Transgenic Disruption of Aromatase Using the Daughterless Construct to Alter Sex Ratio in Common Carp, *Cyprinus Carpio*

by

Dan Zhang

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 6, 2016

Keywords: pDC1, transgenic, common carp, daughterless, sex ratio, aromatase

Approved by

Rex Dunham, Chair, Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Allen Davis, Alumni Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Charles Chen, Associate Professor, School of Crop, Soil and Environmental Sciences

Abstract

The common carp (*Cyprinus Carpio*) is considered as invasive alien species (IAS) in North America and is particularly ecologically damaging in Australia. Traditional methods, biological means, harvesting and chemicals, used to control these aggressive invasive species do not adequately reduce population numbers. A Daughterless construct, which uses a shRNAi approach to silence aromatase, and then subsequently a reduction in estrogen, has been successfully tested in medaka (*Oryzias latipes*) and zebrafish (*Danio ratio*). The P1 transgenic common were produced by electroporation of a Daughterless Carp construct, pDC1, containing a shRNAi sequence targeting common carp aromatase and driven by the common carp aromatase promoter into sperm and eggs. Exposure to this construct either from the plasmid containing this sex reversal gene, integration of the transgene or both, resulted in a skewness towards maleness in the P1 as expected. P1 transgenic common carp males were mated with non-transgenic females to produce 42 F1 transgenic families. Paradoxically, the sex ratio for the F1 transgenic common carp progeny was biased towards femaleness, 3.0♀:1.0, compared to that of the non-transgenic F1 transgenic common carp progeny, 1.3♀: 1.0 ♂ . Genetic, environmental or epigenetic effects resulted in a population that was gravitating towards sonless. Theoretically, this approach or result might be used for long-term population control.

Acknowledgments

I would like to express my deep appreciation to my advisor Dr. Rex Dunham for his patience, encouragement and valuable suggestions throughout my entire study and research at Auburn University. I would also like to thank my committee members, Dr. Allen Davis and Dr. Charles Chen, for their continued support and guidance. I offer my sincere appreciation for the learning opportunities provided by my committee.

I would like to thank CSIRO Australia, who supported this research. I would like to thank Dr. Baofeng Su for his advice and assistance in this project. My gratitude is also extended to all the faculty and staff of the School of Fisheries, Aquaculture and Aquatic Sciences, and all the teachers who have taught me since I came to Auburn. I would like to thank Mrs. Chia-Chen Weng for her support and assistance during the project. I would like to express my sincere gratitude to all my fellow colleagues at the genetics station, Dr. Zhenkui Qin, Dr. Ahmed Alsasqufi, Mr. Ahmed Elaswad, Mr. Zhi Ye, Mr. Hanbo Li, Ms. Qi Cheng, Ms. Guyu Qin, Ms. Sheng Dong, Mr. Ramjie Yusop Odin, and Ms. Nermeen Youssef. Without your help and friendship, I would not have been able to complete my study and research at Auburn University.

Finally, I would like to express my deepest and sincerest gratitude to my dear parents, Yinfa Zhang and Hong Ji, my fiancée Yang Xu, and all my family members for their care, love, support, and encouragement throughout my study.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BW   Body Weight

CPE   Carp Pituitary Extract

CSD   Chromosome Sex Determination

ESD   Environmental Sex Determination

GSD   Genetic Sex Determination

IAS   Invasive Alien Species

MS-222   Tricaine Methanesulfonate

PSD   Polygenic Sex Determination

RIDL   Release of Insects carrying a Dominant Lethal

ShRNAi   Short Hairpin RNA Interference

SIT   Sterile Insect Technique

SVCV   Spring Viremia of Carp Virus

TSD   Temperature-dependent Sex Determination

tTA   Tetracycline-controlled Transcriptional Activation

MT   α-methyltestosterone

Introduction

"The nature of the biological world is very different from what it was before the age of exploration," Mooney, (2005). With the development of human transportation, most species travel across the natural barriers such as mountains and oceans by hitchhiking (Mooney, 2005). Moreover, even before the age of exploration, humans were the main vectors for dispersal of large organisms, which results in the introduction of other species (Davis, 2009). In the recent years, the migration of species is increasing dramatically (Mooney, 2005), and as more species find new habitats, a small amount of them show invasive characteristics and become invasive alien species (IAS). IAS are species that are introduced accidently or purposefully outside their natural geographic range, and their impacts on the local economy, environment or human health are often considered negative. They usually threaten local species by competing for natural resources.

The Burmese python (*Python bivittatus*), is an example of an invasive species that damages the local ecosystem in South Florida, killing many small mammals in the Everglades National Park (Dorcas et al., 2012). Other examples of detrimental invasive species include the lionfish (*Pterois volitans*), in the Caribbean Sea, western Atlantic Ocean and the Gulf of Mexico, which is decimating local fish and coral reefs (Ricardo et al., 2011), and the giant African snail (*Achatina achatina*) which carries parasites that can cause meningitis and harms to more than five hundreds species of plants in the United States (Thiengo et al., 2007). Asian carps are

predicted to destroy a $7 billion sport-fishing industry if they establish themselves in the Great Lakes (Cooke et al., 2010).

The common carp (*Cyprinus Carpio*) is considered Australia's "river rabbits" due to their abundance in some parts of Australia (Thresher, 2014a). They are native to Asia and Europe, and the fossil record for the family cyprinids dates back to the Eocene age (58-37 million years ago) proving that they originated in Asia, and then spread to Europe (Koehn et al., 2000). Common carp have been reared in China for around 3000 years (Li and Moyle, 1993) and Europe since the first century AD, they were first introduced to Australia from Victoria in 1859 and New South Wales in 1865 (Koehn et al., 2000). Due to the gold rush in 1851, Victoria became a self-governing colony with huge numbers of Europeans immigrating. Between 1851 and 1861, the residents desired recreational fishing of common carp as was the European custom (Gillbank, 1996). Hence common carp were released into ponds, but did not spread into the wild (Koehn et al., 2000). Since then, more common carp were imported to Australia (Clements 1988, Hume et al. 1983).

Common carp did not establish in the wild until they were released into the Murray River near Mildura, Victoria from a fish farm at Boolarra, Victoria in 1964 that had initiated carp aquaculture in the late 1950s (Koehn et al., 2000). In the next few years, due to its high reproduction rate and strong adaptability, the numbers of common carp increased rapidly. This process was accelerated by large floods in 1974 and 1975 in the Murray-Darling Basin. The common carp were considered to have become an established species in Victoria by 1976

2

(Wharton, 1978). Now common carp are the most abundant freshwater fish in the Murray–

Darling Basin and are the dominant species in many fish communities in south-eastern Australia
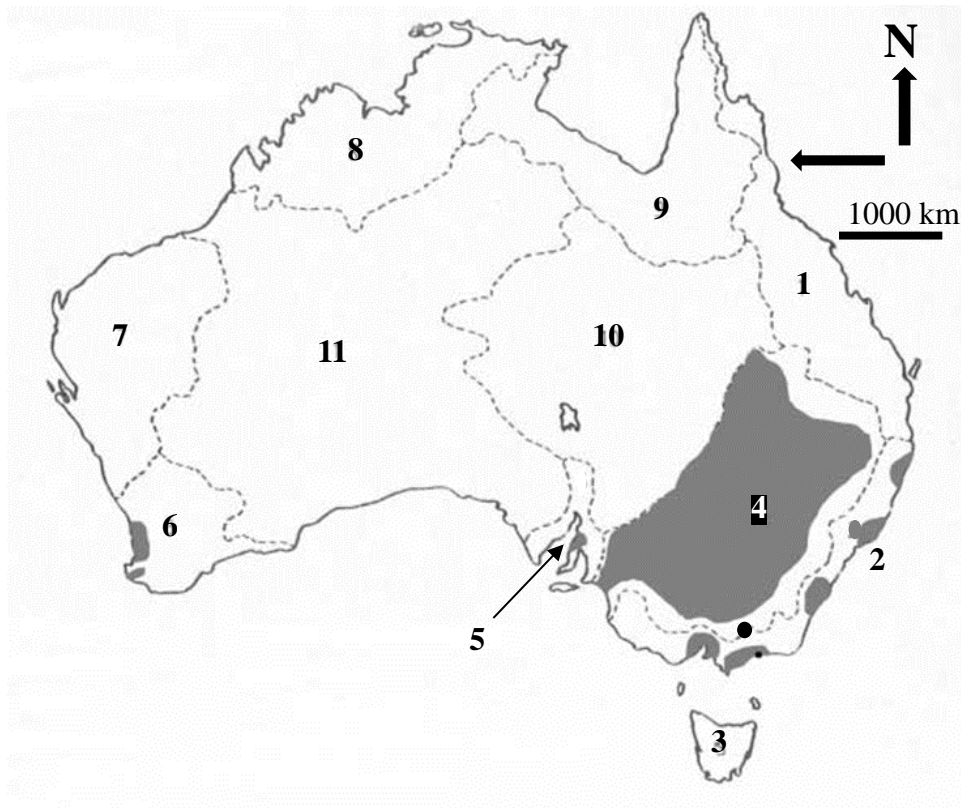
(Koehn et al., 2000). (Fig.1 Koehn, 2004)

Fig 1. Distribution of common carp (*Cyprinus carpio*) (shaded areas) across the major drainage divisions of the Australian continent(after Allen *et al.*, 2002) 1, North-east coast (indicates Burdekin River); 2, South-east coast; 3, Tasmania; 4, Murray–Darling Basin; 5, South Australian gulf; 6, South-west coast; 7, Indian Ocean; 8, Timor Sea; 9, Gulf of Carpentaria; 10, Lake Eyre/Bulloo-Bancannia; 11, Western Plateau, "●" indicates the original introduction site for the 'Boolarra' strain.

Common carp changed the balance of the environment in the Murray-Darling Basin.

They uproot vegetation and stir up sediments, leading to water turbidity, but some researchers

state that common carp do not have a direct relationship with high turbidity and the amount of aquatic vegetation (Fletcher et al., 1985). However, the experiments (Roberts and McCorkelle, 1995) in western New South Wales in the ponds showed that a high stocking density of common carp increases the turbidity of ponds dramatically, and two of five aquatic plants in the ponds were completely lost in one week. Supporting research indicates that aquatic plants are affected by common carp, as the biomass and density of aquatic plants were reduced by common carp feeding habits (Winfield and Townsend 1991). Common carp are bottom feeders that suck small food items from the bottom substrate (Koehn et al., 2000), and fish are not considered as a component of their diets (Sibbing, 1988), however, there is the possibility that they may consume attached fish eggs (Koehn et al., 2000), which may cause harm to other fish species when the density of common carp is large.

Strong adaptability makes common carp an aggressive invasive species in many areas of the world (Koehn, 2004, Lever, 1996). They have become the most notorious fish species for Australians since they conquered the Murray-Darling Basin, and they are included in the list of world's 100 worst invasive species (Roberts & Tilzey, 1996, Lowe et al., 2000). The traditional physical methods for common carp control involves harvesting(capture and removal), angling, draining and drying waterbodies, separating, stocking native fish, which are all time-consuming and have low efficiency considering the high reproduction rate of common carp and the fact that they are mobile (Roberts & Tilzey, 1996, Koehn et al., 2000). There are also biological ways to control the common carp, such as releasing an exotic predator, parasite, and pathogen, which are not widely used as it is difficult to find appropriate agents (Thresher et al., 2014b). Spring

viremia of carp virus (SVCV) was recommended, but the recommendation rescinded because this virus was not species specific (Crane and Eaton, 1997). Another virus, Koi Herpes Virus, is under trial in Australia (Thresher et al., 2014b). Chemicals such as rotenone, endosulfan, antimycin and acrolein can be used to reduce common carp numbers, but it is difficult to determine the appropriate amount for use and there are potential adverse environmental effects when there is large scale application (Sanger and Koehn, 1996).

Progress has been made in controlling invasive pests with environmental and biological techniques, but there are risks and shortcomings when applying these methods (Simberloff & Stiling, 1996). Recent developments in genetics technology provide more options to target certain species and control invasive pests.

Insects pests can be reduced by using lethal gene constructs, causing the deaths of insects in specific developmental stages (Gong et al., 2005, Thomas et al., 2000, Phuc et al., 2007, Horn and Wimmer, 2003). Recombinant DNA and molecular genetics technology can overcome the deficiencies of the original Sterile Insect Technique (SIT), which used irradiation to sterilize males. The disadvantages of this system included negative effects on viability and sperm quality of the insects, reducing fitness of the individuals, which was caused by large doses of radiation (Gong et al., 2005, Horn and Wimmer, 2003, Pedigo, 1996).

Currently, the release of insects carrying a dominant lethal (RIDL) to control insects has been demonstrated using multiple systems (Thomas et al., 2000, Phuc et al., 2007). A sex-specific promoter or enhancer was used in one system, and the other one was non-sex-specific (Thomas et al., 2000). Tetracycline-controlled transcriptional activation method, either Tet-on or

5

Tet-off was used to repress the system to maintain viable lines (Gossen and Bujard, 1992, Gossen et al., 1995).

Experiments were conducted with *Drosophila melanogaster* and with medflies (Gong et al., 2005, Thomas et al., 2000). Female specific promoters were utilized and were linked to cytoxic or lethal genes, for instance, a cell lethal gene which works on the photoreceptors of the compound eyes (Thomas et al, 2000, St Johnston, D., 2002) or tTA which was lethal due to squelching transcription of other genes (Gong et al, 2005). Transgenic females died without tetracycline, whereas, transgenic males lived (Gong et al., 2005, Thomas et al., 2000). In the absence of tetracycline, accumulated tTA level led transgenic tTA Mediterranean fruitfly (*Ceratitis capitata*) to die in larval and pupal stages (Gong et al., 2005). Similar lethal systems can also be applied to control mosquito (*Ae. Aegypti*) (Phuc et al., 2007). Eventually, populations can become extinct because of the elimination of females over time.

Similar to the RIDL approach, a potential method to control and eradicate invasive common carp (Thresher, 2007a) in Australia would be to introduce the fish population with sex-ratio distorting constructs that can progressively skew the sex ratio in the population towards a single sex, which results in the extinction of the population (Thresher et al., 2001). This method was called "the Daughterless approach" (Thresher et al., 2001, Thresher, 2007b).

Daughterless constructs have been successfully tested in medaka (*Oryzias latipes*) and zebrafish (*Danio ratio*) (Thresher, et al., 2001; Thresher, 2007b, Thresher, et al., 2014c). The sex reversal construct pDC1 should block aromatase production in the fish resulting in all males.

6

DC1 constructs electroporated into common carp (*Cyprinus carpio*) embryos shifted sex towards maleness (75%) in P1 individuals (Thresher et al. 2014b).

P1 individuals are likely mosaic, the transfer of the gene across the cell membrane is probably not 100% effective and sometimes phenotypic effects are observed without integration as the transgene has a period of survival in the cytoplasm. The objective of this study was to produce an F1 generation of DC1 common carp and determine the effectiveness of this construct for producing all-male progeny. Hypothetically, F1 progeny inheriting the pDC1 gene would be 100% male.

Materials and Methods

2.1 Daughterless carp construct and primer sets

Theoretically, the daughterless carp construct will result in the degradation of native aromatase, which is required to produce estrogen during sexual differentiation. This disruption of aromatase and estrogen production should result in the sex reversal of genetic females into phenotypic males.

The 7.19kb construct was used as backbone (Fig. 2) (designed and constructed by R Gunasekera and J Patil). The 3.1kb aromatase (cOAr) promoter was from the *Cyprinus carpio* genome (GenBank: LN590725.1). The cOAr cDNA (220bp) and its reverse complement was from *Cyprinus carpio* aromatase (cyp19a) mRNA, complete cds (NCBI GenBank: EU375455.1), and used to produce a shRNAi (short hairpin RNA interference) for degradation of aromatase mRNA. Two sets of primers were used to detect transgenes using PCR with one set amplifying the SV40 region and aromatase knockout region, and one set amplifying the promoter region (Table 1.).

Table 1. Primer sequences for detecting pDC1 transgene with PCR for common carp, *Cyprinus carpio.* (The M13 primer amplifies the promoter region, and cAromPro2723 primer amplifies the SV40 region and aromatase knockout region.)

Gene	Forward Sequence	Reverse Sequence	Size(bp)
M13	TGTAAAACGACGGCCAGT	GTTTTAGCACACCACCTT	700
cAromPro2723	AGTGTTAGTTAGTTCGCTTAG	GGAAACAGCTATGACCATG	1324

2.2 P1 transgene identification

P1 pDC1 common carp were produced by electroporation (Thresher et al. 2014c). The

sperms of 169 potential P1 transgenic common carp males were examined for transgenes. The

protocol used to extract genomic DNA from sperm samples was from Cheng et al. (2014) with

some modifications. Two sets of 1.5ml tubes were prepared with 600µl cell lysis buffer (100mM

NaCl, 10mM Tris HCL pH 8.0, 25mM EDTA pH 8.0, 0.5% SDS) plus 3µl Proteinase K

combined with each sperm sample for the 1st set and 600µl 100% isopropanol added to the 2nd
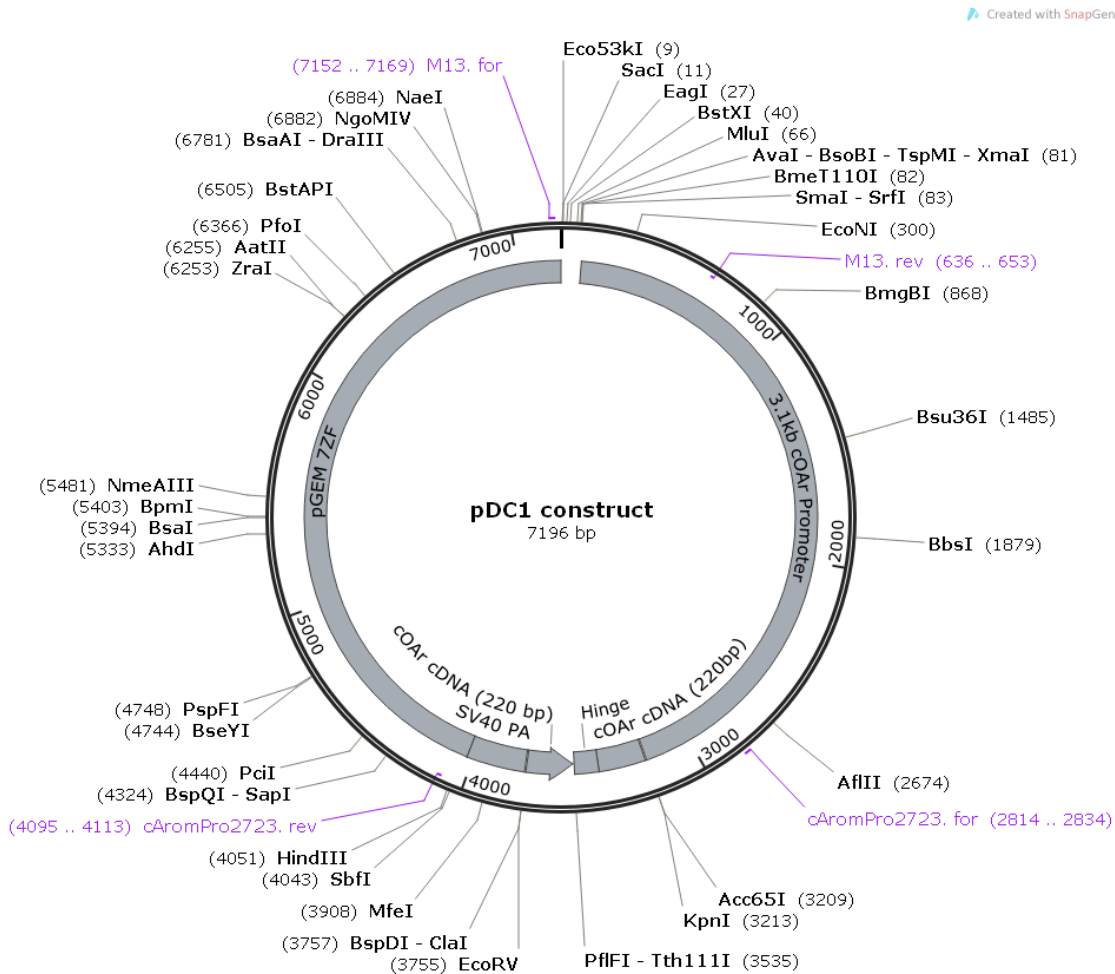
Fig.2 Restriction site map for the Daughterless Carp (pDC1) construct containing the common carp aromatase promoter and shRNAi targeting common carp aromatase.

set for later use. The solutions of the first set of tubes were mixed by inverting tubes 25 times

and vortexing for 10 seconds, followed by incubation in a water bath at 55°C for three hours or

overnight until samples were dissolved. Tubes were then vortexed for an additional 20 seconds,

and returned to the water bath and cooled to room temperature after incubation. Two-hundred µl

of protein precipitation solution (350ml, Qiagen cat# 158912) was added to each tube, and then

the samples were vortexed vigorously for 20 seconds and placed on ice for 15 minutes. Then, the

samples were centrifuged at 15,000 rpm for 10 minutes at 4°C. If there was little or no pellet in

the tube, the vortex-ice-centrifuge steps were repeated to obtain a tight protein pellet. The

supernatant of cell pellet of the first set of tubes was poured to the corresponding labeled second

set of tubes and mixed by inverting gently 50 times. The second set of tubes was centrifuged at

15,000 rpm for 5 minutes at 4°C. Small white DNA pellets were at the bottom of the tubes. The

supernatant was decanted, 600µl of 70% ethanol was added to each tube to wash the pellet by

inverting the tubes several times, and then the solution centrifuged at 16,000 rpm for 2 minutes at

4°C. The ethanol was carefully decanted to avoid disruption of the pellet. The tubes were gently

inverted on paper towels for 30 minutes to air dry the pellet. Then, 20-200µl of sterilized water

was added to dissolve the pellet. DNA samples were incubated overnight in the refrigerator and

the concentration measured using a NanoDrop (UV-Vis spectrophotometer). All the samples had

an A260/280 ratio greater than 1.9, and were diluted to 800 ng/µl. DNA samples were run on

1.0% agarose gel with ethidium bromide (1-2µl of EtBr/100ml gel) to test the DNA quality. PCR

was performed after the quality test with denaturation at 94°C for 5 minutes, then 38 cycles of 94°C for 45s, 58°C for 30s, 72°C for 55s, and the final elongation for 10 minutes at 72°C.

2.3 Production of F1 pDC1 common carp

A total of 24 pDC1 P1 males with transgenic sperm were harvested and acclimated to 26°C in the upstream portion of a holding tank. Three non-transgenic males were placed in the upstream portion of a second tank. One non-transgenic female was placed separately and downstream of the control male, and two non-transgenic females were stocked downstream of the transgenic males. The three females were injected with carp pituitary extract (CPE) with a priming dose of 0.4mg/kg body weight (BW) followed by a resolving dose of 3.6 mg/kg BW 12 hours after the first injection.

Females began to ovulate 14 hrs later. They were anesthetized with 100ppm buffered tricaine methanesulfonate (MS-222), and their eggs stripped into dry, greased small pans. Eggs were divided into multiple pans to allow embryos from each male to be kept separate. Males were anesthetized; their sperm stripped onto individual pans of eggs with most males mated with 2 females, and 25-28°C water added to accomplish fertilization.

Eggs were placed in two replicate 4L-tubs with Holtfreter's solution, 3.5g NaCl, 0.2g NaHCO$_3$, 0.05g KCl, 333µl(300g in 500ml H$_2$O) MgSO$_4$, 333µl (150g in 500ml H$_2$O) CaCl$_2$, in 1.0L dechlorinated water with pH between7.0 and 7.5 for incubation. Embryos were gently agitated with aeration. Dead embryos were removed and the Holtfreter's solution was changed

daily. Treated one part of the embryos with 2ml 10ppm doxycycline, and treated the other part with 0.1ml formalin solution.

2.4 Hatching and Feeding

Embryos hatched in 3 days and the water temperature was 26°C-28°C. Larvae absorbed their yolk sac in one day and then were fed artemia (Brine Shrimp Eggs, 15oz, Carolina Biological) three times a day. At one month of age, the fry were fed Purina® AquaMax® Fry Powder two times per day. Purina® AquaMax® Fry Starter 100 was used as the fry grew larger. Each family was then transferred to three 60L aquaria in a recirculating system and fed Purina® AquaMax® Fry Starter 200 and 300 one or two times a day.

2.5 F1 transgenic fish and gender

Eight fry per family were pooled for DNA extraction one week after hatching. The protocol was the same for analyzing the sperm with little modification.

When fish were one-year-old, surgery was conducted to detect the development status of the gonads and sex of the fish. Scissors, knives, tweezers, povidone iodine10%, 70% ethanol, Vet Cassette Polyamid suture, suture needles, laboratory gloves, a bag of 1.5ml tubes, cotton balls, white towels, 100ppm buffered MS-222, a bucket of water and a hatching trough with water temperature around 26°C were prepared before surgery. Each fish was anesthetized, the area for the incision disinfected using iodine, the incision made and a sample of gonadal tissue excised. After taking samples, fish were sutured, disinfected again with iodine and placed back

12

in tanks for recovery. Samples were placed on ice and then used for DNA extraction. PCR was conducted several times using different primers to detect the presence of the transgene.

2.6 Data Collection and Analysis

Mortality was recorded every day. PCR was conducted several times for each sample using two sets of primers. Microsoft Excel and R were used to organize and analyze data. Chi-squared test of independence ($P<0.05$) and binomial test ($P<0.05$) were conducted in R to test if the fish numbers of different sex was affected by the transgene. Confidence intervals for binomial probabilities were calculated using R.

Results

3.1. Transgenic P1 and F1 common carp

A total of 25% of P1 common carp males were transgenic for pDC1, and 100% of F1 families produced contained transgenic individuals. (Table 2, Fig. 3). A total of 26.8% of the males, 45.5% of the females, and 58.6% of the undetermined sex fish were transgenic in the F1 generation. The sex ratio of the non-transgenic common carp F1 progeny was 1.3♀: 1.0♂, while the sex ratio for the transgenic common carp F1 progeny was 3.0♀:1.0. (Table 3)

The pDC1 transgene affected sex ratio. The percentage of females was greater for the transgenic population compared to the non-transgenic full-siblings ($P<2.2e-16$, Fig.4).

Table 2. Percentage (%) of transgenic P1 male individuals and transgenic F1 families for the sex-ratio biasing gene construct pDC1 in common carp, *Cyprinus carpio*.

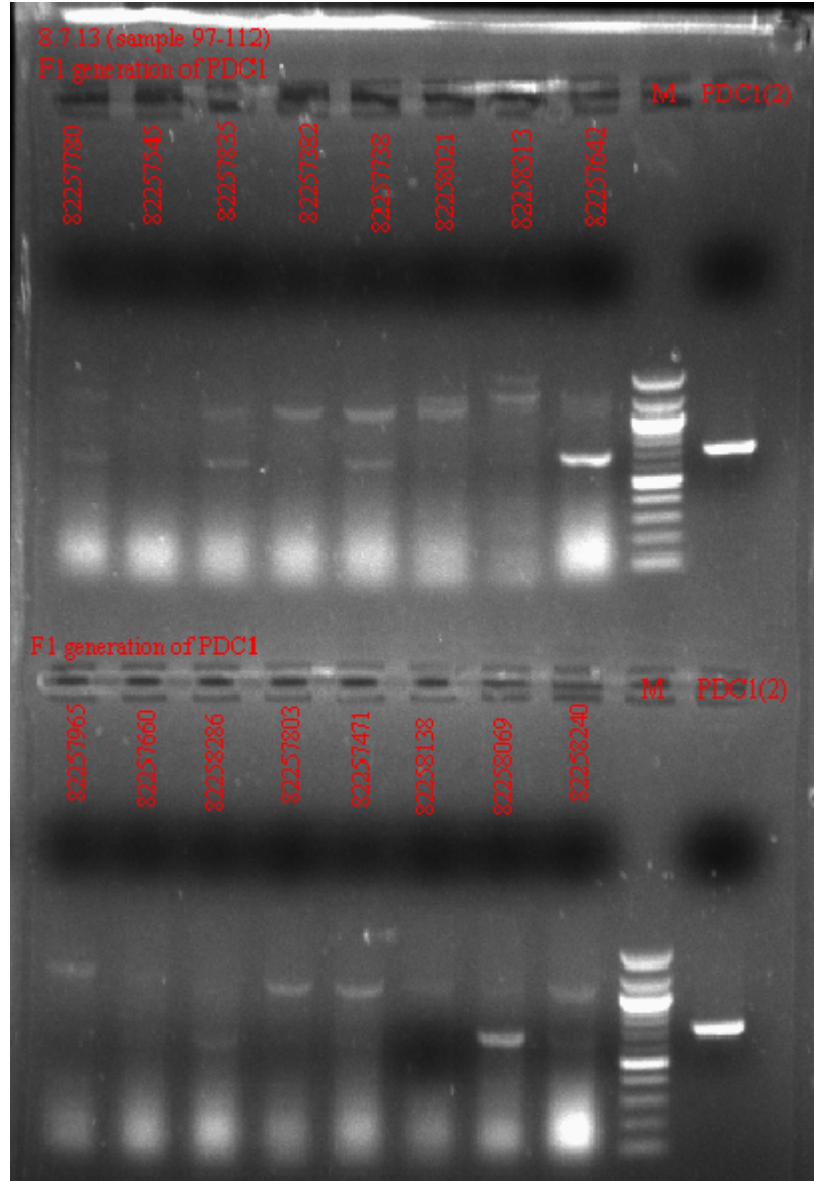| Gene constructs	P1 males			F1 families		
	NT	T	% Transgenic	NT	T	% Transgenic
pDC1	107	42	25	0	42	100

Fig 3.   PCR of results for putative F1 pDC1 common carp, *Cyprinus carpio*, fry. On the far right is the positive transgene control. Top row, positive families are found in lanes 1, 3, 5 and 8 starting on the left, and in the bottom row, positive families are found in lane 3 and 7.

Table 3. Sex ratios of F1 transgenic (T) (pDC1) and non-transgenic (NT) full-sibling common carp, *Cyprinus carpio.* Transgenic common carp had a greater percentage of females than non-transgenic common carp (chi-squared test of independence and binomial test; P<2.2e-16).

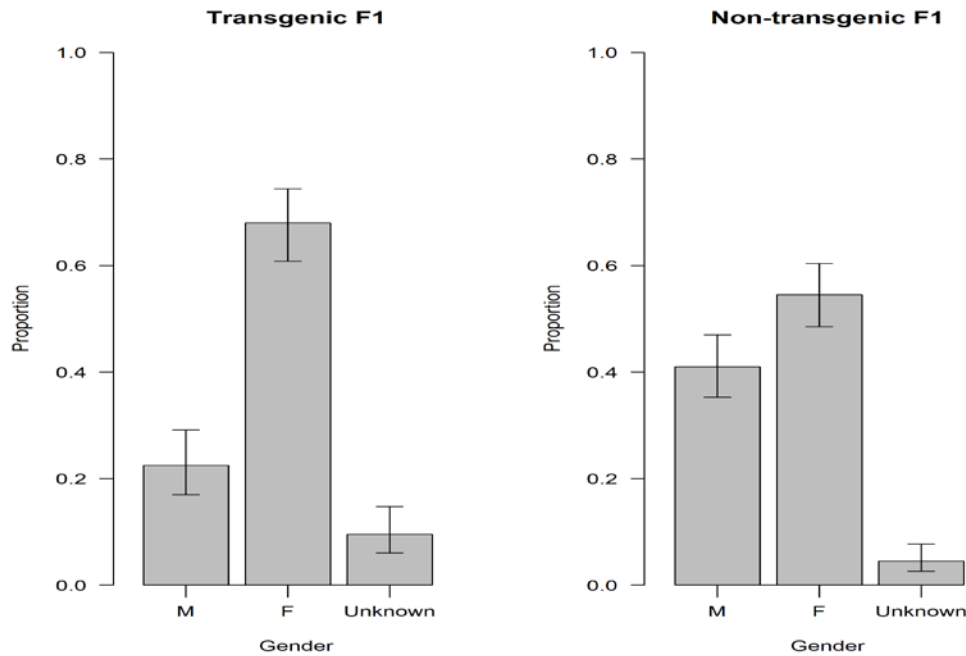|	T	NT	%T
Male	40	109	26.8
Female	121	145	45.5
Unknown	17	12	58.6
Sex Ratio	3.0♀:1.0♂	1.3♀: 1.0♂	

Fig.4. Comparison of gender proportions of pDC1 (shRNAi targeting the aromatase gene driven by the aromatase promoter) transgenic and non-transgenic common carp, *Cyprinus carpio*, from 42 families.

Discussion

The construct pDC1, which was designed to disrupt aromatase expression needed to produce females, was introduced into common carp. A large percentage of pDC1, female transgenic common carp transgenic were present in the F1, which was opposite of expectations. Skewed sex ratios can be influenced by environmental, genetic, behavioral, and physiological factors (Devlin and Nagahama, 2002, Stelkens and Wedekind, 2010, Budd et al. 2015).

Several mechanisms are used for fish to control sex differentiation (Devlin and Nagahama, 2002). The sex determination process is a complicated process for which each step may vary among populations, such as Japanese flounder *Paralichthys olivaceus* and channel catfish *Ictalurus punctatus* (Devlin and Nagahama, 2002, Budd et al., 2015, Kitano et al., 1999).

Two primary types of genetic sex determination (GSD), chromosome sex determination (CSD) and polygenic sex determination (PSD) have been elucidated in fish (Budd et al., 2015). In the case of CSD, sex-related genes are located on specific chromosomes (Budd et al., 2015). In fish, amphibians and reptiles, both male (XX/XY) and female (ZZ/ZW) heterogamety exists (Budd et al., 2015, Stelkens and Wedekind, 2010, Devlin and Nagahama, 2002). The common carp has a CSD system with XX/XY (Nagy et al., 1984). In the case of PSD, sex-related genes are found on more than one chromosome set (Budd et al., 2015, Stelkens and Wedekind, 2010, Devlin and Nagahama, 2002), and the genetic sex is determined by multiple loci (Stelkens and Wedekind, 2010, Bull, 1983). Among all the fish species evaluated, only 7% have differentiated sex chromosomes (Budd et al., 2015, Penman and Piferrer, 2008, Oliveira et al., 2009), which means only 7% are truly GSD, others are possible PSD. Only a few experiments have

demonstrated the existence of PSD in fish (Kosswig, 1964, Liew and Orbán, 2014, Vandeputte et al., 2007).

In genetically male cells, the aromatase gene is silent, while in genetically female cells, the aromatase gene is activated (Devlin and Nagahama, 2002). Cytochrome P450 aromatase, which is encoded by *cyp19* gene (Simpson, 1994), is vital for female fish to convert androgens to estrogens, thus have a large influence on gonadal sex differentiation (Guiguen et al., 2010, Barney et al., 2008). Two different *cyp19* genes, which can encode two different aromatase isoforms (*cyp19a* and *cyp19b*), were discovered in many teleost fish, including common carp (Cheshenko et al., 2008, Barney et al., 2008). In the common carp, both isoforms are expressed in the ovary and brain of adult fish, while *cyp19a* is mainly expressed in the ovary and *cyp19b* is mainly expressed in the brain (Barney et al., 2008). Damages from exogenous sources such as chemicals and radiation and intrinsic sources of errors in DNA repair may disable the function of adjacent genes (Devlin and Nagahama, 2002). For example, the harmful recessive mutations on sex-related chromosomes in PSD systems may have little effect on sex determination, as long as undamaged homologous chromosomes still participate in sex determination (Devlin and Nagahama, 2002). However, as in some cases only few chromosomes are sex-related or even single chromosomes, the genetic effects of mutation on individual chromosomes cannot be excluded (Devlin and Nagahama, 2002). When the sex-related chromosome (W or Y) is in the heterozygous state (ZW or XY), and does not have a homologous chromosome, the recessive genetic mutations will be accumulated and affect the phenotype (Devlin and Nagahama, 2002).

Knowledge of epigenetics, studying of changes in gene function that cannot be explained by changes in DNA sequence, is developing rapidly (Russo et al., 1996, Piferrer, 2013). Epigenetics may be a key mechanism for investigating the interactions between environmental factors and genomic information and the influences on sex determination and differentiation process (Piferrer, 2013, Shao et al., 2014, Budd et al., 2015).

For true environmental sex determination (ESD) mechanism, sex is not determined mainly by genes, but the environment (Budd et al., 2015). The most common environmental factor is temperature and is widely a sex determining mechanism in reptiles (Janzen and Paukstis, 1991, Valenzuela and Lance, 2004, Stelkens and Wedekind, 2010). Temperature can affect aromatase activity and estradiol synthesis in females, and steroid receptors in both females and males (Crews and Bergeron, 1994, Crews, 1996, Devlin and Nagahama, 2002). This particular temperature-dependent sex determination (TSD) also exists in fish like Atlantic silverside (*Menidia menidia*) (Conover and Kynard, 1981) and pejerrey (*Odontesthes bonariensis*) (Karube et al., 2007). In many fish species, temperature treatment can shift the sex ratio to produce more females or males in the population, regardless of TSD or GSD being affected by environmental factors (Ospina-Alvarez and Piferrer, 2008). For instance, temperature treatments on European seabass (*Dicentrarchus labrax*) of 20°C can produce a 73% male population and treatment of 15°C can produce a 77% female population (Socorro et al., 2007). Moreover, with increasing temperatures, aromatase mRNA levels and estradiol levels decrease in Nile tilapia (*O. niloticus*) and Japanese flounder (*P. olivaceus*) (Kitano et al., 1999, D'Cotta et al., 2001).

Other environmental factors include exogenous hormones, behavioral interactions, etc. (Devlin and Nagahama, 2002). Treatment of fish with exogenous androgen has proven effective for induction of masculinization (Hunter and Donaldson, 1983). Steroid treatment on fish can alter the gonad development in many fish species (Devlin and Nagahama, 2002). Immersion of coho salmon larvae with of 400μg/L estradiol-17P for two hours can produce a population of nearly 97% females (Hunter et al., 1986, Budd et al., 2015). However, treatment of androgenic substances can lead to paradoxical feminization of some fish (Cheshenko et al., 2008). For instance, treating zebrafish with aromatizable androgen α-methyltestosterone (MT) causes feminization instead of masculinization (Fenske & Segner, 2004). Treating channel catfish with non-aromatizable androgen dihydrotestosterone also leads to feminization (Davis et al., 1992). Hermaphroditic fish can change sex because of social interactions when the dominant fish cannot control the population (Devlin and Nagahama, 2002, Budd et al., 2015, Munday et al., 2006, Mitcheson et al., 2008).

In nature, GSD species normally have sex ratios of 1:1, but these sex ratios can be influenced by many factors (Stelkins and Wedekind, 2010). In the fish species with TSD, the frequency of the males is strongly influenced by the variation of the temperature (Devlin and Nagahama, 2002, Baroiller et al. 1999, Stelkins and Wedekind, 2010). In aquaculture, the stocking density of some species can change the pathways of sex differentiation, especially in *Anguillidae* spp., a larger number of males are produced when the stocking density is high (Budd et al., 2015, Davey and Jellyman, 2005). When the culture water is acidic, the sex ratio may bias towards males (Budd et al., 2015, Rubin, 1985). The fitness of the fish can affect the sex ratio

(Stelkins and Wedekind, 2010). For instance, when females are in poor health condition, more females would be produced to maintain a balanced fitness, thus when females are in good condition, more males would be generated (Stelkins and Wedekind, 2010, Komdeur et al., 1997, Nager et al., 1999, Whittingham and Dunn, 2000, Proffitt et al., 2008). In addition, supplementary feedings can bias sex ratios (as the sex ratio is biased towards males (1:2.3) in Kakapo (*Strigops habroptilus*), an endangered parrot species in New Zealand, when supplementary feed is supplied (Clout et al., 2002, Tella., 2001, Stelkins and Wedekind, 2010). Sex allocation theory gives another possible explanation of skewed sex ratio (Stelkins and Wedekind, 2010). When mating with attractive males, females would produce more sons than with less attractive males to to ensure maximized reproductivity (Williams, 1966, Ellegren et al, 1996, Griffith et al., 2003, Røed et al., 2007, Saino et al., 1999, Leitner et al., 2006, López-Rull and Gil, 2009).

The pDC1 transgenic F1 generation had an unexpected skewed sex ratio towards femaleness rather than maleness. Temperature, pH, stocking density and other environmental factors may have a large and unexpected effect during the critical period of sex determination, which even override the influence of the transgene. In this case, the effectiveness of this transgene needs to be considered before application in the field, since the natural environment is more variable than in the laboratory. In the current experiment, the environmental conditions and water quality were maintained with little variability.

The desired action of the construct was to knock out the aromatase expression. One explanation for the contradictory results is that the knockout sequence actually increased the

production of aromatase mRNA, causing the increase in femaleness. There are two isoforms of *cyp19* genes in common carp that are functionally and structurally different (Cheshenko et al., 2008), producing *cyp19a* protein which is 44% different from *cyp19b* protein (Barney et al., 2008). Downregulation of the aromatase gene could lead to a higher expression of *cyp19a*, causing a biased sex ratio (Chang et al., 2005) or if *cyp19a* gene is blocked, *cyp19b* gene may increase its expression and influence sex differentiation (Cheshenko et al., 2008). The expression of aromatase mRNA was not measured or compared between the P1 and F1 generation, but it is likely that the expression levels in these two generations were different. Also, the transgenic gene expression can be affected by the chromosome position and almost half of insertions cannot express properly (Day et al., 2000). It is possible that the promising result of P1 generation may have resulted from influences of non-integrated plasmids in the cytoplasm, and the biased sex ratio of F1 generation was caused by the influences of negative insertion locations.

Theoretically and initially, an extremely high copy number of the transgene would be in the cytoplasm of the developing embryo in the P1 generation. These copies should degrade over time and be gone by the time the common carp reach the size of sexual differentiation and presumably the excess mRNA has degraded by that time point as well. In the F1 generation , the copy number would be initially lower, but theoretically, would be higher than the P1 at the time of sexual differentiation. Once integrated, three-dimensional changes in the chromosome are key for proper expression (Ma et al., 2016). Additionally, it has been recently discovered that N^1-methyladenosine mRNA has widespread epitranscriptomic effects on mammalian gene expression (Hoemes et al., 2016), and perhaps similar mechanisms exist in fish that could affect

transgene expression. Thus, these various factors could lead to differences in electroporated P1 individuals compared to F1 integrated transgenic progeny.

Although the results were opposite of expectations, they still might be useful for achieving the overall goal of controlling and eradicating nuisance species. If sex ratios can be highly skewed towards femaleness or matings result in all-female progeny, a lack of males could still result in reduced population numbers.

Reference

Allen, G. R., Midgley, S. H., & Allen, M. (2002). *Field guide to the freshwater fishes of Australia*: Western Australian Museum.

Barney, M. L., Patil, J. G., Gunasekera, R. M., & Carter, C. G. (2008). Distinct cytochrome P450 aromatase isoforms in the common carp (Cyprinus carpio): sexual dimorphism and onset of ontogenic expression.*General and comparative endocrinology*, *156*(3), 499-508.

Baroiller, J. F., Guiguen, Y., & Fostier, A. (1999). Endocrine and environmental aspects of sex differentiation in fish. *Cellular and Molecular Life Sciences CMLS*, *55*(6-7), 910-931. doi:10.1007/s000180050344

Betancur-R, R., Hines, A., Acero P, A., Ortí, G., Wilbur, A. E., & Freshwater, D. W. (2011). Reconstructing the lionfish invasion: insights into Greater Caribbean biogeography. *Journal of Biogeography, 38*(7), 1281-1293. doi:10.1111/j.1365-2699.2011.02496.x

Budd, A. M., Banh, Q. Q., Domingos, J. A., & Jerry, D. R. (2015). Sex control in fish: approaches, challenges and opportunities for aquaculture. *Journal of Marine Science and Engineering*, *3*(2), 329-355.

Bull, J. J. (1983). *Evolution of sex determining mechanisms*. The Benjamin/Cummings Publishing Company, Inc..

Chang, X., Kobayashi, T., Senthilkumaran, B., Kobayashi-Kajura, H., Sudhakumari, C. C., &
Nagahama, Y. (2005). Two types of aromatase with different encoding genes, tissue
distribution and developmental expression in Nile tilapia (Oreochromis niloticus). *General
and Comparative Endocrinology, 141*(2), 101-115.
doi:http://dx.doi.org/10.1016/j.ygcen.2004.11.020

Cheng, Q., Su, B., Qin, Z., Weng, C. C., Yin, F., Zhou, Y., Fobes, M., Perera, D. A., Shang, M.,
Soller, Fabio., & Shi, Z. (2014). Interaction of diet and the masou salmon Δ5-desaturase
transgene on Δ6-desaturase and stearoyl-CoA desaturase gene expression and N-3 fatty acid
level in common carp (Cyprinus carpio). *Transgenic research*,*23*(5), 729-742.
doi:10.1007/s11248-014-9812-1

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., & Eggen, R. I. L. (2008). Interference of endocrine
disrupting chemicals with aromatase CYP19 expression or activity, and consequences for
reproduction of teleost fish. *General and Comparative Endocrinology, 155*(1), 31-62.
doi:http://dx.doi.org/10.1016/j.ygcen.2007.03.005

Clements, J. (1988). *Salmon at the antipodes: a history and review of trout, salmon and char and
introduced coarse fish in Australasia*. John Clements.

Clout, M. N., Elliott, G. P., & Robertson, B. C. (2002). Effects of supplementary feeding on the
offspring sex ratio of kakapo: a dilemma for the conservation of a polygynous parrot.
Biological Conservation, 107(1), 13-18.
doi:http://dx.doi.org/10.1016/S0006-3207(01)00267-1

Conover, D. O., & Kynard, B. E. (1981). Environmental Sex Determination: Interaction of

    Temperature and Genotype in a Fish. *Science, 213*(4507), 577-579.

    doi:10.1126/science.213.4507.577

Cooke, S. L., & Hill, W. R. (2010). Can filter-feeding Asian carp invade the Laurentian Great

    Lakes? A bioenergetic modelling exercise. *Freshwater Biology*, *55*(10), 2138-2

Crane, M. S., & Eaton, B. T. (1996). Spring Viraemia of Carp Virus (Rhabdovirus carpio): a

    biological control agent?. *Controlling Carp*, *22*, 87.

Crews, D. (1996). Temperature-Dependent Sex Determination: The Interplay of Steroid Hormones

    and Temperature. *Zoological Science, 13*(1), 1-13. doi:10.2108/zsj.13.1

Crews, D., & Bergeron, J. M. (1994). Role of reductase and aromatase in sex determination in the

    red-eared slider (Trachemys scripta), a turtle with temperature-dependent sex

    determination. *Journal of Endocrinology*, *143*(2), 279-289.

Davey, A. J. H., & Jellyman, D. J. (2005). Sex Determination in Freshwater Eels and Management

    Options for Manipulation of Sex. *Reviews in Fish Biology and Fisheries, 15*(1), 37-52.

    doi:10.1007/s11160-005-7431-x

Davis, K. B., Goudie, C. A., Simco, B. A., Tiersch, T. R., & Carmichael, G. J. (1992). Influence of

    dihydrotestosterone on sex determination in channel catfish and blue catfish: Period of

    developmental sensitivity. *General and Comparative Endocrinology, 86*(1), 147-151.

    doi:http://dx.doi.org/10.1016/0016-6480(92)90136-8

Davis, M. A. (2009). *Invasion biology*. Oxford University Press on Demand.

Day, C. D., Lee, E., Kobayashi, J., Holappa, L. D., Albert, H., & Ow, D. W. (2000). Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. *Genes & development*, *14*(22), 2869-2880.

D'Cotta, H., Fostier, A., Guiguen, Y., Govoroun, M., & Baroiller, J.-F. (2001). Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia Oreochromis niloticus. *Molecular Reproduction and Development, 59*(3), 265-276. doi:10.1002/mrd.1031

De Mitcheson, Y. S., & Liu, M. (2008). Functional hermaphroditism in teleosts. *Fish and Fisheries, 9*(1), 1-43. doi:10.1111/j.1467-2979.2007.00266.x

Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture, 208*(3–4), 191-364. doi:http://dx.doi.org/10.1016/S0044-8486(02)00057-1

Dorcas, M. E., Willson, J. D., Reed, R. N., Snow, R. W., Rochford, M. R., Miller, M. A., Meshaka, W. E., Andreadis, P. T., Mazzotti, F. J., Romagosa, C. M., & Hart, K. M. (2012). Severe mammal declines coincide with proliferation of invasive Burmese pythons in Everglades National Park. *Proceedings of the National Academy of Sciences*, *109*(7), 2418-2422. doi:10.1073/pnas.1115226109

Ellegren, H., Gustafsson, L., & Sheldon, B. C. (1996). Sex ratio adjustment in relation to paternal attractiveness in a wild bird population. *Proceedings of the National Academy of Sciences*, *93*(21), 11723-11728.

Fenske, M., & Segner, H. (2004). Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). *Aquatic Toxicology, 67*(2), 105-126. doi:http://dx.doi.org/10.1016/j.aquatox.2003.10.008

Fletcher, A., Morison, A., & Hume, D. (1985). Effects of carp, Cyprinus carpio., on communities of aquatic vegetation and turbidity of waterbodies in the lower Goulburn River basin. *Marine and Freshwater Research, 36*(3), 311-327. doi:http://dx.doi.org/10.1071/MF9850311

Gillbank, L. (1996). A tale of two animals: alpaca and camel: zoological shaping of Mueller's Botanic Gardens. *Victorian Historical Journal*, *67*(1), 83.

Gong, P., Epton, M. J., Fu, G., Scaife, S., Hiscox, A., Condon, K. C., Condon, G. C., Morrison, N. I., Kelly, D. W., Dafa'alla, T., Coleman, P. G., & Alphey, L. (2005). A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature biotechnology*, *23*(4), 453-456.

Gossen, M., & Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences*, *89*(12), 5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., & Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. *Science, 268*(5218), 1766-1769. doi:10.1126/science.7792603

Griffith, S. C., Örnborg, J., Russell, A. F., Andersson, S., & Sheldon, B. C. (2003). Correlations between ultraviolet coloration, overwinter survival and offspring sex ratio in the blue tit. *Journal of evolutionary biology*, *16*(5), 1045-1054.

Guiguen, Y., Fostier, A., Piferrer, F., & Chang, C. F. (2010). Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *Gen Comp Endocrinol, 165*(3), 352-366. doi:10.1016/j.ygcen.2009.03.002

Hoernes TP., Hüttenhofer A., Erlacher MD. (2016). mRNA modifications: Dynamic regulators of gene expression? *RNA Biology*. 2016 Jun 28:0. [Epub ahead of print] doi:10.1080/15476286.2016.1203504

Horn, C., & Wimmer, E. A. (2003). A transgene-based, embryo-specific lethality system for insect pest management. *Nature biotechnology*, *21*(1), 64-70.

Hume, D. J., Fletcher, A. R., & Morison, A. K. (1983). Interspecific hybridization between carp (Cyprinus carpio L.) and golfish (Carassius auratus L.) from Victorian Waters. *Marine and Freshwater Research*, *34*(6), 915-919.

Hunter, G. A., & Donaldson, E. M. (1983). 5 Hormonal Sex Control and its Application to Fish Culture. In D. J. R. a. E. M. D. W.S. Hoar (Ed.), *Fish Physiology* (Vol. Volume 9, Part B, pp. 223-303): Academic Press.

Hunter, G. A., Solar, I. I., Baker, I. J., & Donaldson, E. M. (1986). Feminization of coho salmon (Oncorhynchus kisutch) and chinook salmon (Oncorhynchus tshawytscha) by immersion of alevins in a solution of estradiol-17β. *Aquaculture, 53*(3), 295-302. doi:http://dx.doi.org/10.1016/0044-8486(86)90360-1

Janzen, F. J., & Paukstis, G. L. (1991). Environmental Sex Determination in Reptiles: Ecology, Evolution, and Experimental Design. *The Quarterly Review of Biology, 66*(2), 149-179.

Karube, M., Fernandino, J. I., Strobl-Mazzulla, P., Strüssmann, C. A., Yoshizaki, G., Somoza, G. M., & Patiño, R. (2007). Characterization and expression profile of the ovarian cytochrome P-450 aromatase (cyp19A1) gene during thermolabile sex determination in pejerrey, Odontesthes bonariensis. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *307*(11), 625-636. doi:10.1002/jez.416

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., & Abe, S. I. (1999). Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (Paralichthys olivaceus). *Journal of Molecular Endocrinology*, *23*(2), 167-176.

Koehn, J. D. (2004). Carp (Cyprinus carpio) as a powerful invader in Australian waterways. *Freshwater Biology, 49*(7), 882-894. doi:10.1111/j.1365-2427.2004.01232.x

Koehn, J., Brumley, A., & Gehrke, P. (2000). *Managing the impacts of carp.*

Komdeur, J., Daan, S., Tinbergen, J., & Mateman, C. (1997). Extreme adaptive modification in sex ratio of the Seychelles warbler's eggs. *Nature, 385*(6616), 522-525.

Kosswig, C. (1964). Polygenic sex determination. *Experientia, 20*(4), 190-199.

Leitner, S., Marshall, R. C., Leisler, B., & Catchpole, C. K. (2006). Male Song Quality, Egg Size and Offspring Sex in Captive Canaries (Serinus canaria). *Ethology, 112*(6), 554-563. doi:10.1111/j.1439-0310.2006.01195.x

Lever, C. (1996). *Naturalized fishes of the world*: Academic Press.

Li, H. W., & Moyle, P. B. (1993). Management of introduced fishes. *Inland fisheries management in North America. American Fisheries Society, Bethesda, Maryland*, 287-307.

Liew, W. C., & Orbán, L. (2014). Zebrafish sex: a complicated affair. *Briefings in Functional Genomics, 13*(2), 172-187. doi:10.1093/bfgp/elt041

López-Rull, I., & Gil, D. (2009). Do female spotless starlings Sturnus unicolor adjust maternal investment according to male attractiveness? *Journal of Avian Biology, 40*(3), 254-262. doi:10.1111/j.1600-048X.2009.04553.x

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000). *100 of the world's worst invasive alien species: a selection from the global invasive species database*.

Ma, H., Tu, L.-C., Naseri, A., Huisman, M., Zhang, S., Grunwald, D., & Pederson, T. (2016). Multiplexed labeling of genomic loci with dCas9 and engineered sgRNAs using CRISPRainbow. *Nat Biotech, 34*(5), 528-530. doi:10.1038/nbt.3526

Mooney, H. A. (2005). *Invasive alien species: a new synthesis* (Vol. 63): Island press.

Munday, P. L., Wilson White, J., & Warner, R. R. (2006). A social basis for the development of primary males in a sex-changing fish. *Proceedings of the Royal Society B: Biological Sciences, 273*(1603), 2845-2851. doi:10.1098/rspb.2006.3666

Nager, R. G., Monaghan, P., Griffiths, R., Houston, D. C., & Dawson, R. (1999). Experimental demonstration that offspring sex ratio varies with maternal condition. *Proceedings of the National Academy of Sciences, 96*(2), 570-573.

Nagy, A., Csanyi, V., Bakos, J., & Bercsenyi, M. (1984). Utilization of gynogenesis and sex-reversal in commercial carp breeding: growth of the first gynogenetic hybrids. *Aquacult. Hung*, *4*(7).

Oliveira, C., Foresti, F., & Hilsdorf, A. W. S. (2009). Genetics of neotropical fish: from chromosomes to populations. *Fish Physiology and Biochemistry, 35*(1), 81-100. doi:10.1007/s10695-008-9250-1

Ospina-Álvarez, N., & Piferrer, F. (2008). Temperature-Dependent Sex Determination in Fish
Revisited: Prevalence, a Single Sex Ratio Response Pattern, and Possible Effects of Climate
Change. *PLoS ONE, 3*(7), e2837. doi:10.1371/journal.pone.0002837

Pedigo, L. P. (1996). *Entomology and pest management* (No. Ed. 2). Prentice-Hall Inc..

Penman, D. J., & Piferrer, F. (2008). Fish Gonadogenesis. Part I: Genetic and Environmental
Mechanisms of Sex Determination. *Reviews in Fisheries Science, 16*(sup1), 16-34.
doi:10.1080/10641260802324610

Phuc, H. K., Andreasen, M. H., Burton, R. S., Vass, C., Epton, M. J., Pape, G., Fu, G., Condon, K.
C., Scaife, S., Donnelly, C. A., Coleman, P. G., White-Cooper, H. & Alphey, L. (2007).
Late-acting dominant lethal genetic systems and mosquito control. *BMC biology*, *5(1), 1-11.*
doi:10.1186/1741-7007-5-11

Piferrer, F. (2013). Epigenetics of sex determination and gonadogenesis. *Developmental Dynamics,
242*(4), 360-370. doi:10.1002/dvdy.23924

Proffitt, K. M., Garrott, R. A., & Rotella, J. J. (2008). Variation in Offspring Sex Ratio among
Individual Weddell Seal (Leptonychotes weddellii) Females of Different Quality. *Behavioral
Ecology and Sociobiology, 62*(10), 1679-1687.

Roberts, J., & McCorkelle, G. (1995). *Impact of carp (Cyprinus carpio L.) on channel banks in New
South Wales* (No. 95/41). Consultancy Report.

Roberts, J., & Tilzey, R. (1996). *Controlling carp: exploring the options for Australia.*

Røed, K. H., Holand, Ø., Mysterud, A., Tverdal, A., Kumpula, J., & Nieminen, M. (2007). Male phenotypic quality influences offspring sex ratio in a polygynous ungulate. *Proceedings of the Royal Society B: Biological Sciences, 274*(1610), 727-733. doi:10.1098/rspb.2006.0214

Rubin, D. A. (1985). Effect of ph on Sex Ratio in Cichlids and a Poecilliid (Teleostei). *Copeia, 1985*(1), 233-235. doi:10.2307/1444818

Russo, V. E., Martienssen, R. A., & Riggs, A. D. (1996). *Epigenetic mechanisms of gene regulation*: Cold Spring Harbor Laboratory Press.

Saino, N., Ellegren, H., & MØLler, A. P. (1999). No evidence for adjustment of sex allocation in relation to paternal ornamentation and paternity in barn swallows. *Molecular Ecology, 8*(3), 399-406. doi:10.1046/j.1365-294X.1999.00574.x

Sanger, A. C., & Koehn, J. D. (1996). *Use of chemicals for carp control.* Paper presented at the Proceedings of the Workshop on Controlling Carp: Exploring the Options for Australia.

Shao, C., Li, Q., Chen, S., Zhang, P., Lian, J., Hu, Q., Sun, B., Jin, L., Liu, S., Wang, Z., Zhao, H., Jin, Z., Liang, Z., Li, Y., Zheng, Q., Zhang, Y., Wang, J., & Zhang, G. (2014). Epigenetic modification and inheritance in sexual reversal of fish. *Genome research*, *24*(4), 604-615.

Sibbing, F. A. (1988). Specializations and limitations in the utilization of food resources by the carp, Cyprinus carpio: a study of oral food processing. *Environmental Biology of Fishes, 22*(3), 161-178. doi:10.1007/bf00005379

Simberloff, D., & Stiling, P. (1996). How Risky is Biological Control? *Ecology, 77*(7), 1965-1974. doi:10.2307/2265693

Simpson, E. (1994). Aromatase Cytochrome-P450-Structure, Function and Regulation. In *Faseb Journal* (Vol. 8, No. 7, pp. A1241-A1241). 9650 Rockville Pike, Bethesda, MD 20814-3998 USA: Federation Amer Soc Exp Biol.

Socorro, S., Martins, R. S., Deloffre, L., Mylonas, C. C., & Canario, A. V. M. (2007). A cDNA for European sea bass (Dicentrachus labrax) 11β-hydroxylase: Gene expression during the thermosensitive period and gonadogenesis. *General and Comparative Endocrinology, 150*(1), 164-173. doi:http://dx.doi.org/10.1016/j.ygcen.2006.07.018

Stelkens, R. B., & Wedekind, C. (2010). Environmental sex reversal, Trojan sex genes, and sex ratio adjustment: conditions and population consequences. *Molecular Ecology, 19*(4), 627-646. doi:10.1111/j.1365-294X.2010.04526.x

Tella, J. L. Sex-ratio theory in conservation biology. *Trends in Ecology & Evolution, 16*(2), 76-77. doi:10.1016/S0169-5347(00)02075-9

Thiengo, S. C., Faraco, F. A., Salgado, N. C., Cowie, R. H., & Fernandez, M. A. (2007). Rapid spread of an invasive snail in South America: the giant African snail, Achatina fulica, in Brasil. *Biological Invasions, 9*(6), 693-702. doi:10.1007/s10530-006-9069-6

Thomas, D. D., Donnelly, C. A., Wood, R. J., & Alphey, L. S. (2000). Insect Population Control Using a Dominant, Repressible, Lethal Genetic System. *Science, 287*(5462), 2474-2476. doi:10.1126/science.287.5462.2474

Thresher, R. (2014a). Male-only gene trick could leave invasive fish species floundering. *ECOS*, *2014*(195).

Thresher, R. E. (2007a). Genetic options for the control of invasive vertebrate pests: prospects and constraints.

Thresher, R. E., Hayes, K., Bax, N. J., Teem, J., Benfey, T. J., & Gould, F. (2014b). Genetic control of invasive fish: technological options and its role in integrated pest management. Biological invasions, 16(6), 1201-1216. doi:10.1007/s10530-013-0477-0

Thresher, R., Grewe, P., Patil, J., & Hinds, L. (2001). *U.S. Patent Application 10/398,482*.

Thresher, R., van de Kamp, J., Campbell, G., Grewe, P., Canning, M., Barney, M., Bax, N. J., Dunham, R., Su, B., & Fulton, W. (2014c). Sex-ratio-biasing constructs for the control of invasive lower vertebrates. *Nature biotechnology*, *32*(5), 424-427. doi:10.1038/nbt.2903

Thresher, R.E. (2007b). Daughterless technology – Genetic approaches to controlling carp in the M-D Basin. Pages 7-10, In: Lintermans, M. (ed) (2008). *Proceedings of the MDBC Native Fish Strategy Forum 2007*. MDBC pub no 02/08. 29-30 August 2007, Mildura, Victoria, Australia.

Valenzuela, N., & Lance, V. (2004). *Temperature-dependent sex determination in vertebrates*: Smithsonian Books Washington, DC.

Vandeputte, M., Dupont-Nivet, M., Chavanne, H., & Chatain, B. (2007). A polygenic hypothesis for sex determination in the European sea bass Dicentrarchus labrax. *Genetics, 176*(2), 1049-1057. doi:10.1534/genetics.107.072140

Wharton, J. C. F., & Division, V. F. a. W. (1979). *Impact of Exotic Animals, Especially European Carp Cyprinus Carpio, on Native Fauna*: Fisheries and Wildlife Division, Ministry for Conservation.

Whittingham, L. A., & Dunn, P. O. (2000). Offspring sex ratios in tree swallows: females in better condition produce more sons. *Molecular Ecology*, *9*(8), 1123-1129.

Williams GC. (1966) Natural selection costs of reproduction and a refinement of Lack's principle. American Naturalist, 100, 687–690.

Winfield, I. J., & Townsend, C. R. (1991). The role of cyprinids in ecosystems. In I. J. Winfield & J. S. Nelson (Eds.), *Cyprinid Fishes: Systematics, biology and exploitation* (pp. 552-571). Dordrecht: Springer Netherlands.