control problem carp populations in Murray-Darling basin of Australia. However, homing gene drive technologies have the potential to genetically modify whole populations and even entire species. There is therefore much concern about the potential environmental and security challenges associated to this potentially powerful technology (Esvelt et al. 2014; Oye et al. 2014; Esvelt and Gemmell 2017; Kohl et al. 2019; Rode et al. 2019).

There is, for example, the danger that a gene drive cannot be sufficiently controlled and spreads across political borders or into populations where the genetic construct could cause unwanted damage and even lead to extinction (Deredec et al. 2008). Other potential problems are that the trait that is propagated causes unwanted ecological problems that may have been difficult to foresee, that mutations that give rise to unwanted traits could accidentally be propagated by the gene drive, or that cross-breeding or gene flow allows the drive gene to cross species barriers. Gene flow is, for example, possible between many of the different species and even different genera within the cyprinid family (Delomas et al. 2017; Wang et al. 2017; Konopinski and Amirowicz 2018; Wang et al. 2018; Warner et al. 2018). Using target-species specific DNA sequences could significantly reduce such risks.

To the best of my knowledge, no engineered gene drives have ever been released into a wild population (Gould et al. 2019). I am also not aware of any research programs that aims at developing gene drive technologies for controlling carp or any other fish populations. Despite the potentially high efficacy of these technologies, the possibility that they can induce unintended consequences that may be hard to control, such as, for example, accidentally driving a target species and non-target species to extinction, may eventually render engineered gene drives socially and legally unacceptable.

Various ideas are currently being developed in response to concerns about an unintended spread of gene drive insertions from target populations into non-target populations. "Tethered homing gene drives" (Dhole et al. 2019), for example, are based on three different engineered constructs. Two of them are toxin-suppressor underdominance constructs. The third forms a homing component that contains a payload gene that, for example, reduces female fitness. The homing is then driven by the presence of the two underdominance constructs, i.e. the underdominance constructs first have to reach high frequency in the target population before the homing component can become effective. Dhole et al. (2019) recently provided a very general proof-of-concept model. However, the fact that two underdominance constructs first need to reach high frequency in a population makes this technology probably less promising for controlling problem fish populations, especially fish with large generation times such as the carp.

Another recent idea, the "Locally Fixed Allleles" approach (Sudweeks et al. 2019) exploits the high degree of genetic specificity that is achievable using engineered homing drives. Sometimes, a problem (i.e. target) population is genetically distinct from neighbouring populations, for example, because of genetic drift has driven some alleles to fixation in the problem population while multiple alleles are still maintained in neighbouring non-target populations. If so, such locally fixed alleles could be used as target for the gene drive to potentially eradicate a problem population. Sudweeks et al.'s (2019) models focus on the example of controlling rodents on island. The potential of this or similar ideas for controlling large carp populations still needs to be demonstrated and may in general be small, given the large genetic diversity that has been observed among in carp in Australia.

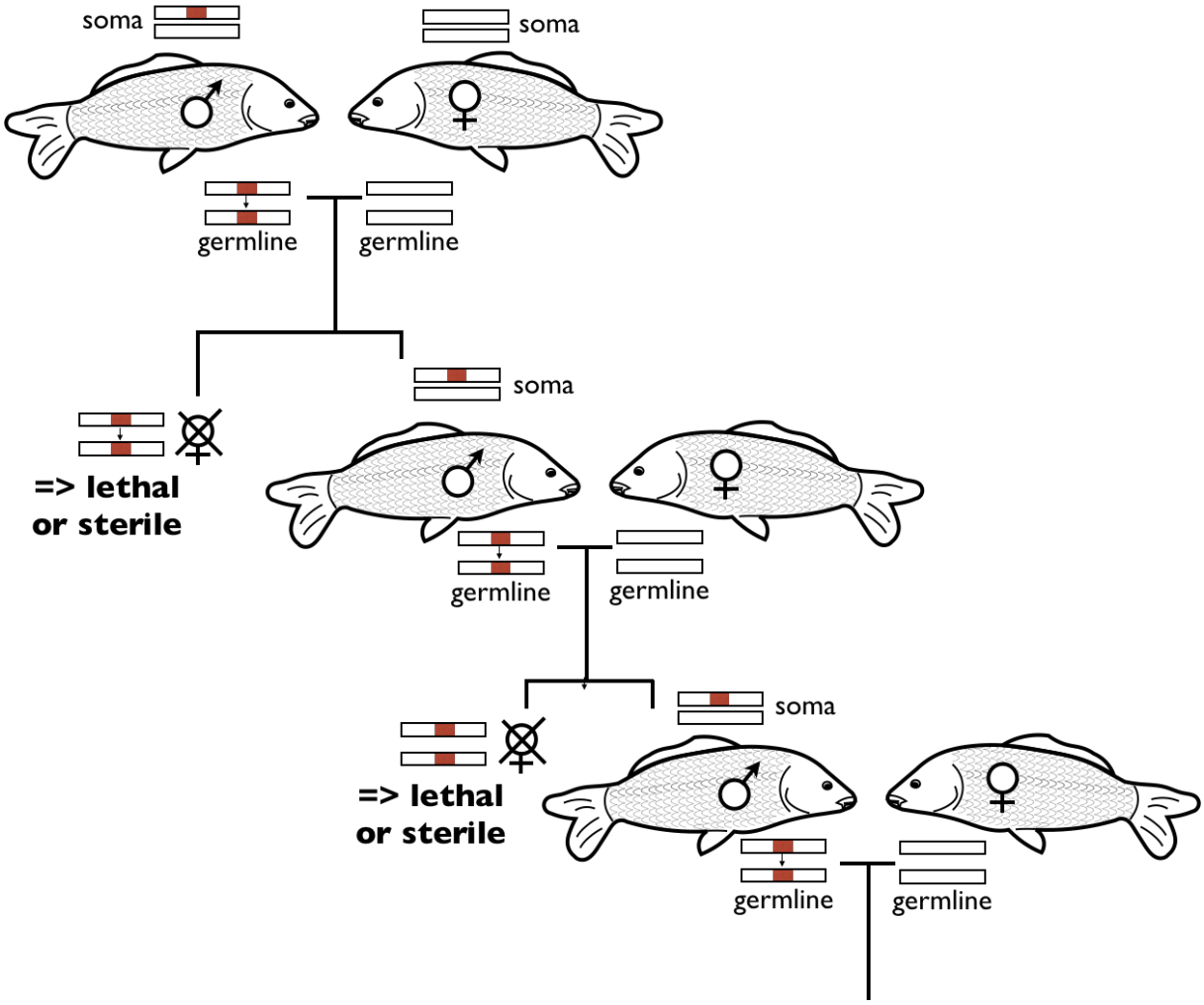

Figure 4. Example of how an engineered gene drive could be used to change the sex ratio of a population

An RNA-guided endonuclease cuts the chromosomes at a specific site to induce a repair mechanism that copies a drive sequence (red) into the damaged chromosome via homologous recombination. Inheritance thereby becomes 100% for the drive sequence, i.e. the drive sequence will rapidly spread in a population. Population sex ratio will be affected if the drive sequence reduces female reproduction (e.g. is lethal or renders them sterile). The rapid spread of the drive and the change in sex ratio will eventually lead to population crash. Adapted from Figure 2c of Champer et al. (2016).

4. Marking carriers

4.1 Introduction

Monitoring the frequency of carriers of chromosomal or genetic constructs will be easiest if there are phenotypic differences between carrier and naturally born carp. Moreover, genetic biocontrol technologies would probably be most efficient if carriers could be spared from angling and fishing, i.e. if carriers of chromosomal or genetic constructs would be clearly distinguishable from non-carriers. Phenotypic marks of genetically modified fish may potentially even enhance the public acceptability of genetic biocontrol technologies that would be based on genetic constructs.

The chromosomal or genetic constructs that are discussed here are, by themselves, unlikely to lead to morphological changes that allow to distinguish them from wild types. Therefore, markers would have to be bred into these carriers, or further genetic modification of these carriers would have to ensure that they are morphologically distinguishable from the wild types without suffering significantly from reductions of the vital rates. There are two types of potential genetic markers that seem promising and will be discussed in the given context, namely the “mirror” scale pattern and various skin colour. I will also briefly discuss non-genetic markers especially with regard to their applicability when very large numbers of fish need to be marked, as would be undoubtedly the case for controlling carp in the Murray-Darling basin.

4.2 Scale patterns

While common carp have an even, regular scale pattern, so-called “mirror” or “scattered” carp (Casas et al. 2013) have irregular and patchy scaling. Figure 5 shows an example of a “mirror” carp. The morph is linked to the ss genotype, i.e. to homozygosity of one of several mutations on the paralog of the fibroblast growth factor receptor 1, fgfr1a1 (Maderspacher 2009; Rohner et al. 2009). The heterozygous +s and the wild-type ++ genotypes give rise to the regular scale pattern (Casas et al. 2013). The “mirror” phenotype was selected for during domestication of the carp because it made kitchen handling easier while not leading to significant reductions in growth, survival, and fertility (Bohl 1999; Geldhauser and Gerstner 2002; Maderspacher 2009). It introgressed into wild populations in Europe and can still be observed in the wild (Figure 5). The fact that “mirror” carp are often raised in aquaculture and that they can be found in the wild together with wild-type scale pattern supports the view that the mirror geno- and phenotype is associated with no or minor reductions of fitness-relevant traits such a survival and reproduction in the wild. However, reports from carp introduced into Madagascar suggest the wild-type scaling pattern has a minor selective advantage over the “mirror” type. The carp that were originally introduced into Madagascar

about a century ago and again in the 1950s were mostly of the “mirror” phenotype (Hubert et al. 2016). Records from the 1950s reveal that some carp in Madagascar developed a full-scale cover that is based on fewer scales. This new full-scale cover is phenotypically distinct from the wild type (Hubert et al. 2016). Hubert et al. (2016) found that the carp in Madagascar developed their full-scale cover despite still being homozygous for the fgfr1a1 gene that would normally lead to the mirror phenotype. The authors suggested that natural selection against the mirror phenotype lead to the evolution of the full-scale cover from standing variation alone and in less than 40 generations. However, they still found much variation between populations, with rates of fully scaled carp ranging from 13.3% to 93.2%. Therefore, even if the full-scale cover may provide a selective advantage, selection has not been strong enough to be fully purifying within the observational period. Overall, Hubert et al. (2016) observations support the assumption that the fitness effects of the mirror phenotype are small. Combining the mirror phenotype with other genetic characteristics is therefore not expected to be problematic with respect to the carps’ growth and survival (Tadmor-Levi et al. 2017).

Other well-known morphs are the so-called “leather” or “nude” carp that has no scales, and the “line” carp that has only a single line of scales along the flanks or dorsal line. These morphs are linked to another locus, the so-called “N” locus (Bohl 1999; Casas et al. 2013). “Leather” and “line” carp are therefore genetically distinct from the “mirror” carp (Maderspacher 2009; Casas et al. 2013). They typically grow significantly slower than wild-type and “mirror” carp and often have increased offspring mortality that seems, however, strain-specific (Casas et al. 2013). Because of these reductions in important fitness parameters, the “leather” and the “line” carp morphs are not further discussed here.

The karyotype of the domesticated carp strain that Xu et al. (2014) studied has 2n=100 chromosomes. The fgfr1a1 mutation that gives rise to the mirror phenotype is on chromosome 34 (Xu et al. 2014). If the fgfr1a1 mutation could be moved from chromosome 34 to the Y chromosome, the homozygous ss genotype that leads to the “mirror” phenotype would then specifically mark the YY genotype, i.e. the most effective male or female Trojan Y carriers. However, the Y chromosome first needs to be identified, and moving a gene from one chromosome to another is time-consuming and technically challenging. This is especially true for Y chromosomes.

Introduction of a species into a new environment is likely to be followed by rapid evolution, i.e. local adaptation (Kinnison et al. 2001; Hendry et al. 2008; Hendry et al. 2011). Using foreign “mirror” carp strain to produce carriers of chromosomal or genetic constructs would therefore be expected to lead to genotypes that are less adapted to the ecology of the Murray-Darling basin than the feral normal-scaled fish, even if the ss genotype that leads to the “mirror” phenotype is likely to cause no or only minor fitness effects. Therefore, instead of using “mirror” carp strain to produce carriers of chromosomal or genetic constructs, CRISPR/Cas9 technology could potentially be used to directly edit the paralog of the

fibroblast growth factor receptor 1 in order to induce the fgfr1a1 mutation in individuals of the local carp strain that would be used for a breeding program outlined in Figure 2 and 3. Using CRISPR/Cas9 technology to mark fish with the mirrow phenotype should then lead to locally well-adapted carriers of chromosomal or genetic constructs that would be clearly distinguishable from non-carriers.

If the "mirror" phenotype is used to mark Trojan YY females that are released into the wild, the mutations on the fgfr1a1 locus will be inherited to the next generations. However, the frequency of naturally produced "mirror" phenotypes can be expected to be low, because the "mirror" phenotype requires homozygosity on the fgfr1a1 locus. If the next generations do not breed with the marked Trojan YY females again, "mirror"-type XX females or XY males (i.e. not Trojan Y carriers) will not appear before generation F_3. The frequency of these non-Trojan Y carriers is expected to be very low (Figure 6) because they result at a frequency of 25% from matings of heterozygotes only. If, however, the sons of Trojan YY females mate with sex-reversed Trojan YY females, some of their offspring will be YY males, the other type of Trojan Y carriers. First "mirror"-type XY males, i.e. non-Trojan Y carriers, can then be expected from generation F_3 on and again at low frequency (Figure 7). In conclusion, non-Trojan "mirror" phenotypes will only appear two to three generations after the first release of sex-reversed Trojan YY females and their frequency will be low. The "mirror" phenotypes will therefore remain a useful marker of Trojan Y carriers over several generations, especially if sex-reversed Trojan YY females are continuously released over longer periods.

4.3 Colour morphs

Domestic carp were recently, i.e. during the past two centuries, bred for prettiness to so-called "koi" (Maderspacher 2009). The genetic basis of carp colours could therefore be exploited for producing markers that help to distinguish carriers from wild types. Jiang et al. (2018) used the orange colour of the "Xingguo red carp" (a recessive inherited trait) and the black colour of the "Yellow River carp" (a dominant inherited trait) to mark YY individuals that they produced by androgenesis. These YY individuals could then be distinguished by their orange colour type (Jiang et al. 2018).

It is still unclear whether changes in colours affect survival and reproduction of carp in the wild. However, even if colours are selectively in the case of carp, using non-locally adapted koi carp, individuals of the "Xingguo red" or the "Yellow River" strain, or any other well-distinguishable foreign strain would be expected to lead to genotypes that are less adapted to the ecology of the Murray-Darling basin than feral normal-scaled fish from the region (Hendry et al. 2008; Hendry et al. 2011). The CRISPR/Cas9 technology could potentially be used here to directly edit genes in individuals of a local carp strain before they are used for one of the breeding protocols outlined in Figure 2 and 3. Before doing such molecular editing, it may be necessary to test the possibility that the editing of the genes and/or the

coloration itself reduce fitness-relevant traits such as survival and reproduction to a degree that renders the carriers ineffective, for example, by increasing the predation pressure.

4.4 Other marking procedures

There are many possible marks that are not genetic and that could be applied to carriers of chromosomal or genetic constructs before introduction into the wild. Such marks should ideally have a high retention rate, have no or little effects on survival, growth and reproduction (for example, would not attract predators), and would be fast to apply and of low cost, especially given the high number of carp that would need to be marked in a control program in the Murray-Darling basin. None of the currently available non-genetic marking techniques seem to fulfil all these criteria (Lukas and Baras 2001; McKenzie et al. 2012).

Arguably, the method that comes closest to this ideal is the fluorescent pigment mass marking technology (Moffett et al. 1997; Friman and Leskelä 1998; Schumann et al. 2013; von Siebenthal et al. 2017). Von Siebenthal et al. (2017), for example, used differently coloured pigment granules (Swada, Chelshire, UK) to spray-mark about 15,000 grayling (*Thymallus thymallus*) at the fingerling stage before releasing about 200 of them into a protected pond and the remaining ones into the wild. The retention rate could be determined in the pond fish after one year (Figure 8) and turned out to be 73.5%, depending on which of the four types of granules was used (the observed retention rate could potentially be increased with improved protocols). In total, 28 of the marked fish were recaptured from the wild either as four- or as five-year-old fish. The pattern of the marks of recaptured fish suggested selection against some type of granules when applied dorsally, probably because their colours (in this case yellow) attract piscivorous birds that spot their prey from above. Other colours like green or red seemed less problematic. Von Siebenthal et al. (2017) concluded that retention rate is not 100% and not all the marks are selectively neutral, but spray marking was cost effective (less than 100 $ of material costs in total), and the marking procedure itself caused no significant mortality (total mortality during marking and the following 3 weeks was 0.45 %), contrary to most other marking techniques (Lukas and Baras 2001; McKenzie et al. 2012). Last but not least, the fluorescent pigment mass marking technology proved to be very efficient, with 3 workers marking 15,000 fish in only one afternoon. This latter point seems important for application on a range that would be required in the case of carp in Australia. However, identification of marked fish requires examination under an UV-A lamp (that let the pigments shine) installed in a room that minimized ambient light (for example, a dark tent) to improve visibility. Therefore, the fluorescent pigment mass marking technology would only allow to monitor the frequency of carriers of chromosomal or genetic constructs but may not be sufficiently practical to spare carriers from angling and fishing.

Figure 5. The "mirror" phenotype
The phenotype that could be used to mark, for example, sex-reversed YY females. © C. Wedekind.

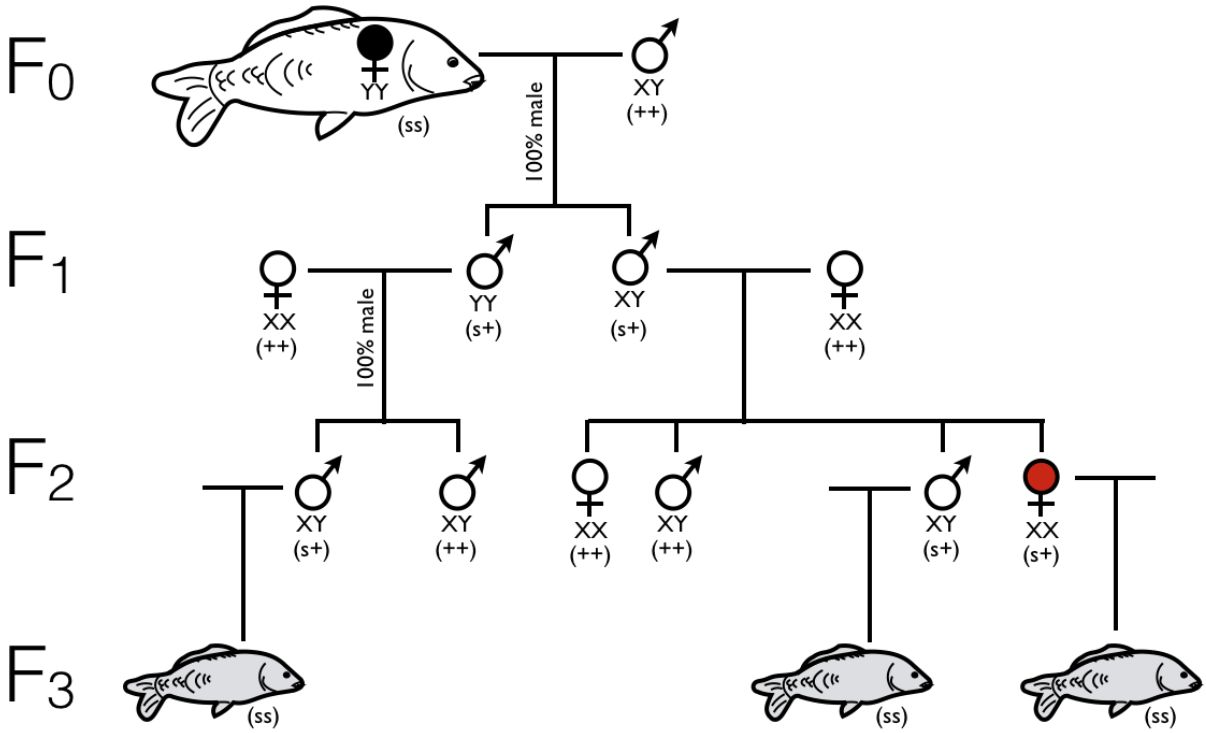

Figure 6. Later appearance of "mirror" carp that are not Trojan Y carriers
Appearance of "mirror" carp (the homozygous ss genotype) that are not Trojan Y carriers (drawn here in grey) after introduction of "mirror"- marked Trojan YY females (drawn in white) followed in the F_1 generation by mating with wild born females only. First females with the s+ genotype would be expected at low frequency from generation F_2 on (gender symbol in red). First "mirror" carp that are not Trojan Y carriers (XX females or XY males) could then be expected at low frequency from generation F_3 on.

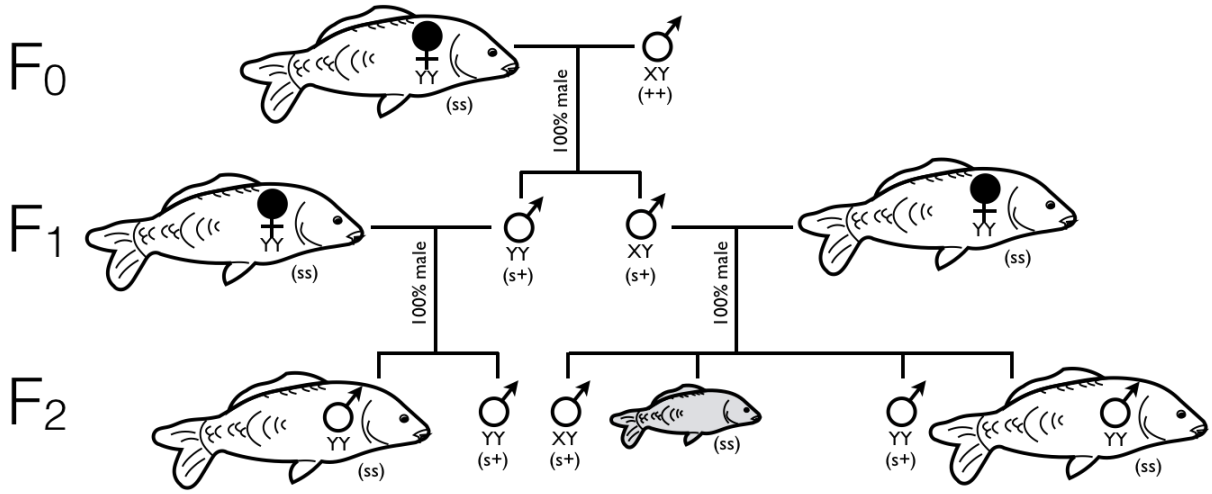

Figure 7. Natural production of "mirror"-type Trojan Y carriers
Trojan Y carriers (white, YY males) and non-carriers (grey; XY males) in the wild after release of "mirror"- marked Trojan YY females (white) and mating with YY females again in generation F_1. First "mirror" XY males (not Trojan Y carriers) could be expected at low frequency from generation F_2 on.

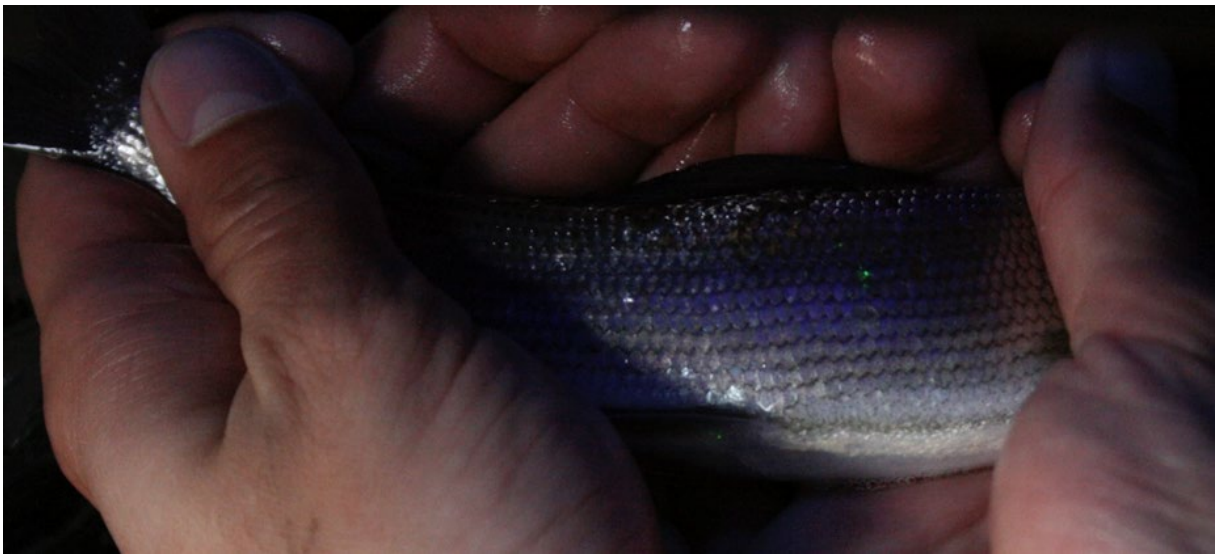

Figure 8. Spray marking with fluorescent pigments
A green fluorescent pigment granule found on the body side of a juvenile grayling (*Thymallus thymallus*) that was spray-marked one year earlier at a body length of around 9 cm. The pigments glow under long-wave ultraviolet light.

5. Population models

The potential efficiency of various genetic biocontrol technologies has been extensively analyzed in different population models (for example, Gutierrez and Teem 2006; Bax and Thresher 2009; Cotton and Wedekind 2009; Senior et al. 2013; Thresher et al. 2013; Teem and Gutierrez 2014; Prowse et al. 2017; Wedekind 2017). In some cases, experiments or meta-analyses suggested that some of these models were based on partially unrealistic assumptions about certain parameter. For example, Cotton and Wedekind (2009) have probably overestimated the importance of the effects of sex reversal on viability and fertility (Senior et al. 2012; Holleley et al. 2016; Senior et al. 2016). Such findings underline the importance of linking empirical work with modelling.

Arguably the potentially most effective genetic biocontrol would be based on the gene drive technology. However, as discussed above, this technology is not sufficiently developed for application in fish, and it comes with considerable risks that may make it legally unacceptable. Some of the other technologies that are based on genetic constructs, for example the "daughterless carp" technology, are less powerful and arguably less risky than methods based on engineered gene drives. With regard to their efficiency, they seem more comparable to the Trojan Y technology (Teem and Gutierrez 2014; Thresher et al. 2014b). Teem and Gutierrez (2014) analysed a combination of the Trojan Y technology and the "daughterless carp" technology and found that such a combined approach could lead to a "… *modest reduction in the time required for female eradication*" but that "… *the effort and expense of a combined strategy may not be warranted if the fitness cost of the … autocidal fish is significant*." These fitness costs clearly are a key factor in population models.

The fitness costs of the sex reversal, i.e. the induction of a phenotype-genotype mismatch in XY females or YY females, may typically be much smaller than previously assumed, to the degree that they may even be ignored (Senior et al. 2012; Holleley et al. 2016; Senior et al. 2016). Among the three types of Trojan Y carriers, sex-reversed XY females would then be expected to suffer least from fitness reductions. The effects of the aberrant YY karyotype on viability and fertility can be significant and may have to be newly determined for every species. This fitness cost is a crucial parameter for the Trojan Y chromosome technology to work, because sex-reversed YY females are expected to have the strongest effect on population sex ratio (Figure 1) if they do not suffer from significant reduction in viability and fertility. Using procedures that avoid inbreeding and that enhance purging of deleterious mutations as discussed in chapter 2.6 may therefore be important.

In order to illustrate the potential demographic effects of the Trojan Y technology, let us assume that only sex-reversed YY females are released into a population of carp, and that they then also produce YY male offspring, i.e. the third type of Trojan Y carriers (Figure 1). The viability and fertility of these YY females will depend on the effectiveness of synergistic measures that, for example, spare YY females from exploitation because they are

phenotypically marked and can be released again after being caught by anglers or commercial fishery. The fitness of YY females (v_{YY}) can then be high.

There are various possibilities to increase v_{YY} through synergistic biocontrol measure, apart from higher fishing pressure on non-Trojan Y carriers than on Trojan Y carriers (regardless of whether fishing is for human consumption or serves other purposes (David et al. 2018)), and apart from the breeding protocols that have been discussed in chapter 2 that would increase the quality of Y chromosomes and avoid inbreeding depression in stocked fish. When Cyprinid herpesvirus 3 (CyHV-3) is applied on feral populations, high v_{YY} could also be achieved by breeding genetic resistance against CyHV-3 into the Trojan Y carriers (Tadmor-Levi et al. 2017), or by using "mirror" strains that show a high resistance to CyHV-3 (Jia et al. 2018). It may even be possible to time the stocking of YY females such that they are least affected by an induced CyHV-3 epidemic. Another measure that could potentially increase v_{YY} is stocking individuals at a time and at a size that makes them more likely to outcompete naturally born fish.

In the following I will describe a simple model that shall illustrate the importance of increased v_{YY} on the likelihood of extinction of XX females. The model assumes that the population sizes remains at carrying capacity. Compensatory population growth can then be avoided (Weber et al. 2016).

Let us assume that phenotypically marked Trojan YY females are continuously produced and released into a wild population of carp, while the wild population is expected to have sporadic recruitment, i.e. there will be years when mortality of naturally spawned eggs and fry approach 100% (Thresher et al. 2014b). The accumulated stocking of YY females between such natural recruitment years could make the stocking more efficient, because juvenile YY females may then be more likely to outcompete their naturally born competitors simply because of their size and age. Therefore, if the rate of stocking is, for example, at 1% of the carrying capacity, this rate is likely to underestimate the demographic effect of well-designed stocking protocols. The effective rate of stocking would be somewhat higher and would have to be determined empirically. These potential effects are ignored in the present model, i.e. the effectiveness of the Trojan Y technology is conservatively estimated.

In the model, stocking of phenotypically marked YY females is continued over a period of 20 years and ceases thereafter. The following information was used to parametrize the model: Szabo et al. (2000) determined the weight of each egg mass that could be stripped from in total 2,086 carp of various weights and found these egg mass to be on average 16.3 % of the body mass (Figure 9a). Figure 9a suggests that this relative egg mass increases with increasing body weight. For simplicity, I assume a constant rate of 16.3%, i.e. conclusions about the effectiveness of the Trojan Y technology will again be conservative.

Because Szabo et al. (2000) did not give the weight-length relationship, this relationship was taken from Aera et al. (2014) (Figure 9b):

$$\log(\text{body weight}) = -0.0127 \log(\text{body length})) \quad (1)$$

Using the length-at-age relationships taken from 931 carp that could be age-determined by Brown et al. (2005) (Figure 9c), and using their von Bertalanffy growth model for females:

$$\text{body length} = 594 (1 - e^{-0.177 (\text{age}-0.609)}) \quad (2)$$

(Brown et al. 2005), the relationship between egg weight and age class can be estimated with an exponential growth function as

$$\text{egg weight} = 1128.71 - (1533.538 / 2^{(\text{age} / 5.266042)}) \quad (3)$$

(Figure 9d).

For simplicity, exploitation of non-marked carp, i.e. of XY or YY males and XX females, is assumed to not significantly affect their frequency in the population. Exploitation then only affects the average age of these genotypes. Their number will be determined by the carrying capacity, i.e. carp that are removed from a population will be replaced by younger unmarked fish or by phenotypically marked YY females. The stronger the fishing pressure on non-marked carp, the lower their average age and size.

Marked YY females will largely be spared from exploitation, but a yearly mortality will reduce their number and frequency in the population. This is different from XX females whose frequency is determined the carrying capacity.

Figure 10 shows what could then be expected from a yearly stocking of YY females at 0.5% (Figure 10a, c) or 1% of the carrying capacity (Figure 10b, d), an average age of XX females of 5 years (Figure 10a, b) or 7 years (Figure 10c, d), and various annual mortalities of YY females. During the first years after the start of the stocking program, the frequency of YY females will increase but will have virtually no effect on the frequency of XX females because YY females start reproducing only 4 years after the first stocking. The average age of YY females will constantly increase and reach, depending on the scenario plotted in Figure 10, between 7.9 and 10.3 years at the time when stocking would cease. From that moment on, the average age of YY females will increase more rapidly while mortality will reduce their frequency. The frequency of XX females will continuously decline from the moment YY females start to reproduce, because they will increasingly be replaced by XY and YY males. Several parameter settings illustrated in Figure 10 predict that XX females will go extinct few years after the ceasing of the stocking. All else being equal, natural reproduction would then stop after the last YY female has died.

Each panel in Figure 10 gives at least one parameter setting that predicts extinction of XX females, and at least one parameter setting where the frequency of XX females would recover after the ceasing of the stocking because of either a too low rate of stocking, a too low exploitation rate of non-marked fish, or too high mortality of YY females.

A key to these scenarios is that synergistic biocontrol measures spare YY females and let them grow old, large, and increasingly fecund (because of equations 2 and 3). The longevity of carp and the increased fecundity and increasing size and age is then a carp-specific characteristic that supports a genetic biocontrol technology based on phenotypically marked Trojan Y carriers.

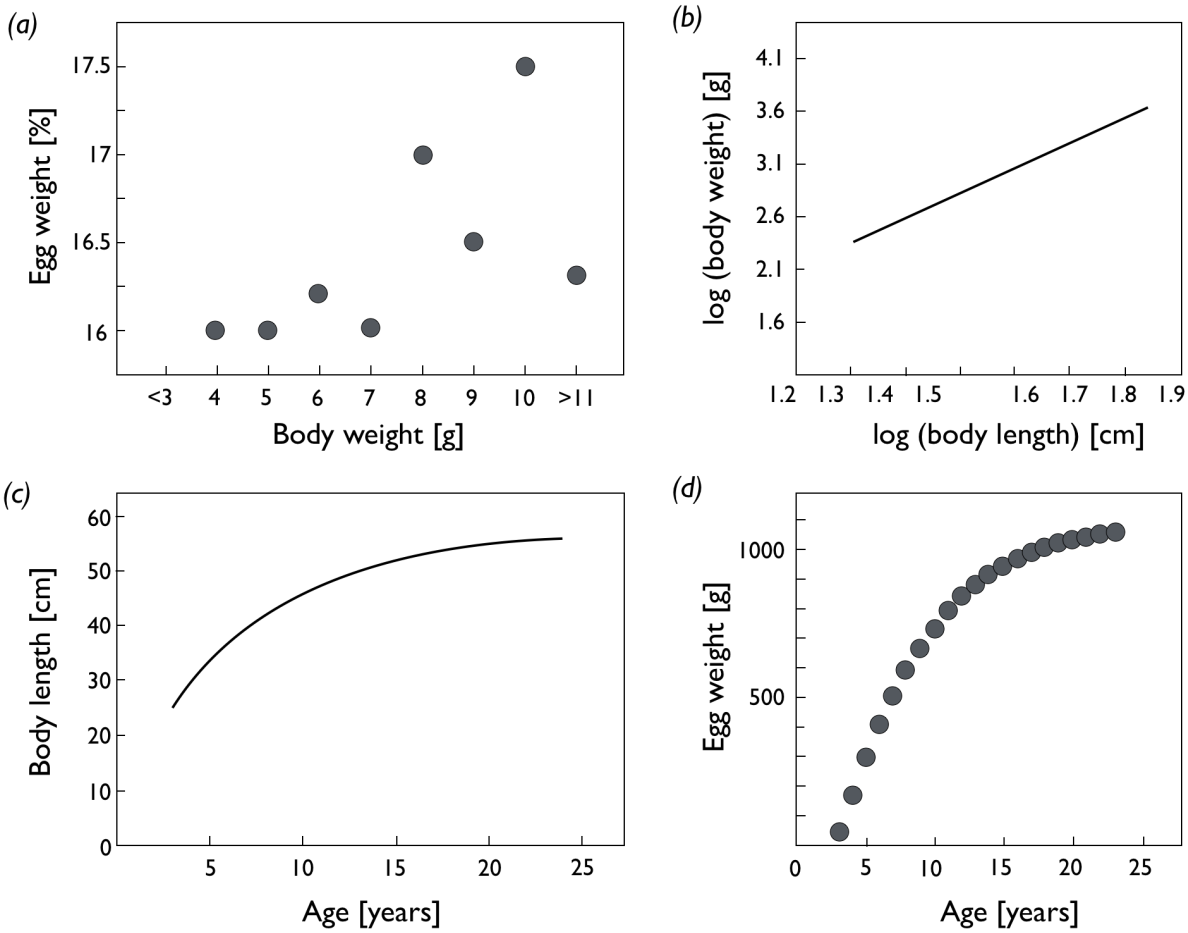

Figure 9. Observations used to parameterize the population model

(a) Average weight of eggs that could be stripped from 2,086 carp of different weight categories. Data taken from Table 2 of Szabo et al. (2000). (b) Regression line linking body weight and body length in Aera et al. (2014) (redrawn from their Figure 2b). (c) Length-at-age relationship for female carp as described in Brown et al. (2005) (redrawn from their Figure 4). (d) Relationship between egg weight and age class as derived from Brown et al.'s (2005) von Bertalanffy length-at-age growth model, Aera et al.'s (2014) weight-to-length relationship, and Szabo et al. 's (2000) average egg weight per body weight.

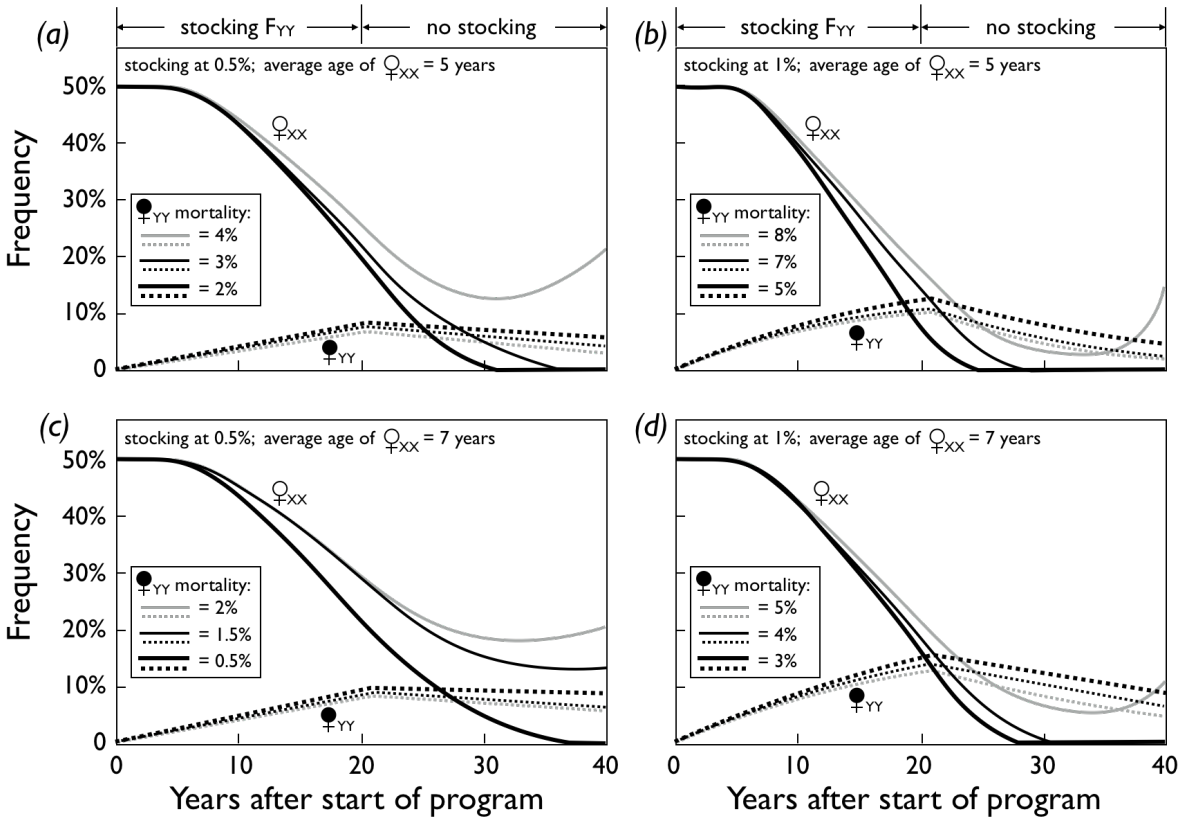

Figure 10. Simulating the effects of stocking rate, average age of XX females, and mortality of YY females on the frequencies of XX and YY females

The effects of stocking Trojan YY females into a carp population that remains at carrying capacity and starts with an equal population sex ratio. In the present model, stocking happens over period of 20 years and ceases after that, but significant effects of Trojan YY females on the frequency of XX females can be expected for many more years because of their longevity and as a consequence of sparing marked Trojan YY females from exploitation and hence allowing them to grow larger and spawn more eggs than XX females. Panels (a) and (b) show the effects of stocking on the frequencies of XX and YY females when the average age of XX females can be kept at 5 years, while in (c) and (d), the average age of XX females is allowed to be 7 years. In panels (a) and (c), the stocking rate of marked YY females is at 0.5%, while in (b) and (d) the stocking rate is 1%. The mortality of the Trojan YY females then decides whether XX females go extinct or their frequency recovers after the ceasing of the stocking. Each panel shows at least one scenario where XX females would go extinct and one where their frequency would recover.

6. Conclusions and general recommendations

Genetic biocontrol technologies that are based on genetically modified individuals provide potentially powerful options for controlling invasive carp in the Murray-Darling basin and elsewhere. Most of these technologies are at an early developmental stage and are mostly untested in fish. Arguably the most advanced technology in this context is the “daughterless carp” strategy. Potential biocontrol technologies based on engineered DNA sequences will require a careful risk analysis and risk management, especially those based on gene drives. It remains to be seen if such genetic biocontrol technologies can obtain the social acceptability and the legal support that would be required for implementation on problem populations.

Among the genetic biocontrol technologies that are not based on engineered DNA sequences are the “sterile male” and the “Trojan Y chromosome” technologies. In the case of carp, the former would probably have to be based on the production of triploid males whose reproductive competitiveness in the wild is not sufficiently understood yet. Moreover, the strategy would require very high stocking intensities that could lead to undesirable ecological effects.

The Trojan Y chromosome technology seems more promising. However, this technology may only be effective when Trojan Y chromosome carriers are of high viability and fecundity. The production of (ideally sex-reversed) YY individuals to be stocked into the wild should therefore include measures to minimize inbreeding depression and to allow for purging of deleterious mutations from the Y chromosome. Moreover, such breeding programs would ideally be based on locally adapted strains. It would then be important to combine the Trojan Y chromosome technology with synergistic measures to increase the viability and fecundity of Trojan Y carriers. These supportive measures include phenotypic marking (for example, using the “mirror” phenotype) to allow sparing Trojan Y carriers from angling and fishing so that the extra-ordinary longevity of carp and the increased fecundity at increasing size and age can be exploited for biocontrol measures. Other synergistic measures that have the potential to support the Trojan Y chromosome technology and that would have to be further discussed include enhancing resistance against CyHV-3 or other pathogens, for example, by breeding genetic resistances into the Trojan Y carriers, by immunizing Trojan Y carriers before stocking, or by timing the stocking in response to dynamics of an induced epidemics.

7. Recommended next steps

There are several protocols that could be followed to implement the Trojan Y technology (as outlined in chapter 2), and there are various types of challenges that would have to be dealt with. First, questions about social acceptability and legal support would have to solved. Arguably the most critical ones in this context are: (i) would stocking of sex-reversed YY individuals be acceptable, and (ii) would it be acceptable to use the CRISPR/Cas9 technology on local carp strains to directly edit the paralog of the fibroblast growth factor in order to promote local adaptation of mirror-type Trojan Y carriers? The latter question may turn out as not as important as the former if mirror-type strains from other origins are able to establish themselves well in the wild. However, the potential of the Trojan Y technology critically depends on whether sex-reversed, i.e. hormone-treated, individuals can be released into the wild. The facts that the hormone treatment would be confined to a short period during larval stages, and that hormone-treated Trojan Y carriers would be of the mirror phenotype and hence clearly distinguishable from wild-type carp, may potentially make the induced sex reversal more acceptable for the public and for legislation.

A second challenge is the management of the genetic background of Trojan Y carriers, for example the avoiding of inbreeding depression or the fostering of certain potentially useful genetic characteristics. As long as the question about the use of the CRISPR/Cas9 technology is not solved, different strains of mirror-type carp could be obtained from various sources to avoid losing time and opportunities. Starting with different strains would be necessary to allow for active outbreeding in the final step in the production of Trojan Y carriers (see protocols in Wedekind (2019)). Also, strains of mirror carp that are bred to be resistant to CyHV-3 (Tadmor-Levi et al. 2017; Jia et al. 2018) could be used to keep the option of later combining the Trojan Y technology with a controlled release of CyHV-3.

A third challenge is that the production of large number of Trojan Y carriers takes time. If the first-generation mirror-type carp were mature and ready to spawn, the program could quickly start with the first breeding steps to produce the F1 generation. Ideally, conventional breeding could be used to produce sex-reversed XY individuals (step A in Figure 2) and XY males, and androgenesis could be used in parallel to produce both YY males and YY females (G1 and G2 in Figure 3). The androgenetically produced fish are expected to suffer from inbreeding depression and should not be used as stocking material. Instead, all F1 fish would have to be raised to maturity before they could be used for the 2nd breeding step. Crossing androgenetically produced YY males with YY females would then already produce 100% YY individuals that, if sex-reversed, could be used for stocking (C2 in Figure 3). The F1 males and females used in this second breeding step would have to originate from different strains to avoid inbreeding depression and to promote hybrid vigour in the F2 generation. Importantly, androgenesis would allow producing large numbers of YY females after only one carp generation, i.e. only few years after the start of the program. In parallel, conventional

breeding could be used to produce YY males and YY females in the F2 generation (B1 and B2 in Figure 2) that would have to be identified as such and then raised to maturity to allow the large-scale production of conventionally bred YY females to be stocked into the wild (C2 in Figure 2). This conventional breeding program would take two carp generations, i.e. twice the amount of time that an androgenesis-based protocol would require, before large numbers of Trojan Y carriers could be released into the wild. However, running this parallel breeding program may be required, on the one hand, to manage the risks associated with androgenesis, and, on the other hand, to promote recombination between the X and Y chromosomes in the female phenotype and hence to foster the purging of the Y chromosome from deleterious mutations. One of the risks of androgenesis is, for example, that it causes too high levels of inbreeding depression and that the raising of large numbers of F1 to maturity may hence be difficult. However, if androgenesis is successful, conventionally produced XY females (A in Figure 2) could also be crossed with androgenetically produced YY males to produce, for example, YY males (E1 in Figure 2) or YY females (E2 in Figure 2) in a twice as high frequencies than what the 2nd conventional breeding step would offer. Alternatively, or in parallel, eggs of conventionally produced XY females could be used to gynogenetically produce YY males and YY females (analogous to G1 and G2 in Figure 3) to profit from the expected recombination between the X and Y chromosomes in the XY females and enhance to profit from the purging of the Y chromosome from deleterious mutations (see chapter 2.6). These YY males and YY females could then be used to produce the YY females for stocking (C2 in Figure 2 or 3). In conclusion, while androgenesis may allow producing large numbers of YY females after only one carp generation (i.e. 3 to 4 years), conventional breeding or a breeding program that includes gynogenesis require at least two carp generations (i.e. 6 to 8 years) to start producing Trojan Y carriers on an industrial scale. When both techniques are used in parallel, first Trojan Y carriers could potentially be released already 3 to 4 years after the start the program.

A fourth challenge is the identification of Y chromosomes that would be required to separate androgenetically produced YY females from XX females (e.g. G2 in Figure 3), conventionally produced and sex-reversed XY females from XX females (e.g. A in Figure 2), YY males from XY males (e.g. B1 in Figure 2), or YY females from XY females (e.g. B2 in Figure 2). Reliable sex-specific genetic markers still need to be developed, as outlined in chapter 2.3. It is, however, not necessary to wait with the production of the F1 following protocols A (Figure 2) or G1 and G2 (Figure 3) until these sex-specific genetic markers have been developed. The F1 can be sorted at any stage whenever the sex-linked markers are available.

Obviously, the production of large numbers of Trojan Y carriers will require infrastructure and trained personnel. The requirements in this context will depend on the scale at which this technology would be implemented and would have to be determined.

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FRDC FINAL REPORT CHECKLIST

Project Title:			
Principal Investigators:	XXXX (include all recognised authors -)		
Project Number:	XXXX/XXX		
Description:	Brief one/two paragraph overview of what the project did and achieved.		
Published Date:	XX/XX/XXXX (if applicable)	**Year:**	XXXX
ISBN:	XXXXX (if applicable)	**ISSN:**	XXXXXXXXXXXXX (if applicable)
Key Words:	Needs to include key subject areas and species name (see www.fishnames.com.au)		

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	Is it included (Y/N)	**Comments**
Foreword (optional)		
Acknowledgments		
Abbreviations		
Executive Summary		
– **What the report is about**		
– **Background – why project was undertaken**		
– **Aims/objectives – what you wanted to achieve at the beginning**		
– **Methodology – outline how you did the project**		
– **Results/key findings – this should outline what you found or key results**		
– **Implications for relevant stakeholders**		
– **Recommendations**		
Introduction		
Objectives		
Methodology		
Results		
Discussion		
Conclusion		
Implications		
Recommendations		
Further development		
Extension and Adoption		
Project coverage		
Glossary		
Project materials developed		

Appendices		

NATIONAL CARP CONTROL PLAN

The National Carp Control Plan is managed by the
Fisheries Research and Development Corporation

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Post: Locked Bag 222, Deakin West ACT 2600

www.carp.gov.au