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## **POPULATION CONSEQUENCES OF RELEASING SEX-REVERSED FISH: APPLICATIONS AND CONCERNS**

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### **1. Abstract**

Sex differentiation is generally more labile in gonochoristic fish than it is, for example, in birds and mammals. Environmentally induced sex reversal is therefore often possible and creates genotype-phenotype mismatches that can be useful in population management. Interestingly, sex chromosomes of fish are typically not significantly decayed, i.e. all types of offspring of sex-reversed individuals may be viable, including YY and WW individuals that can then often be sex reversed again. Releasing sex-reversed fish, or releasing untreated YY and WW offspring of sex-reversed fish, into natural populations can therefore affect sex ratio of the population in the following generations and hence affect population growth. Some types of releases are likely to boost population growth if the number of females are limiting (e.g. WW individuals to create female-biased population sex ratios), others can be used to reduce growth of undesired population (e.g. YY individuals to create male-biased sex ratios). However, these ideas remain still largely untested. Key variables in respective population models are the effects of sex reversal and of unusual karyotypes on viability and reproduction under natural conditions. These key variables need to be determined to allow for data-based models that can serve as basis for field trials.

### **2. Introduction**

#### **2.1 The threats of distorted population sex ratios**

Sexual reproduction creates strong frequency-dependent selection on the production of sons and daughters [1] which explains why we can usually expect about 1:1 sex ratios in undisturbed populations, at least at some early life-history stages. However, adult sex ratios often deviate significantly from 1:1 [2]. Among the factors that can bias sex ratios in one direction or another are sex-specific life histories and life-history association mortality rates [2], non-random harvest [3, 4], or sex-specific tolerance to anthropogenic stress. Any deviations from equal sex ratios can be a threat to natural population because they increase effects of stochasticity. For example, the last individuals of the Dusky Seaside Sparrow (*Ammodramus maritimus nigrescens*) that were meant to be used in a breeding program turned out to be all male [5]. Deviations from equal sex ratios also reduce the genetically effective population size ( $N_e$ ) relative to the census size ( $N_c$ ), because  $N_e = 4N_mN_f/(N_m+N_f)$ , with  $N_m$  and  $N_f$  being the number of mature males and females, respectively [6]. Sex ratio biases therefore reduce the genetic diversity and, hence, the evolutionary potential of a population and may contribute to an extinction vortex especially in small or declining populations [7].

The potentially damaging effects of shifted sex ratios may be more obvious in a male-biased than in a female-biased population if the available number of eggs constrains population growth. One of the most spectacular examples of this is the case of the critically endangered Kakapo (*Strigops habroptilus*) that typically lay only one or two eggs per season: it appears that management measures have unintentionally affected parental strategies and

thereby caused an overproduction of sons [8]. The lack of daughters then further threatened the survival of the Kakapo as a species [8]. Fish, with their usually high reproductive potential (e.g. high number of eggs per female), may seem less susceptible to these kinds of threats. However, fish typically show high embryo, larval, and juvenile mortality, and male-biased shifts in sex ratios have been discussed as a possible cause of further declines of already protected populations [9].

It seems often possible to manage population sex ratios by manipulating ecological or social factors that affect sex-specific growth and survival or that affect maternal life-history and hence, family sex ratio [10]. Such measures could aim to support small and endangered populations, either by preventing distorted sex ratios or by inducing small female-biased sex ratio distortions to increase  $N_c$  of the later generations, even if this means to first reduce  $N_e$  in the F1 and possibly F2. The immediate negative effect of such an induced genetic bottleneck would have to be compensated by the additional population growth as a consequence of the manipulation [11, 12].

**Box 1.** Terms used:

- $N_c$ : census population size
- $N_e$ : genetically effective population size, i.e. the size of an idealized model population that loses genetic diversity at the same rate as the study population [6].
- Environmental sex reversal: mismatch between genetic and phenotypic sex that is induced by environmental factors (e.g., extreme temperatures or micropollutants) during a sensitive period in life.
- “Trojan” genetic element: genetic factor that can change the demography of a population.

Distorted population sex ratios are not only an important topic in the management of threatened populations. Sometimes, intentionally distorting sex ratios may help us to control populations that have been identified as problematic for a given ecosystem; for example, an exotic and invasive species that has successfully established in a system. This is especially the case if manual or chemical eradication of such undesirable populations are not practical [13].

## 2.2 Sex determination and sex differentiation fish

Fishes show a great diversity of gonadal development and sex differentiation that include gonochoristic species with individuals developing either testis or ovaries, simultaneous hermaphrodites, sequential hermaphrodites that mature as males or as females first and may change sex later in life, and all-female species that reproduce gynogenetically [14, 15]. The mechanisms of sex determination are very diverse in fish too [16], and sex differentiation is typically more labile as compared to birds and mammals [17, 18]. The diversity ranges from purely genetic sex determination, with males or with females as the heterogametic sex, to purely environmental sex determination [14, 15]. In fish and amphibia, this range can be seen as a continuum, with phenotypic sex as a threshold trait that is dependent on the interaction between genetic and environmental factors that may influence physiological processes during sex differentiation [19]. Importantly in this context, sex determination then also includes environmental sex reversal, i.e., a mismatch between genetic and phenotypic sex that is caused by an environmentally induced sex reversal. The environmental factor that induces the sex change can be, for example, extreme water temperatures or temperature variation [20-23], municipal wastewater effluents that contain endocrine disrupting chemicals [24, 25], or exogenous hormones such as the synthetic ethinylestradiol (EE2), the natural 17-estradiol (E2), or 11-ketotestosterone (KT) applied during a sensitive period in ontogeny to manage population sex ratios [26]. It even seems that, in many teleost fish, environmental sex reversal

occurs regularly over evolutionary time and has contributed to the maintenance of homomorphic sex chromosomes [27, 28].

### **3. Sex reversal and “Trojan” genetic elements**

Genetic elements are called “Trojan” if they have the potential to change the demography of populations and potentially even drive them to extinction [29]. There are various types of genetically engineered organisms whose Trojan elements are used or could potentially be used in controlling problem populations, including, for example, sex-specific lethality constructs [30] or a genetically engineered aromatase inhibitor gene (D) that, when introduced into a population, may lead D-gene carriers to phenotypically develop into males regardless of their sex chromosome [31]. For many fish, arguably the most promising methods in this context are based on the “Trojan sex chromosome” idea originally suggested and modelled by Gutierrez and Teem [32, 33] and is not based on such a recombinant approach but on sex reversal [34]. It therefore avoids the danger of gene constructs jumping to other species [35] and may hence be more likely to be accepted by the public and approved by local authorities [36, 37].

The basic idea of the Trojan sex chromosomes hypothesis is to change the frequencies of sex chromosomes in natural populations in order to influence population demography. This may be possible if the target species is gonochoristic and has sex chromosomes that are not significantly decayed as a result of suppressed recombination between the sex chromosomes, i.e. not like in most mammals [38]. Interestingly, sex chromosomes of fish and amphibians are indeed typically not significantly decayed [16]. Therefore, in fish and amphibians with an X-Y sex determination system, i.e., with normally XX females and XY males, individuals with a YY genotype are mostly viable. Such unusual genotypes can be produced by mating, for example, a feminized XY individual with a wild-type XY male (Figure 1). YY individuals would be males who, when mated with a wild-type XX female, would only produce XY sons. Feminized YY individuals would, when mated with a wild-type XY male, produce 50% XY sons and 50% YY sons who themselves could only produce sons. Therefore, when YY males or females are released into the wild, the Y-chromosome would act as a Trojan element to reduce the frequency of females in the following populations, assuming that the Trojan chromosome carriers successfully reproduce and produce viable offspring. Analogous Trojan chromosome carriers can be constructed in a W-Z sex determination system, and the Trojan element can be used to create both male- or female-biased sex ratios in the following generation. While male-biased sex ratios would usually aim to control the growth of an undesired population [32], an induced female-biased sex ratio could potentially be used to boost population growth [39].

[Insert figure 1 here]

Figure 1 illustrates the expected demographic and genetic effects of the release of various types of Trojan chromosome carriers in a X-Y and a W-Z sex determination system, assuming that all mating types are possible and all types of offspring have the same viability. The Trojan chromosome carriers with their unusual karyotypes would either be offspring of sex-reversed individuals but not hormone-treated themselves, or they would be individuals that have been sex-reversed before release into the wild.

### **4. Trojan chromosome carriers produced in brood stocks**

Population management based on Trojan sex chromosomes is ideally based on brood stocks. If the release Trojan sex chromosome carriers is meant to lead to male-biased population sex ratios in the following generation, the brood stock would ideally consist of YY males and YY females if males are normally the heterogametic sex, and of ZZ males and ZZ females if

females are normally the heterogametic sex (Figure 1). If the release of Trojan sex chromosome carriers is meant to lead to female-biased population sex ratios in the following generation, the brood stock would ideally consist of XX males and XX females or of WW males and WW females, respectively (Figure 1). The establishment of such brood stocks is greatly simplified if genetic sex markers are available. At the time Gutierrez and Teem [32] suggested their idea, such genetic sex markers were not available for many fishes. Meanwhile, master sex-determining genes have been found in various fish [40, 41], including the sdY locus in rainbow trout (*Oncorhynchus mykiss*) [42] that proved to be highly conserved among many salmonids [43]. However, there are examples of within-species variation in sex determination in other taxa [44]. It may therefore often be necessary to verify a sex-linked marker for a given population. The latest developments in restriction-site associated DNA sequencing technology (RAD-seq) allow for cost-effective identification of sex-specific markers in fish with no reference genome [45, 46].

Recently, Schill et al. [47] successfully produced a brood stock of brook trout (*Salvelinus fontinalis*) that can now be used to produce and release Trojan chromosome carriers to possibly eradicate brook trout populations outside of their native range. The brook trout has a X-Y sex determination system and is sensitive to steroids during the sensitive stage in sex differentiation, i.e. during early larval stages. Schill et al. [47] therefore produced a YY brood stock to produce untreated YY males for release into the wild (the first scenario in Figure 1). The authors followed the three-step approach that was originally suggested by Gutierrez and Teem [32] (see also Figure 2). Phase 1: They fertilized eggs, incubated the embryos at standard hatchery conditions, and exposed half of the resulting swim-up fry to estrogens over a period of 60 days. For exposure, they used food pellets that had been sprayed with the natural  $17\beta$  estradiol to create an estradiol concentration of 20 mg/kg diet, following recommendations from Johnstone et al. [48] except that they did not defatten the diet pellets prior to treatment. The fish were then raised to about fingerling size when fin clips could be taken for genetic sex identification (based on the sdY genotype [43]). Hormone-treated and untreated XY individuals were then raised to maturity. Phase 2: Eggs of hormone-treated XY females were fertilized with sperm from untreated XY males, the embryos raised, the clutches split, and half of the swim-up fry again exposed to estradiol-treated food pellets as in phase 1. After genetic screening (again based on fin clips taken from fingerlings), YY females from the hormone-treated group and YY males from the untreated group were raised to maturity. Phase 3: Eggs of YY females were fertilized with sperm from YY males to produce and maintain a YY brood stock.

[Insert figure 2 here]

Analogous procedures are likely to work in many fish species. In aquaculture, monosex cultures are often economically advantageous, for example, because they avoid the problems of early maturation and uncontrolled reproduction [49]. There are therefore a number of species for which the large-scale production of monosex progeny has already been established, including the Nile Tilapia (*Oreochromis niloticus*) [50] and the rainbow trout [51, 52], and many estrogenic substances have been tested on many different fish species in this context [49]. However, instead of producing a YY brood stock, masculinization of XX individuals is often used to eradicate Y chromosomes and hence produce a female monosex culture (the third scenario in Figure 1).

In the case of the brook trout, reaching phase 3, i.e. the production of YY offspring only, takes at least four years, because the minimal generation time in this species is two years. Schill et al. [47] describe the investment in terms of manpower and financial costs as “modest,” despite the fact they worked with rather large sample sizes and kept families separate until PIT-tagging the fish to avoid crossing full-sibs later. The total financial costs

for the development of their YY brood stock that produced 5,000 YY males at the beginning of phase 3 and 15,000 YY males two years later were "...less than US \$10,000" [47], including genetic testing ("ca US \$5/fish"), feed, and labor. Manpower needs, apart from maintaining the fish stock, included only 2-3 days per spawning period at the end of each phase, and a day per generation for fin clipping and PIT tagging.

The sex-reversal protocol that Schill et al. [47] used proved very effective in the first phase, with 99.6% feminization of XY individuals. Feminization of YY individuals in phase 2 was less successful, with 93.8% of the hormone-treated YY individuals showing intersex characteristics. In order to avoid self-fertilization during stripping, Schill et al. [47] had to bisect the body cavity and to remove ovulated eggs by hand. Hence, the production of YY-females that could be released into the wild (scenario 2 in Figure 1) seems not sufficiently established yet for brook trout. However, Schill et al. [47] discussed techniques that could be tried to potentially achieve better feminization rates of YY individuals, including immersion in estradiol-treated water around the time of hatching from eggs has been found before to lead to very high rates of sex reversal [53, 54]. Moreover, attempts to feminize YY individuals have been successful in several other species [55, 56].

## **5. Consequences of releasing sex-reversed fish**

The demographic and genetic consequences of releasing Trojan chromosome carriers are not well understood yet. Figure 1 only lists the expected consequences of various types of releases under the assumption that there is no reduction in viability and reproductive capacity in any sex-reversed fish or any of the unusual karyotypes, as compared to the wild types. This assumption is currently not well supported, and in fact there are various indications that sex reversal and especially unusual karyotypes (the chromosomally aberrant YY and WW) reduce viability or reproductive potential [57]. As mentioned above, intersex characteristics are frequently observed, possibly as a result of non-complete sex reversal. However, among various taxa, masculinized fish generally show similar sperm characteristics as wild-type males [58], suggesting that sex-reversed female genotypes have reproductive success comparable to genotypic males. Moreover, Schill et al. [47] found no reduction of fecundity of sex-reversed XY females as compared to XX females.

Theoretical analyses of the effects of environmental sex reversal and/or the release of sex-reversed individuals or of offspring of sex-reversed individuals are either based on strong assumptions about viability and fertility, or they include treatment-induced effects on viability and fertility in their models as further factors [32, 59-62]. Laboratory-based estimates of these key variables are scarce and potentially misleading if not confirmed by field studies. Therefore, data-based modelling is currently constrained and may give only rough ideas about whether a certain stocking strategy could drive undesired populations toward extirpation or support declining population whose population growth is constrained by their number of females.

## **6. Public and legal acceptance of releasing sex-reversed fish**

I currently do not know of any experiments that include the release of carriers of Trojan sex chromosomes into a natural population. However, public and legal acceptance of field trials based on Trojan sex chromosomes may not pose a major challenge. First, introducing Trojan sex chromosomes into a population is a method that specifically targets the undesired population, with little risk of direct ecological collateral damage [63]. Second, the release of hormone-treated individuals can be avoided if necessary from a food safety standpoint, for example if the targeted species has a X-Y sex determination system and male-biased sex ratios are the aim (scenario 1 in Figure 1). Third, a management measure based on the release of certain types of fish is quickly reversible and genetic long-term effects are unlikely. Last but not least, the Trojan sex-chromosome approach to population management may often be

the only realistic chance to eradicate a problem population, as long as manual or chemical measures are not practical.

## 7. Acknowledgements

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	X-Y sex determination			W-Z sex determination		
released type:						
mating in the wild with:						
F1 males	100%*	100% (50%  )	0%	0%	0%	100%*
F1 females	0%	0%	100%*	100%*	100% (50%  )	0%
X-chromosomes in F1	50%	75%	100%			
Y-chromosomes in F1	50%	25%	0%			
W-chromosomes in F1				50%	75%	0%
Z-chromosomes in F1				50%	25%	100%

\* only regular karyotypes

Figure 1. The expected consequences of the release of different types of Trojan chromosome carriers into natural populations with an X-Y or an W-Z sex determination system. Trojan chromosome carriers are individuals with karyotypes that can result from sex reversal in the parental generation (grey symbols) and/or that have been sex-reversed themselves (black symbols). The expected frequency of males and females in the F1 generation are based on the assumption that all mating types are possible and have the same effect on the viability of all types of offspring. The figure gives the expected frequencies of wild-type males and females, the frequencies of offspring males and females with unusual karyotypes, and the expected frequencies of X-, Y- W- and Z-chromosomes.

	X-Y sex determination		W-Z sex determination		
released type:					
mating in the wild with:					
F1 males	100%*	100% (50%  )	0%	0%	100%*
F1 females	0%	0%	100%*	100% (50%  )	0%
X-chromosomes in F1	50%	75%			
Y-chromosomes in F1	50%	25%			
W-chromosomes in F1			50%	75%	0%
Z-chromosomes in F1			50%	25%	100%

\* only regular karyotypes

Figure 2. Production of outbred Trojan Y carriers that are not hormone treated. In Phase 1, various sibgroups are produced. Some individuals per sibgroup are estradiol treated to induce sex reversal. Sex reversal is verified via genetic screening (of the sdY genotype) and phenotypic sexing. In Phase 2, sex-reversed XY females are crossed with XY males from other families. Some individuals per sibgroup are again estradiol treated to induce sex reversal, and sex reversal is verified via genetic screening and phenotypic sexing. In Phase 3, sex-reversed YY females are crossed with YY males from other families to produce YY males. The figure only shows combinations of genotypes and phenotypes that are essential for the brood-stock production of Trojan Y carriers.