
THE UNIVERSITY OF ARIZONA
University Libraries

UA CAMPUS
REPOSITORY

The Development of Trojan Sex Chromosome Carrying Green Sunfish Lepomis Cyanellus and Red Shiner Cyprinella Lutrensis to Control Their Nuisance Populations

Item Type	text; Electronic Dissertation
Authors	Teal, Chad
Citation	Teal, Chad. (2022). The Development of Trojan Sex Chromosome Carrying Green Sunfish Lepomis Cyanellus and Red Shiner Cyprinella Lutrensis to Control Their Nuisance Populations (Doctoral dissertation, University of Arizona, Tucson, USA).
Publisher	The University of Arizona.
Rights	Copyright © is held by the author. Digital access to this material is made possible by the University Libraries, University of Arizona. Further transmission, reproduction, presentation (such as public display or performance) of protected items is prohibited except with permission of the author.
Download date	21/08/2023 01:31:02
Item License	http://rightsstatements.org/vocab/InC/1.0/
Link to Item	http://hdl.handle.net/10150/667275

THE DEVELOPMENT OF TROJAN SEX CHROMOSOME CARRYING GREEN SUNFISH *LEPOMIS CYANELLUS* AND RED SHINER *CYPRINELLA LUTRENSIS* TO CONTROL THEIR NUISANCE POPULATIONS

by

Chad Nicolas Teal

Copyright © Chad Nicolas Teal 2022

A Dissertation Submitted to the Faculty of the

SCHOOL OF NATURAL RESOURCES AND THE ENVIRONMENT

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2022

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by: Chad Nicolas Teal, titled: The Development of Trojan Sex Chromosome Carrying Green Sunfish *Lepomis Cyanellus* and Red Shiner *Cyprinella Lutrensis* to Control Their Nuisance Populations

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Scott A. Bonar — Date: 11/14/22

Daniel J. Schill — Date: 11/14/22

Melanie Culver — Date: 11/14/22

Kevin Fitzsimmons — Date: 11/14/22

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Scott A. Bonar — Date: 11/14/22
Dissertation Committee Chair
Wildlife and Fisheries Program, Natural Resources and the Environment

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Scott A Bonar. His confidence in my abilities and consistent encouragement pushed me to confront challenges head-on and emboldened me to conduct technically intimidating research. His guidance was invaluable throughout my time at the University of Arizona. I cannot thank you enough for always being my champion and supporting me through this challenging and rewarding endeavor. I look forward to our continued collaborations.

I want to thank my committee member, Dr. Daniel Schill. Without Dr. Schill's involvement this project would not have produced the extent of crucial information it has. Thank you Dr. Schill for your consistent enthusiasm and for acting as a role model for a creative and passionate scientist. I look forward to continuing my work with you.

I want to thank my committee member, Dr. Melanie Culver. Dr. Culver was boundless in her guidance and support through the genetic aspects of this project. With this acknowledgement, I would also like to thank her incredible lab, Alex Erwin, Karla Vargas, Ellie Wisely, and Natalie Mercer for their patience and time in assisting with my project. Dr. Culver and her lab adopted me as one of their own and taught me more about genetics research than I ever thought I would learn.

I want to thank my committee member, Dr. Kevin Fitzsimmons. Dr. Fitz consistently provided me useful, 10,000 ft, perspectives on my research and shared novel applications for our findings. His technical support also allowed me to challenge my aquaculture skill set by building a massive research system.

I want to thank Katharine Coykendall, Matthew Campbell, Thomas Delomas, and Daniel Eardley with the Dept. of Idaho Fish and Games Eagle Genetics Facility. Their expertise proved essential in the success of this project. Their generosity and patience with me showed me how collaborative research can be so rewarding and fruitful. I am a better scientist because of your guidance through this project.

I want to thank Dr. Susan Fogelson and Elizabeth Mamer for their time and expertise with histology. Dr. Fogelson was a mentor for me during my undergraduate education and collaborating with her throughout this project has led to amazing publications and beautiful histology images!

Thank you to the SNRE professors and administrative staff. Thank you to Dr. Michael Bogan, Dr. Robert Stiedl, Dr. David Walker, and Dr. Peter Reinthal for their incredible instruction, for reviewing my manuscripts, and for vastly improving my research. A special thanks to Dr. Javan Bauder for spending so much of his time going over my data and helping me restructure the way I approach data analysis. Also, a special thanks to USGS AZ Coop Administrator, Ashleigh Grogan. She facilitated my transition into life as a UA PhD student and always made the logistics of my project run smoothly.

I would like to thank James Shira with the University of Arizona Genetics Core. His expertise and innovative thinking vastly improved my research.

I want to thank Heidi Blasius with the Bureau of Land Management and Nate Berg with Arizona Game and Fish Dept. for assistance in broodstock and genetic sample collections. I always have a blast working with you both.

I would like to thank all my research technicians and volunteers: Amy Heiling, Kaitlyn Tyler, Jesus Bustamante, Hunter Henry, Colby Roberts, Kim Smoot, Cora Thomas, Suhailal Hussein, Kylee Rath, Gabriella Buonavolonta, Matthew Murphy, and Stephen Ferrar. These students showed inspiring commitment and grit during this project. Thank you for keeping me smiling and keeping our fish babies alive.

None of this research would have been possible without funding from the U.S. Bureau of Reclamation. Bill Stewart with U.S.B.R. has been extremely dedicated to the success of this project and has worked incessantly to financially support my research. Due to his dedication, the odds of my son growing up to see native fishes in Arizona are much better.

Thank you to my lab members and friends Chris Jenney, Kaitlyn Gahl, Zach Nemec, Taylor Ulrich, Stephen Ingram, Joshua Grant, Erin Tracy, and Emily Freed. I am a more well-rounded scientist due to all of your unique perspectives, expertise, and guidance. You all made this endeavor enjoyable and enriching. I am especially thankful for Chris Jenney for consistently reminding me that I love nature, which is why we devoted our lives to aquatic resource conservation.

A special thank you to my wife, Savannah Rocker. You are the best fish-wife a fish-man could ask for. Thank you for always being interested, or at least faking interest, in my histology slides, my rants about sex determination systems, and my existential crises. You have always given me the strength and confidence to challenge myself. You have sacrificed time with friends, proximity to family, and financial well-being so that I can pursue my passions. Thank you Sweetness. I love you.

I owe all my accomplishments to my parents, Tricia Teal and Mike Teal, and my brother, Kyle, and my sister, Alex. Thank you all for supporting me in so many ways throughout my education. Thank you for instilling in me a drive to follow my interests and passions. Thank you for teaching me environmental stewardship and compassion for wildlife. Who I am and everything I have done is because of your love and support. I am forever grateful. I love you all.

Finally, thank you little Arlo. You are my motivation to protect the beautiful places and critters that make this world magical. This is for you buddy.

LAND ACKNOWLEDGEMENT

We respectfully acknowledge the University of Arizona is on the land and territories of Indigenous peoples. Today, Arizona is home to 22 federally recognized tribes, with Tucson being home to the O'odham and the Yaqui. Committed to diversity and inclusion, the University strives to build sustainable relationships with sovereign Native Nations and Indigenous communities through education offerings, partnerships, and community service.

DEDICATION

I am dedicating my dissertation to the research animals used in this study. I will never know what the experience of being a Red Shiner or Green Sunfish is like, but I hope that under my care and use, the individuals that were a part of my research experienced a life with minimal suffering. It is my hope that this research will allow for humane extirpation of these species' invasive populations. Thank you for your ultimate sacrifice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Green Sunfish *Lepomis cyanellus* and Red Shiner *Cyprinella lutrensis* are considered highly invasive when introduced outside of their native range. Trojan sex chromosome eradication models have shown the release of YY males (or ZZ females) to be effective at suppressing and eradicating invasive populations of species across genera. Developing Trojan sex chromosome carrying individuals requires aquaculture protocols, knowledge about gonadal development, identification of sex determination systems, and sex reversal methods for the species of interest. We have developed aquaculture protocols for Green Sunfish and Red Shiner. Our spawning methods provided year-round volitional spawns from Green Sunfish and Red Shiner broodstock. Our larval rearing methods resulted in consistent year-round production of Green Sunfish and Red Shiner, allowing us to identify the timing of their gonadal differentiation through histological assessment. Green Sunfish and Red Shiner are gonochoristic, with testes and ovaries differentiating directly from undifferentiated gonads. Green Sunfish's gonadal differentiation was observed between 39 days post hatch (dph) – 69 dph. Red Shiner's gonadal differentiation was observed between 45 dph – 105 dph. To investigate these species' sex determination systems, we used restriction-site associated DNA sequencing (RAD-Seq) for SNP discovery and genotyping of known-sex Green Sunfish and Red Shiner DNA samples to search for sex-diagnostic single nucleotide polymorphisms (SNPs) and restriction-site associated sequences present in one sex and absent in the other. In Green Sunfish, the bioinformatic analyses discovered candidate SNPs and sex-specific restriction-site associated sequences that fit patterns of male or female heterogametic sex determination systems. However, when primers were developed and tested for Green Sunfish, no candidates reliably identified phenotypic sex. The top performing Green Sunfish SNP candidate (ZW_218) correlated with phenotypic sex

63.0% of the time and the presence-absence loci universally amplified in both sexes. Genetic investigations that interrogate a larger fraction of the Green Sunfish genome could uncover a reliable sex identification marker. Additionally, studies on environmental influences on Green Sunfish sex determination systems may be necessary since we observed heavily male-skewed sex ratios of control groups (82.61% male) during aquaculture protocol development and sex reversal trials for this species. For Red Shiner, we used RAD-Seq in addition to a series of breeding experiments with sex reversed males (neofemales) to uncover their sex determination system. All candidate sex-linked SNPs that fit our selection criteria exhibited a pattern of male heterogamety. We developed two sex identification (sex-ID) marker assays, XY_248 and XY_170, which showed a phenotype-genotype concordance score of 77.00% and 84.35%, respectively. These sex-ID markers exhibited a relatively high phenotype-genotype concordance in Red Shiner females (XY_248 = 96.30%, XY_170 = 98.61%) which allowed for selective breeding of neofemales. We observed a 3:1 male to female sex ratio in spawns from neofemales and wild-type males, indicative of a male heterogametic sex determination system (i.e., XY-male/XX-female) within this species. For the sex reversal trials, a low-dose (100 E2 mg per kg of diet) and a high-dose (150 E2 mg per kg of diet) experimental E2 treatment were fed to juvenile Green Sunfish from 30 to 90 dph. Both E2 treatments resulted in 100% feminization, with no morphological or histological differences detected between E2 treated ovaries and those from a control group. Overall, there was no effect of E2 on survival ($P = 0.310$) and growth rate data suggested no statistical differences ($P = 0.0805$). However, the growth rate of the high-dose group increased slightly higher after the treatment ended than the other treatments ($P = 0.042$), suggesting that E2 might suppress growth in Green Sunfish. In addition, the control group did not exhibit a lower mortality rate after the treatment period ended ($P = 0.266$), whereas both E2

treated groups did (P = 0.0003–0.0050). We found that the low-dose, 100 E2 mg per kg of diet, was sufficient for fully feminizing Green Sunfish if administered during development from 30 to 90 dph and E2 dosages may result in deleterious effects on Green Sunfish's health and growth. We observed 100% feminization of Red Shiner fed either 50 mg E2 per kg of diet from 2-120 dph or 100 mg E2 per kg of diet from 20-120 dph. Among Red Shiner given the control diet, the 50 mg of E2 per kg of diet, or the 100 mg of E2 per kg of diet from 2-62 dph, the gonadosomatic index and mortality rate were significantly higher in fish given the 100 mg of E2 per kg of diet than the other treatments ($P < 0.01$). Infrequent occurrences of follicular atresia and/or inflammation were found in ovaries of all Red Shiner E2 treatment groups. Differences in mean total lengths and mean total weights among E2 treated groups of Red Shiner and their respective control groups were small and not statistically significant ($P > 0.2163$). Putative YY Red Shiner that resulted from spawning neofemales with wild-type Red Shiner were viable and differences in fecundity among YY individuals and wild-type individuals were insignificant as exhibited by mean egg counts per day when YY individuals were crossed with wild-type counterparts ($P > 0.05$). In the 20 spawns observed from crossing either YY-females with YY-males, YY-males with XX-females, or YY- females with XY-males, all the offspring developed into males except for one female that developed from a possibly inbred cross between a putative YY-daughter and an XY-father. The presence of this female may be indicative of autosomally-derived sex modifying genes that produce female phenotypes due to inbreeding homozygosity. Despite the presence of a female from one YY verification cross, the prevalence of all-male progeny from all other YY crosses suggests that the use of a Trojan sex chromosome eradication strategy will be effective at extirpating nuisance Red Shiner populations.

INTRODUCTION

Competition and predation from nonnative fishes have had substantial impacts on native fish populations in the southwestern United States (Meffe 1985; Clarkson et al. 2005). When quantifying threats to imperiled species in the United States, nonnative species introductions are second only to habitat loss (Wilcove et al. 1998). Traditional removal efforts of nuisance fish populations can be ineffective, costly, and indiscriminate at the species level. Solely relying on mechanical removal of nonnative fish populations has had varied success depending on the targeted species and the size and complexity of the waterbody (Franssen et al. 2014). Chemical removal methods, such as rotenone treatments, are more effective, but impact non-targeted species and are best suited for use in smaller, enclosed water bodies (Britton et al. 2010).

Genetic approaches for pest control are species specific and show potential for being highly effective. The Sterile Insect Technique (SIT) and CRISPR-Cas9 gene drive systems have been successful at suppressing mosquito populations through the release of sterile males or through the spread of female sterility (Wilke and Toledo 2012; Hammond et al. 2016). Skewing sex ratios to the point of extirpation is another genetic approach for population control. Skewing sex ratios of invasive fish populations to extirpate them has been modeled in multiple species (Gutierrez and Teem 2006; Senior et al. 2013; Teem et al. 2014; Schill et al. 2017; McCormick et al. 2021). The daughterless technique skews the sex ratio in a population through the inheritance of an aromatase blocking gene which inhibits the development of females in subsequent progeny (Thresher and Bax 2003; Teem et al. 2014). Although the daughterless technique was modeled to be effective, eradication of a large population using this technique would take many decades (Thresher and Bax 2003; Teem et al. 2014). Another method for skewing sex ratios in a population to the point of extirpation is through the development and

release of Trojan sex chromosome carriers (Guttierez and Teem 2006; Schill et al. 2016). Trojan sex chromosome carriers (TSC) are males or females that are homozygous for the male-coding region(s) of the genome. These TSC carriers have all male progeny when they spawn with wild-type males or females. The continual release of TSC carriers, YY males or ZZ females, shows promise at extirpating nuisance populations and existing models show it could lead to extirpation at least twice as quickly the daughterless strategy (Teem et al. 2014; Schill et al. 2017). Another advantage to using TSC carriers is that unforeseen ecological impacts can be easily reversed by stopping their release into the system (Schill et al. 2017; Beauregard et al. 2020). Trojan sex chromosome carriers are the result of rearranging preexisting sex chromosomes, not the result of gene editing, which means the accidental release of these fish into its native range will not have a deleterious effect on the genetics or health of that population (Cotton and Wedekind 2007).

The basic methods needed for producing TSC carriers (i.e. YY males or ZZ females) have been practiced in aquaculture for decades (Varadaraj and Pandian 1989; Beardmore et al. 2001). In fish with an XY-male/XX-female sex chromosome compliment, production of YY males involves a series of sex reversals and selective breeding. Larval fish are sex reversed during their development (male to female) through exposure to some form of estrogen, usually via immersion or feed. These treated fish are either progeny tested or genetically screened to determine which of the phenotypic females are genetic males (XY-female). The XY-females are crossed with wild-type males which produces three diplotypes; XX, XY, and YY. Half of the larvae from that cross are sex reversed, resulting in some fish to be YY females. These YY females are crossed with untreated YY-males and their progeny are the TSC carriers that will be stocked into nuisance populations. YY brood stock Brook Trout (*Salvelinus fontinalis*) have been produced and the first field trials of YY-male Brook Trout are underway (Schill et al. 2016;

Kennedy et al. 2018; Teem et al. 2020). The production of TSC carriers is also possible if the species has a ZZ-male/ZW-female sex determination system, where the female is the heterogametic sex (Figure 1).

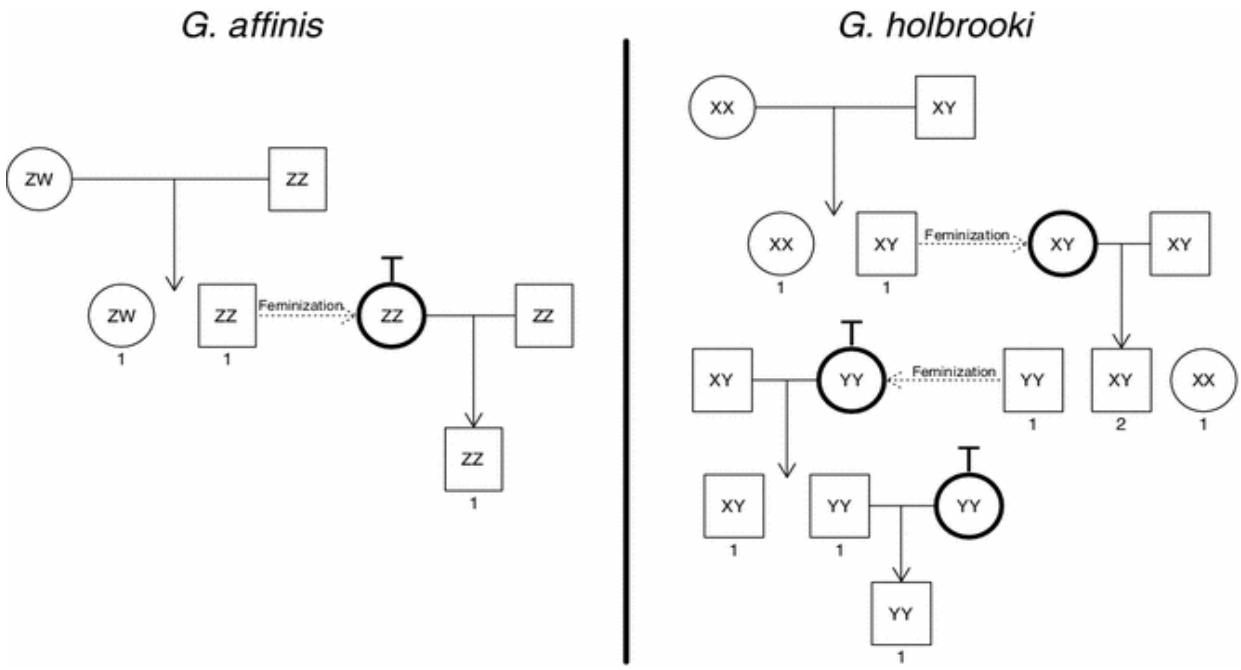

Figure 1. Senior et al. (2013) techniques for producing Trojan sex chromosome carriers, YY males or YY females, in two closely related species of mosquitofish with different sex determination systems. Circles represent phenotypic females and squares represent phenotypic males. In *G. affinis* the ZZ female is the Trojan sex chromosome carrier and in *G. holbrooki* the YY males or YY females are the Trojan sex chromosome carriers.

In the Southwest, Red Shiner (*Cyprinella lutrensis*) have reduced native fish populations through predation on larvae and habitat displacement of other small-bodied cyprinids (Douglas et al. 1995; Gido et al. 1999; Rinne 1991; Ruppert et al. 1993). Green Sunfish (*Lepomis cyanellus*) are another highly invasive species that have been shown to prey on endemic Southwestern

species and reduce recruitment of native species in streams (Dudley and Matter 2000; Lohr and Fausch 1996). Red Shiner and Green Sunfish are among the fastest expanding invaders in the Southwest and are among the most invasive fish species due to their negative impacts on native fish communities (Olden and Poff 2005). Their persistence and fast maturation times makes them appealing candidates for the use of the TSC eradication strategy.

The production of TSC carrying Red Shiner and Green Sunfish requires the following: the development of lab based rearing techniques, the discovery of their sex determination systems, the development of genetic sex identification markers, the discovery of their labile period during sexual development, and the development of an effective sex reversal technique. The methods used to develop TSC carriers in species with none of this prior knowledge available is discussed in the following dissertation composed of six appendices. This dissertation features my efforts at developing TSC carrying Green Sunfish, which are published in various, peer-reviewed, science journals (Appendix A-C), as well as my efforts at developing TSC carrying Red Shiner which are either published (Appendix D), in press (Appendix E), or prepared for (Appendix F) the North American Journal of Aquaculture. Each appendix is prepared and presented in the format appropriate for its respective journal.

REFERENCES

Beardmore, J. A., G. C. Mair, and R. I. Lewis. 2001. Monosex male production in finfish as exemplified by Tilapia: Applications, problems and prospects. Aquaculture 197:283-301.

Beauregard, M.A., Parshad, R.D., Boon, S., Conaway, H., T. Griffin, and J. Lyu. 2020. Optimal control and analysis of a modified trojan Y-Chromosome strategy. Ecological Modelling, 416, p.108854.

Britton, J. R., R.E. Gozlan, and G.H. Copp. 2010. Managing non-native fish in the environment. Fish and Fisheries 12:256–274.

Clarkson, R. W., P. C. Marsh, S. E. Stefferud, and J. A. Stefferud. 2005. Conflicts between native fish and nonnative sport fish management in the southwestern United States. Fisheries 30:20-27.

Cotton, S., and C. Wedekind. 2007. Control of introduced species using Trojan sex chromosomes. Trends in Ecology and Evolution 22:441–443.

Douglas, M. E., P. C. Marsh, and W. L. Minckley., 1994. Indigenous fishes of western North America and the hypothesis of competitive displacement: *Meda fulgida* (Cyprinidae) as a case study. Copeia 9-19.

Dudley, R., and W. Matter. 2000. Effects of small Green Sunfish (*Lepomis cyanellus*) on recruitment of Gila Chub (*Gila intermedia*) in Sabino Creek, Arizona. The Southwestern Naturalist 45:24-29.

Franssen, N.R., Davis, J.E., D.W. Ryden, and K.B. Gido. 2014. Fish community responses to mechanical removal of nonnative fishes in a large southwestern river. Fisheries 39:352-363.

Gido, K., Schaefer, J., Work, K., Lienesch, P., E. Marsh-Matthews, and W. Matthews, 1999. Effects of Red Shiner (*Cyprinella lutrensis*) on Red River Pupfish (*Cyprinodon rubrofluviatilis*). The Southwestern Naturalist 44:287-295.

Gutierrez, J.B., and J.L.Teem. 2006. A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology 241:333-41.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., Gribble, M., Baker, D., Marois, E., Russell, S., Burt, A., Windbichler, N., A. Crisanti, and T. Nolan. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. Nature Biotechnology 34:78–83.

Kennedy, P.A., Meyer, K.A., Schill, D.J., Campbell, M.R., Vu, N.V., 2018. Survival and reproductive success of hatchery YY male Brook Trout stocked in Idaho streams. Transactions of the American Fisheries Society 147:419–430.

Lohr, S., and K. Fausch. 1996. Effects of Green Sunfish (*Lepomis cyanellus*) Predation on Survival and Habitat Use of Plains Killifish (*Fundulus zebrinus*). The Southwestern Naturalist 41:155-160.

McCormick, J.L., D.J. Schill, and K.A. Meyer. 2021. Simulated use of YY male stocking and suppression for eradicating Common Carp populations. North American Journal of Fisheries Management 41:366–382.

Meffe, G. K. 1985. Predation and species replacement in American southwestern fishes: a case study. Southwestern Naturalist 30:173-187.

Olden, J. D. and N.L. Poff. 2005. Long–term trends of native and non–native fish faunas in the American Southwest. Animal Biodiversity and Conservation 28:75–89.

Rinne, J. N. 1991. Habitat use by Spikedace, *Meda fulgida* (Pisces: Cyprinidae) in southwestern streams with reference to probable habitat competition by Red Shiner, *Notropis lutrensis* (Pisces: Cyprinidae). Southwestern Naturalist 36:7-13.

Ruppert, J. B., R. T. Muth, and T. P. Nesler. 1993. Predation on fish larvae by adult Red Shiner, Yampa and Green rivers, Colorado. Southwestern Naturalist 38:397-399.

Schill, D. J., J. A. Heindel, M. R. Campbell, K. A. Meyer, and E. R. J. M. Mamer. 2016. Production of a YY male Brook Trout broodstock for potential eradication of undesired Brook Trout populations. North American Journal of Aquaculture 78:72–83.

Schill, D.J., K.A. Meyer, and J. Hansen. 2017. Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. North American Journal of Fisheries Management 37:1054-1066

Senior, A. M. N., M. Krkosek, and S. Nakagawa. 2013. The practicality of Trojan sex chromosomes as a biological control: An agent based model of two highly invasive Gambusia species. Biological Invasions 15:1765–1782.

Teem, JL., J.B. Gutierrez, J.B., and R. Parshad. 2014. A Comparison of the Trojan Y chromosome and daughterless carp eradication strategies.
Biological Invasions 16:1217-1230

Teem, J., Alphey, L., Descamps, S., Edgington, M., Edwards, O., N. Gemmell, and A. Roberts. 2020. Genetic biocontrol for invasive species. Frontiers in Bioengineering and Biotechnology 8, 1–18.

Thresher, R.E. and N. Bax. 2003. The science of producing daughterless technology; possibilities for population control using daughterless technology and maximizing the impact of carp control. In: Proceedings of the National Carp Control Workshop (ed. K. Lapidge). Cooperative Research Centre for Pest Animal Control, Canberra, Australia, pp. 19–24.

Varadaraj, K. and T. J. Pandian. 1989. First report on production of supermale Tilapia by integrating endocrine sex reversal with gynogenetic technique. Current Science 58:434-441.

Wilcove, D. S., D. Rothstein, J. Dubow, A. Phillips, and E. Losos. 1998. Quantifying threats to imperiled species in the United States. Bioscience 48:607-615.

Wilke, A.B.B., and M.T. Marrell. 2012. Genetic Control of Mosquitoes: population suppression strategies. Revista do Instituto de Medicina Tropical de São Paulo, 54:287-292.

APPENDIX A

DEVELOPMENT OF AQUACULTURE PROTOCOLS AND GONADAL DIFFERENTIATION OF GREEN SUNFISH (*LEPOMIS CYANELLUS*)

Aquaculture 547 (2022) 737515

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Development of aquaculture protocols and gonadal differentiation of green sunfish (*Lepomis cyanellus*)

Chad N. Teal [a,*], Daniel J. Schill [b], Susan B. Fogelson [c], Colby M. Roberts [d], Kevin Fitzsimmons [e], Scott A. Bonar [f]

[a] Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2 Rm N315, 1064 E. Lowell St., Tucson, AZ 85721, USA
[b] Fisheries Management Solutions, Inc., 9950 W. Prairie Road, Boise, ID 83714, USA
[c] Fishhead Labs, LLC, 5658 SE Pine Ave, Stuart, FL 34997, USA
[d] University of Arizona, School of Natural Resources and the Environment, 1064 E Lowell St., Tucson, AZ 85721, USA
[e] University of Arizona, Department of Environmental Science, Forbes 306, 1140 E. South Campus Drive, Tucson, AZ 85719. USA
[f] U.S. Geological Survey, Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2 Rm N315, 1064 E. Lowell St., Tucson, AZ 85721, USA

ARTICLE INFO

Keywords:
Lepomis cyanellus
Aquaculture
Gonadal development
Sex differentiation
Larval rearing

ABSTRACT

We provide detailed rearing methods and describe green sunfish (*Lepomis cyanellus*) gonadal development and histological differentiation for both sexes. Developing in-depth aquaculture protocols and describing the gonadal differentiation of green sunfish could facilitate strategies to control nuisance populations, enhance stocking programs, and provide information for this species' use in bioassay trials or toxicology studies. Our methods resulted in consistent year-round production of green sunfish and allowed us to identify the timing of their gonadal differentiation through histological assessment. Our spawning methods provided year-round volitional spawns from green sunfish broodstock. Our rearing methods involved weaning larval green sunfish off live nauplii and onto only artificial diets by 37 days post-hatch (dph). Most of the offspring generation reached sexual maturity by 213 dph. Green sunfish are gonochoristic, with testes and ovaries differentiating directly from undifferentiated gonads. Ovaries begin to differentiate by 39 dph and testes begin to differentiate by 69 dph. This information can provide biologists consistent means to produce this Centrachid and understand their gonadal development.

1. Introduction

Green sunfish (*L. cyanellus*) is a North American Centrarchid species native to the Great Lakes, Hudson Bay and the Mississippi River basins (Fuller et al., 2021). Green sunfish are considered invasive in other areas of the world (Yun-Chang et al., 2008) and have been found in most U.S. states outside of their native range (Lemly, 1985; Dudley and Matter, 2000; Fuller et al., 2021). Their widespread distribution can be attributed to aquaculture practices, stocking as game fish, and releases as forage fish for largemouth bass (*Micropterus salmoides*) (Welcomme, 1988; Halos et al., 2004). Green sunfish have been implicated in the decline of many native aquatic species, including: Gila chub (*Gila intermedia*) and Chiricahua leopard frog (*Rana chiricahuensis*) in Arizona (Rosen et al., 1995; Dudley and Matter, 2000), California roach (*Hesperoleucus symmetricus*) and Ranid frogs in California (Moyle, 1976; Hayes and Jennings, 1986), and multiple native fish populations in North Carolina (Lee et al., 1980). Their aggressive behavior also negatively impacts sport fisheries (McKechnie and Tharratt, 1966). For example, their presence has been shown to cause stunting and competition with more desirable game fish, such as bluegill *Lepomis macrochirus* (Werner and Hall, 1977). Conversely, green sunfish are a desirable bait for catfish (Brunson and Morris, 2000) and Green Sunfish x Bluegill hybrid fisheries have been developed because this hybrid is a fast growing and aggressive sportfish (Lewis and Heidinger, 1971; Brunson and Robinnette, 1985).

Controlling invasive green sunfish populations is challenging and

* Corresponding author.
E-mail addresses: chadteal@email.arizona.edu (C.N. Teal), dschill@fishmgmtsolns.com (D.J. Schill), susan.fogelson@fishheadlabs.com (S.B. Fogelson), kevfitz@ag.arizona.edu (K. Fitzsimmons), sbonar@ag.arizona.edu (S.A. Bonar).

https://doi.org/10.1016/j.aquaculture.2021.737515
Received 12 July 2021; Received in revised form 20 September 2021; Accepted 22 September 2021
Available online 27 September 2021
0044-8486/© 2021 Elsevier B.V. All rights reserved.

successful removals have required extensive efforts (Reinthal et al., 2020), sometimes coupled with indiscriminate methods such as piscicide treatments (Blasius, 2002; Ward et al., 2015). Genetic biocontrol methods, such as the Trojan sex chromosome strategy (TSC), are species-specific population control strategies that were modeled to effectively suppress or extirpate nuisance populations of various fishes (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2017; Teem et al., 2020; McCormick et al., 2021). The TSC eradication strategy works through the continuous release of YY males (males with two Y chromosomes instead of an XY chromosome complement) or ZZ females (sex reversed males in a ZZ-male/ZW-female sex determined species) into a nuisance population. When these TSC carrying fish spawn with wild-type females the resulting progeny are 100% male. With significant introductions of TSC carriers, the sex-ratio of the population can theoretically be skewed towards all male resulting in a population decline and, ultimately, extirpation (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2017; Teem et al., 2020).

As with any eradication strategy, understanding the basic biology of the invasive species is critical for success (Kolar et al., 2010). An initial assessment of the feasibility of using the TSC strategy for green sunfish requires identifying this species' sex determination system. Male heterogametic sex determination systems (XY-male/XX-female) and female heterogametic sex determination systems (ZZ-male/ZW-female) have been discovered in fishes by spawning sex-reversed individuals with wild-type individuals and then assessing the sex ratios of the progeny (Desprez et al., 1995; Gomelsky et al., 2002). These methods require effective sex-reversal treatments and aquaculture practices.

Fish are most susceptible to sex reversal by the administration of exogenous steroids if steroidal treatments are applied prior to gonadal differentiation and finish when gonadal differentiation is first observable through histology (Hackmann and Reinboth, 1974; Piferrer, 2001). A prior study investigated gonadal differentiation of green sunfish, sampling gonads on a monthly time-scale and consolidating 2–3 month old green sunfish into one group for analysis (Yun-Chang et al., 2008). Although Yun-Chang et al. (2008) research generally described gonadal development in green sunfish, it did so with relatively coarse temporal resolution and lacked a report of the percentage of the cohort that had undergone gonadal differentiation in each age sampled. Knowing the percentage of a cohort that is undifferentiated at each age, coupled with more frequent sampling of gonads and length-at-age data, is essential for designing a steroid treatment regimen that could result in 100% sex reversal (Malison et al., 1986; Gao et al., 2009).

Sex-reversal treatments and overall aquaculture operations can benefit from having aquaculture protocols that transition fish onto artificial diets early in their development. Weaning fish onto artificial diets can facilitate the administration of exogenous steroids (Yamamoto, 1963). In addition, use of artificial diets is less labor intensive than live-feeds and can reduce transmission of parasites and disease derived from live-feed sources (Støttrup, 2003; Gugliandolo et al., 2008; Dhont et al., 2013). Although methods are available for weaning the closely related Bluegill onto artificial feeds for sex-reversal treatments (Wang et al., 2008), green sunfish and bluegill prefer different foods (Werner and Hall, 1977). Our pilot studies showed that bluegill rearing methods proved ineffective for weaning green sunfish onto artificial diets without resultant high mortality rates.

Green sunfish aquaculture protocols and detailing the gonadal differentiation of this species are not just necessary for examining its potential use in a TSC strategy, but can also facilitate their use in toxicology studies (Uguz, 2008) and bioassay trials (Hunn et al., 1968; Carr, 1976; Adams and Johnsen, 1986). This information can also aid managers interested in producing and stocking sunfish hybrids (Mischke et al., 2007) or green sunfish mono-sex cultures (Al-Ablani, 1997). The objectives of this study were to 1) develop and report aquaculture protocols for green sunfish that resulted in consistent spawns and featured early weaning onto artificial diets, and 2) identify the timing and pattern of green sunfish gonadal differentiation. This research will facilitate the initial consideration of a TSC eradication strategy for green sunfish and help with laboratory and hatchery operations using green sunfish for bioassay trials, toxicology studies, or stocking programs.

2. Methods

2.1. Broodstock collection and stocking

On May 22, 2018 and May 21, 2019, we collected 25 and 37 adult green sunfish, respectively, by boat electrofishing from Parker Canyon Lake, Arizona, USA (GPS coordinates 31°25′37.0“ N, 110°27’25.0” W). During transit to the University of Arizona Fisheries Propagation Laboratory, fish were given a one-hour prophylactic treatment with a mixture of 0.05 ppm malachite green and 15 ppm formalin (ProForm C, Koi Care Kennel, Utah, USA). Water used at the UA Fisheries Propagation Laboratory was chlorinated well water that we dechlorinated with 25 ppm of sodium thiosulfate before use in the laboratory's aquaculture systems. Fish were acclimated to quarantine tanks by removing half of the water in the transport coolers and then slowly filling the coolers with quarantine tank water via 4.8-mm diameter airline tubing until the coolers were full. For quarantining, fish were divided randomly into three roughly even groups and stocked in two 437 L aquariums equipped with a Fluval FX6 filter (Rolf C. Hagen Inc., Baie-D'Urfe, Canada) and one 1135.62 L Rubbermaid tank (Rubbermaid, Georgia, U.S.A.) with an AquaClear 110 Power Filter (Hagen, Massachusetts, U.S.A.). The quarantine tanks were bare-bottom and contained large plastic plants for cover. Fish were kept in these tanks for two weeks before being sexed and stocked in broodstock tanks. During those two weeks, fish were fed thawed, previously frozen, mysis shrimp (~6 shrimp/fish) once a day.

After two weeks in quarantine, fish were sexed by applying pressure to the ventral surface of the abdomen to express gametes. Fish that did not express gametes were sedated for 3–5 min using 50 ppm of MS 222 buffered with 150 ppm of sodium bicarbonate. Once sedated, a plastic catheter of 0.97 mm ID × 1.27 mm OD was inserted into the urogenital opening to sample gametes (Brunson and Morris, 2000). Fish of known sex were selected as broodstock and were stocked in three 473 L glass aquariums each containing two 300-W Jager EHEIM drop-in heaters (EHEIM GmbH & Co, Deizisau, Germany) and two AquaClear 110 filters. These broodstock tanks had bare glass bottoms. Each tank was divided into three sections with removable screen partitions. Each section of the tank contained four plastic plants for cover and an artificial nest constructed from a terra cotta drain plate that was 30.5 cm in diameter x 3.8 cm deep filled with pea gravel (particle size 5–15 mm diameter). Each section of the tank contained one male and one female for a total of three males and three females in each tank. Water quality in broodstock tanks was maintained within these parameters: water temperature 23–27 °C, ammonia <0.5 ppm, nitrite <1.0 ppm, and pH 8.0–8.4. For the first two weeks in the broodstock tanks, fish were fed thawed shrimp once a day. Broodstock were transitioned onto Skretting (Nutreco, Amersfoort, Netherlands) Classic Brood 5-mm pellet (46% crude protein, 12% crude fat) over a two-week period. The broodstock's transition from shrimp onto the pellet diet was accomplished by reducing the number of shrimp fed to each tank by half over the course of a week and supplementing with pellets ad libitum. By the end of the second week all tanks were fed only pellets ad libitum. The appropriate rate to feed ad libitum was based on how much the fish could eat in two minutes before the diet settled on the bottom of the tank. Broodstock were fed once a day. The green sunfish broodstock mean total length (TL) was 153.6 mm (SD 47.2 mm).

2.2. Broodstock spawning

Changes in photoperiod and temperature were conducted to induce spawning in the broodstock tanks (Kaya and Hasler, 1972; Smith, 1975; Dupree and Hunter, 1984; Bryan et al., 1994). Over a 2-week period, fish were transitioned from a 12-h photoperiod to an 18-h photoperiod by

increasing the light-on time by 25 min each day and temperature was increased from 23 °C to 29 °C by increasing the temperature ~ 1 °C every two days. After the broodstock were transitioned to an increased photoperiod and temperature, artificial nests were checked for eggs and larvae once a day for four weeks. After four weeks with no spawns, different sex ratios were attempted in each tank section (1:2 male to female, 2:2 male to female, 2:3 male to female) to induce spawns (Smith, 1975; Bryan et al., 1994) and reduce the number of injuries and mortalities occurring with females. The three sex ratios were attempted for four weeks unless patterns of injury or mortalities were observed before the trial period ended. No changes were made to the temperature and photoperiod while the three different sex ratios were attempted. Finally, all screen partitions were removed from tanks and lower stocking densities of one male and two females, two males and two females, and two males and three females per tank were attempted to provide more room for males to perform courtship behavior (Bryan et al., 1994). These lower stocking densities with the different sex ratios were attempted for four weeks.

After four months of unsuccessful spawning trials in the 473 L aquariums, two recirculating aquaculture systems were constructed to facilitate the male's circular courtship behaviors and reduce stress on females. Each system was composed of two 360 L round polyethylene tanks (99.06 cm inside diameter x 50.80 cm depth) connected by 4.83-cm (outside diameter) PVC pipe to a 189 L sump filled with biomedia and a split return for each tank using two submersible magnetic drive pumps (Danner Model 7, Danner Manufacturing Inc., New York, U.S.A.), a 40 W Lifegard UV sterilizer (Lifegard Aquatics, California, U.S.A.) and a ¼ hp. Coralife chiller (Central Aquatics, Wisconsin, U.S.A.). Each of the four broodstock tanks contained two artificial nests with a different design than previously used. These artificial nests were 19 L buckets (27.94 cm inside diameter) that were cut to a 7–10 cm depth and filled with pea gravel (particle size 5–15 mm diameter) (Mischke and Morris, 1997). Two males and three females were transferred to each of the four broodstock tanks. Using methods of Mischke and Morris (1997) except utilizing only ambient light, the broodstock tanks' water temperatures and photoperiods were then manipulated to mimic an artificial winter (~15 °C water temperature with 8 h of light) and returned to an artificial summer (27 °C water temperature and 16 h of light) over six-weeks to induce spawning. Despite courtship behavior and nest guarding by the males, no spawns occurred during the next five months, and traumatic injuries were noted on the females. Even though there already were three large plastic aquatic plants in each tank for cover, a circular laundry basket (50.80 cm inside diameter x 38.10 cm depth) with two ~12.70 cm square holes cut in the sides were placed upside-down in each tank to provide the females more cover and to reduce conspecific aggression. The artificial nests were on the outside of the upside-down laundry baskets. One month after the addition of the laundry baskets, the fish began to spawn regularly, and the artificial nests were checked for eggs once a day for a year.

2.3. Hatching, larval rearing, and growout of offspring generation

Nests with eggs were placed in a 37.9 L plastic tub and given a 30 min 100-ppm formalin bath. Nests were then placed in one of four larval rearing tanks consisting of 37.9 L plastic tubs each outfitted with a 50 W Jager EHEIM drop-in heater, air stone, and QANVEE Bio Sponge filter (Taian Qanvee Aquarium Equipment Co., Ltd., Shandong, China). Each larval rearing tank was randomly assigned one artificial nest with eggs from each broodstock tank resulting in a total of four replicates undergoing the following larval rearing treatment. For the first 30 days of rearing, tank temperatures were maintained between 25 and 28 °C. After 30 days post-hatch (dph), heaters were removed from tanks and water quality parameters were maintained at: temperature 15–24 °C, ammonia <0.25 ppm, nitrite <1.0 ppm, and pH 8.0–8.4. Initially, methods used to wean young bluegill onto artificial diets (Gao et al., 2009) were followed closely. Briefly, the feeding of AP-100 microfeed (Zeigler Bros., INC., Pennsylvania, U.S.A.) starting at 10 dph resulted in nearly 100% mortality and those that survived to 30 dph were stunted. We attribute this to the green sunfish's reluctance to feed on this diet and the concomitant reduction in water quality in the recirculating systems we used.

The failed attempts at rearing green sunfish using artificial diets following the Gao et al. (2009) methods required us to experiment with and develop the following feeding regimen (Table 1). The first feeding was given once yolk sacs were absorbed and larvae started their swim up stage (3–4 dph). Larvae were fed <24-h old brine shrimp nauplii four times per day at a rate of ~125 nauplii/L (estimate based on weight of unhatched cysts and ~ 90% hatching rate). At 25 dph we fed the green sunfish, now post-larvae, nauplii four times a day and began feeding Otohime B1 diet (B1: 200–360 μm, 51% crude protein, 11% crude fat) (Pentair Aquatic Eco-Systems, North Carolina, U.S.A.) twice a day. When juveniles were 30 dph, we fed them nauplii once a day and started feeding B1 diet six times a day using an EHEIM automated fish feeder. At 37 dph, we stopped feeding nauplii and only fed B1 feed 6 times a day. Juvenile green sunfish were transitioned onto Otohime B2 diet (B2: 360–620 μm, 51% crude protein, 11% crude fat) at 100 dph by feeding them half B1 and half B2 for two weeks. This and subsequent transitions in diet were accomplished by mixing the fish's current feed with the next stage of feed. At 122 dph fish were transitioned onto Otohime C1 diet (C1: 580–840 μm, 51% crude protein, 11% crude fat). At 175 dph, fish were transitioned onto Otohime C2 diet (C2: 840–1410 μm, 51% crude protein, 11% crude fat) and were fed six times a day with the automated feeder in addition to being fed ad libitum twice a day. At 285 dph, fish were transitioned onto Skretting 2-mm pellets (46% crude protein, 12% crude fat) and fed this diet ad libitum twice a day for the duration of the rearing trials. The automated feeder administered 66.8 mg (SD = 14.24 mg, n = 10 measurements of diet dispersed), 224.0 mg (SD = 79.89 mg), 390.2 mg (SD = 133.05 mg), 552.2 mg (SD = 245.97 mg) during each feeding for B1, B2, C1, and C2 diets respectively. The estimated feed rates in percent body weight per day were 13%, 6%, and finally 3% for fish 0–30 dph, 31–90 dph, and > 90 dph, respectively.

At 30 dph, all but 50 randomly selected fish were removed from the four larval rearing tanks to reduce crowding during growout. At 285 dph, all surviving fish from each larval rearing tank were transferred to four 757 L round fiberglass tanks that comprised a recirculating aquaculture system (RAS). The RAS was composed of thirty 757 L round fiberglass tanks connected to a filtration system featuring a Lifegard ¾ hp. in-line pump, an Emperor 750 W UV sterilizer (Pentair Aquatic Eco-Systems), a DF-6 Polygeyser bead filter (Aquaculture Systems Technologies, Louisiana, U.S.A.), and a Dayton ½ hp. in-line pump (Dayton Electric Mfg. Co., Illinois, U.S.A.). Aeration was provided to each tank by a blower (WW80 Whitewater, Pentair Aquatic Eco-Systems).

The four tanks were siphoned once a day to remove uneaten food and waste. A 10% water change was performed weekly. Fish mortalities were fixed in 10% neutral buffered formalin and occasionally submitted to the University of Arizona's Aquaculture Pathology Laboratory for investigation. Larval rearing tanks where *Aeromonas* infections persisted

Table A.1.
Diet and feeding regimen for green sunfish during their first year of growth.

DPH	Diet	Quantity
3 (swim up stage) – 25	< 24-h old brine shrimp nauplii	4×/day at ~125 nauplii/L
25–30	Otohime B1 Diet	2×/day at ~66.8 mg
30–37	< 24-h old brine shrimp nauplii	1×/day at ~125 nauplii/L
30–100	Otohime B1 Diet	6×/day at ~66.8 mg
100–122	Otohime B2 Diet	6×/day at ~224.0 mg
122–175	Otohime C1 Diet	6×/day at ~390.2 mg
175–285	Otohime C2 Diet	6×/day at ~552.2 mg + 2×/day ad libitum
285–355	Skretting 2 mm pellet	2×/day ad libitum

after a week of 10–30% daily water changes were given oxytetracycline treated feed (1.12 g oxytetracycline/454 g of diet) for 10 days.

Study fish were periodically measured for TL (mm) and weighed (g) during the rearing period. Between 9 and 22 randomly selected fish from each of the four larval rearing tanks were measured and weighed at 30 dph. At 91 dph, 285 dph, and 355 dph all fish from each of the four larval rearing tanks were measured for TL. Absolute growth rates (AGR) were calculated using the formula $AGR = (TL_2 - TL_1)/T \times 100$. Where TL_1 and TL_2 are the mean fish total lengths at the start and end of the growth period for each of the four larval rearing tanks, and T is the time between measurements. This absolute growth rate formula was adapted from Wang et al. (2008), but we used total lengths instead of weights because weights of fish <30 dph were suspected to be inaccurate due to unstable scale readings. A one-way ANOVA was conducted on the grand mean AGR for each growth period to determine if they differed substantially. A Von Bertalanffy growth curve for fish age 1–155 dph was constructed using FSA package (Ogle, 2016) in R studio. A simple linear regression was used to describe the function of age on the TL in fish aged 1–355 dph.

2.4. Gonadal development and histology

Swim-up larvae were stocked in two tanks with identical configurations as the four 37.9 L larval rearing tanks described above. Stocking densities were determined by counts of swim-up larvae from a sample of 500 mL of water when fish were 5 dph. One tank was stocked at a relatively high density (~20 larvae/L) compared to the other tank (~8 larvae/L). Five fish were collected upon hatching, euthanized by immersion for 10 min in 100 ppm of MS 222 buffered with 150 ppm sodium bicarbonate, and fixed in buffered formalin. Subsequently, five fish were collected and euthanized this way from the high-density tank and the low-density tank every 7 days for the first 30 dph and then every 10 days from 30 dph to 159 dph. In addition, fish were collected at 322 dph from one of the four rearing tanks to ensure gonads from sexually mature fish were sampled. Only abdominal sections or gonads were fixed for histology in fish that were over 18-mm TL. Tissue was fixed at a ratio of 1:10 tissue to 10% neutral buffered formalin. A multiple linear regression with TL as a function of age (dph) and tank density (high vs low) was used to evaluate differences in growth rates between the high-density and low-density larval rearing tanks.

Fixed samples 1 dph – 109 dph were submitted to a commercial laboratory (Animal Reference Pathology, Utah, U.S.A.) for histology processing and hematoxylin and eosin staining. Samples 119–322 dph were submitted to Fishhead Labs (Florida, U.S.A.) for histology processing and hematoxylin and eosin staining. Samples were cut along the sagittal plane to bisect the gonad. Histological sections in fish 109 dph and younger were scanned for digital review by Animal Reference Pathology. In samples between 119 dph and 322 dph histological sections were photomicrographed using an AmScope 40×-2000 × 3 W LED Seidentopf trinocular compound microscope and AmScope 14MP camera (United Scope, LLC, California, U.S.A.).

Gonadal tissue in histological sections were sexed and described using various references (Nakamura et al., 1998; Arezo et al., 2007; Uguz, 2008; Gao et al., 2009; Lowerre-Barbieri et al., 2011; Mazzoni and Quagio-Grassiotto, 2017; van der Ven and Wester, 2021). Sex differentiation of gonadal tissue was described by comparing our histological sections with descriptions and histological images from the references listed above. Cytological comparisons were made with previously described green sunfish gonad differentiation (Yun-Chang et al., 2008) and with bluegill gonad differentiation (Gao et al., 2009). The timing of anatomical differentiation of the gonads could not be compared to bluegill anatomical differentiation because our examination of green sunfish gonads along the sagittal plane, as opposed to the transverse plane, made it difficult to observe certain anatomical changes that may have preceded cytological differentiation (Jensen and Shelton, 1983; Sacobie and Benfey, 2005; Gao et al., 2009).

3. Results

3.1. Broodstock spawning and hatching of offspring generation

After adding the laundry baskets to the broodstock tanks, we observed 27 spawns over a one-year duration. Broodstock spawned throughout the year under the lab's ambient photoperiod and tank temperatures (Table 2). Fish were able to spawn four times in a month, but mean number of days between spawns for each tank was 19 ($n = 4$, SD = 14.15 days, 95% CI = 0 days – 42 days). We observed two spawns within the same week in one broodstock tank.

Eggs hatched 24–36 h post spawn. Larvae were benthic and fed off their yolk-sacs for the first 3–4 days. Swim-up larvae readily consumed <24 h old nauplii.

3.2. Growth rates of offspring generation

Growth rates began to decrease in green sunfish at about 91 dph and a Von Bertalanffy growth curve model suited TL data for fish measured 1–155 dph (Fig. 1). However, mean growth extended to 355 dph (Fig. 2A) exhibited a linear function with age (simple linear regression, $F_{1,18} = 1490$, P value <0.0001). Mean TL (mm) increased by 0.26 (95% CI = 0.244–0.272 mm) with each day (Fig. 2B). Green sunfish mean total lengths were 4.08 mm (SD = 0.04 mm, $n = 4$), 12.11 mm (SD = 1.91 mm, $n = 4$), 26.46 mm (SD = 2.17 mm, $n = 4$), 76.76 mm (SD = 7.51, $n = 4$) and 95.75 mm (SD = 6.38 mm, $n = 4$) at 1, 30, 91, 285 and 355 dph, respectively. Changes in mean AGR during growout were small and insignificant (One-Way ANOVA, $F_{3,12} = 0.43$, P value = 0.735). The AGRs for ages 1–30 dph dph, 30–91 dph, 91–285 dph, and 285–355 dph were 27.69 (95% CI = 17.19–38.19, $n = 4$), 23.45 (95% CI = 15.47–31.44, $n = 4$), 25.91 (95% CI = 21.13–30.69, $n = 4$), and 27.12 (95% CI = 16.24–38.00, $n = 4$). Fish density showed no effect on growth rates between the high-density and low-density larval rearing tanks used for histology (multiple linear regression, $t_{2,126}$ *low density* $\beta = -0.10$, $P = 0.92$). Mature gametes were expressed from most offspring individuals at 213 dph. Volitional spawns of the offspring generation occurred within the first year, but spawns were only detected as larvae and juveniles in the growout system, so no exact date for the onset of spawning can be reported.

3.3. Survival of offspring generation

Cannibalism was observed in the first 30 days of growout. Cannibalistic individuals typically exhibited faster growth rates and were culled once their TL became an outlier among the other larvae (roughly double in TL). *Aeromonas hydrophila* infections were common in larval rearing tanks, especially during the transition from live nauplii onto an

Table A.2.
Conditions present in green sunfish broodstock tanks when spawning began and continued for a year.

Condition	Description
Broodstock tank	360 L, 99.06 cm inside diameter x 50.80 cm depth
Water depth	40.46 cm
Water temperature	23–27 °C
Photoperiod	18 h light, 6 h dark
Filtration	Baffled 189 L sump filled with bioballs, UV sterilizer
Pump and flow rate	Danner Model 7, ~473 L/h
Diet and feeding	Skretting Classic Brood 5-mm pellet ad libitum once a day
In-tank cover	three large plastic aquatic plants (~ 60 cm in length), 1 upside-down laundry basket (50.80 cm inside diameter x 38.10 cm depth) with two ~12.70 cm square holes in the sides
Artificial nests	Two 19 L buckets cut down to 7–10 cm depth filled with pea gravel
Sex ratio	2 males: 3 females

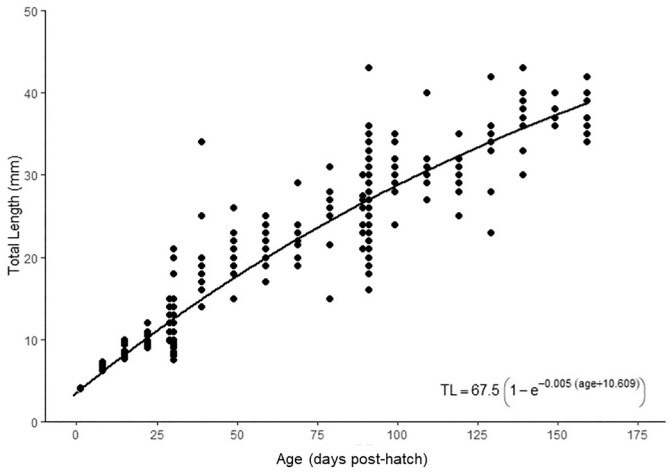

Figure A.1. Von Bertalanffy growth curve for 1–155 dph green sunfish. (For inter- pretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

artificial diet. The infection presented as internal hemorrhaging that could be seen through the skin. This bacterial strain was detected via PCR in the larval and juvenile carcasses submitted to the University of Arizona's Aquaculture Pathology Lab. Oxytetracycline treated diet cured infections and reduced mortalities.

Survival was lowest during the first 30 dph. Since exact counts were not taken of fish younger than 30 dph, precise survival rates for fish less than 30 dph are unknown. By using volume-based tank densities from the high-density and low-density histology tanks, the survival rate from 1 to 30 dph is roughly estimated to range between <10% and up to 69.31%. Mortality rate decreased with age. Mean survival was 49.50% (SD = 23.17%, n = 4), 68.25% (SD = 18.81%, n = 4), 100% (SD = 0%, n = 4) between 30 and 91 dph, 91–285 dph, and 285–355 dph, respectively.

3.4. Gonadal development and histology

Gonads were observable in fish as young as 8 dph (TL = 6.2–7.3 mm). The undifferentiated gonads were observed ventral to the posterior end of the swim bladder and dorsal to the posterior section of the intestine (Fig. 3A). All gonads in fish 8–29 dph (TL = 6.2–15.0 mm) were undifferentiated. Undifferentiated gonads were comprised of primordial germ cells supported by a scant fibrovascular stroma (Fig. 3B). Presumptive ovarian differentiation was first observed at 39 dph (TL = 16–34 mm). The onset of ovarian differentiation (Fig. 4) was subtly characterized by the clustering of oogonia and germ cell meiosis. Oogonia were usually observed alongside various maturation phases of chromatin-nucleolus oocytes (Fig. 4A). Basophilic perinucleolus stage oocytes were observed in females at 49 dph. By 59 dph (TL = 19–23 mm) most females had ovaries predominated by previtellogenic oocytes (Fig. 4B) and the ovarian cavity could be identified. Between 59 and 159 dph (TL = 19–37 mm) ovaries enlarged and there was an increase in the number of previtellogenic oocytes. Various stages of vitellogenesis was observed in females sampled at 322 dph (TL = 62–100 mm) (Fig. 4C).

Although stromal aggregations and subtle lobule formations were observed in two fish at 59 dph (TL = 17 and 20 mm), definitive testicular differentiation was not observed until 69 dph (TL = 20–24 mm). In young testes, spermatogonia were cytologically indistinguishable from undifferentiated primordial germ cells, but the proliferation of the germ cells and their organization in clusters of four or more were suggestive of spermatogonia differentiation (van der Ven and Wester, 2021). Presumptive spermatogonia were also slightly smaller ($\bar{x}$ = 9.51 µm, 95% CI = 8.55–10.47 µm, n = 12) than undifferentiated primordial germ cells ($\bar{x}$ = 12.92 µm, 95% CI = 11.07–14.76 µm, n = 12). By 69 dph some males exhibited spermatogonia and primary spermatocytes forming loose tubular formations lined by seminiferous epithelium (Fig. 5A). Spermatogonia predominated the tissue in the testi at 89 dph (TL = 21–30 mm) and their organization into tubules became more conspicuous. Meiotic activity in testes was not observed in males until they were 99 dph (TL = 30–35 mm). Haploid spermatids resulted from meiotic divisions of spermatocytes. The clustered spermatids were small (~1–2 µm), darkly basophilic stained, and were roughly round in shape (Fig. 5B). Adult males (322 dph) had testes comprised of germ cells organized in tight tubules that were lined by thickened seminiferous epithelium (Fig. 5C). Adult males had spermatocytes at varying levels of maturity and lumina filled with mature spermatozoa (Fig. 5C).

By 99 dph all gonads had differentiated and were easily discernible as testis or ovary via histological examination (Fig. 6). We observed a heavily male-skewed sex ratio in fish sampled between 79 and 322 dph (71.67% male). We did not observe any evidence of protogynous sex differentiation (Shapiro and Rasotto, 1993). Timing of gonadal differentiation in the high-density and low-density tanks was synchronous and the sex ratios in fish older than 79 dph were male skewed from both the high-density (84.62% male) and low-density tanks (76.19% male).

4. Discussion

4.1. Aquaculture of green sunfish

Detailed aquaculture and rearing methods are essential for any

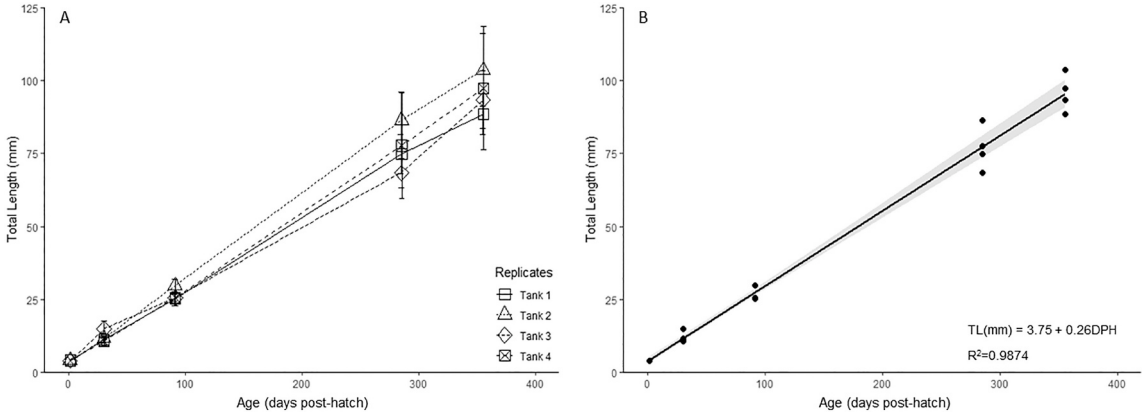

Figure A.2. (A) Growth of green sunfish as depicted by total length (TL mm) versus age (days post-hatch) for each larval rearing tank. (B) Growth of green sunfish from 1 to 355 dph as estimated by linear regression of TL on age with 95% confidence bands for the line of best fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

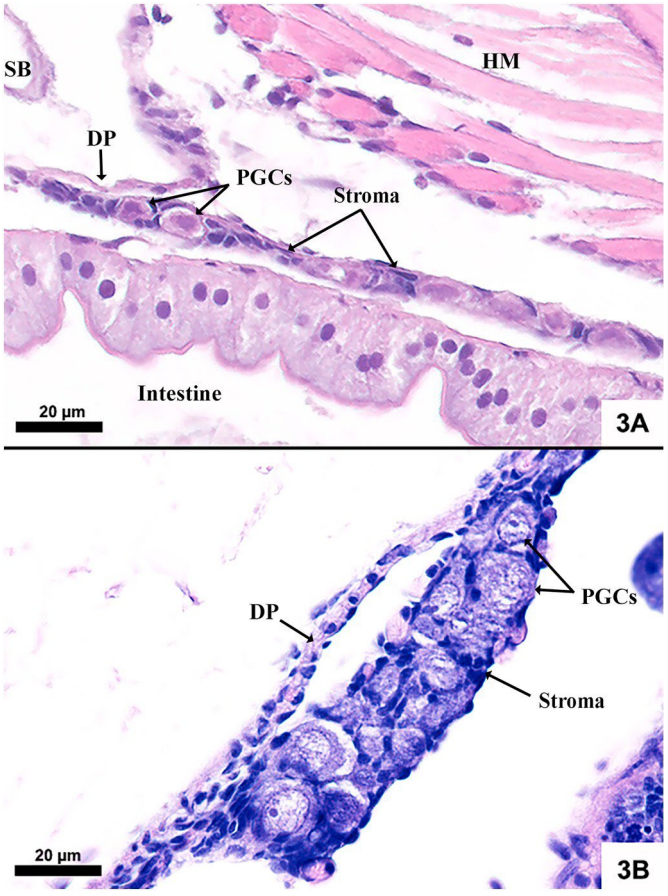

Figure A.3. Photomicrographs of undifferentiated gonad in 8 dph green sunfish (3A). Primordial germ cells (PGCs) were supported by mesenchymal stroma dorsal. The gonad is positioned dorsal to the posterior end of the intestine (DP = dorsal peritoneum, SB = swim bladder, HM = hypaxial muscle). 3B) As evident in this 22 dph green sunfish, gonads grew larger and PGCs were abundant before any histological differentiation was observed. (For interpre- tation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stocking program. In the case of green sunfish, such knowledge could prove useful for enhancing a native stock (Cotton and Wedekind, 2007; Lorenzen, 2008) or to develop and maintain a TSC broodstock for population eradication efforts where the species is invasive (Schill et al., 2016). Protocols are also essential for the maintenance and propagation of fishes for research in a laboratory setting (Lawrence, 2011). Using our feeding regimen (Table 1), we were able to wean green sunfish completely onto artificial diets by 37 dph. Transitioning fish onto arti- ficial diets can help laboratory and hatchery operations by reducing cost and labor (Jones et al., 1993). This species' ability to continuously spawn and ability to be reared in simple, indoor systems, makes them an appealing species for a variety of studies. In addition, this protocol can be scaled up from the laboratory to the hatchery level in order to in- crease production.

The current protocol allowed us to obtain year-round volitional spawns from green sunfish broodstock. Despite collecting wild-caught broodstock in May, at the beginning of their spawning season (Hunter, 1963; Etnier and Starnes, 2001), the first attempts to spawn green sunfish using 473 L rectangular glass aquaria with various male to fe- male ratios and stocking densities were unsuccessful. The glass aquaria we used were double the volume of those used by Smith (1975) and were stocked at the same sex ratio of 2 males to 3 females, yet we noticed male-on-male and male-on-female aggression that resulted in stress and occasional injury. Moving the broodstock to 360 L round tanks enabled

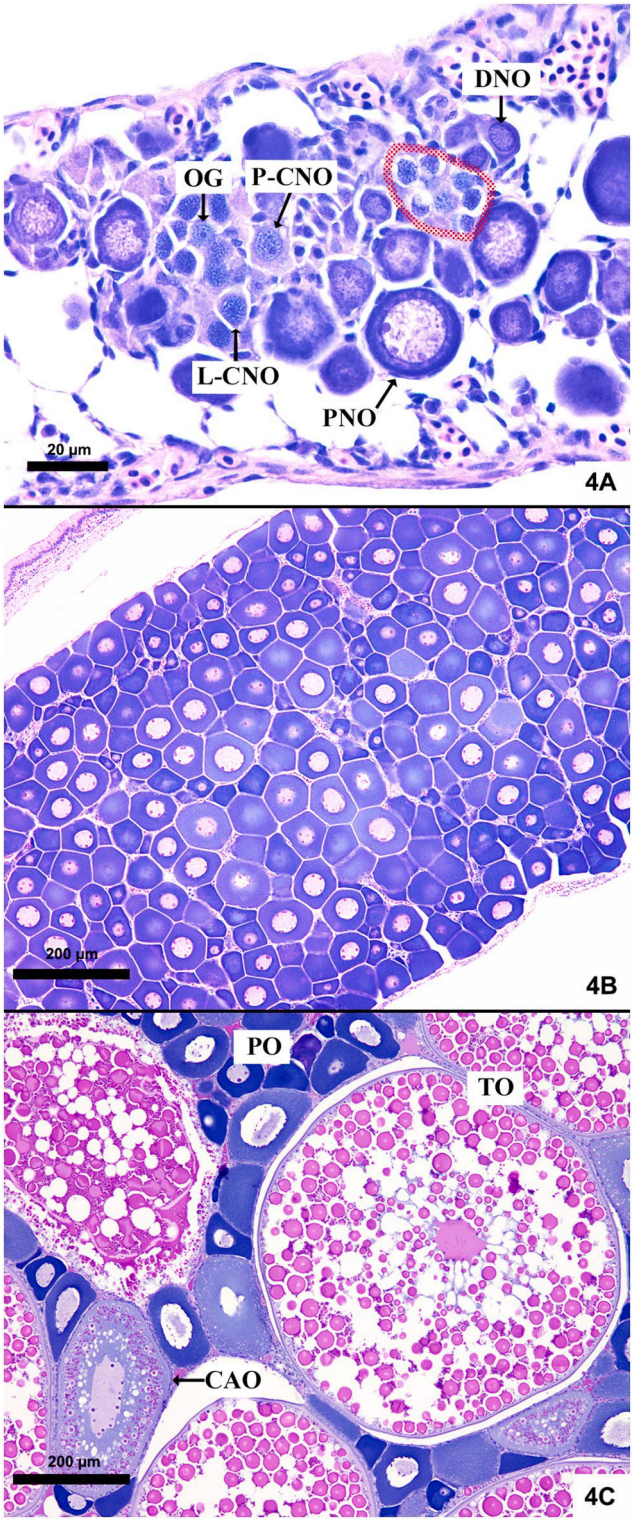

Figure A.4. Photomicrograph 4A shows an oogonium (OG), a cluster of proliferating oogonia (within red circle), a leptotene stage chromatin nucleolus-oocyte (L- CNO), a pachytene stage chromatin nucleolus-oocyte (P-CNO), a diplotene stage perinucleolus ooctye (DNO), and a previtellogenic stage perinucloelus oocyte (PNO) in 49 dph green sunfish. 4B) Ovaries in green sunfish between 59 and 159 dph were predominated by previtellogenic oocytes, as seen in this 99 dph female. 4C) Ovaries of a 322 dph green sunfish contained mature tertiary vitellogenic oocytes (TO), primary vitellogenic oocytes (PO) and intermediate cortical alveolus stage ("oil droplet stage") oocytes (CAO). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

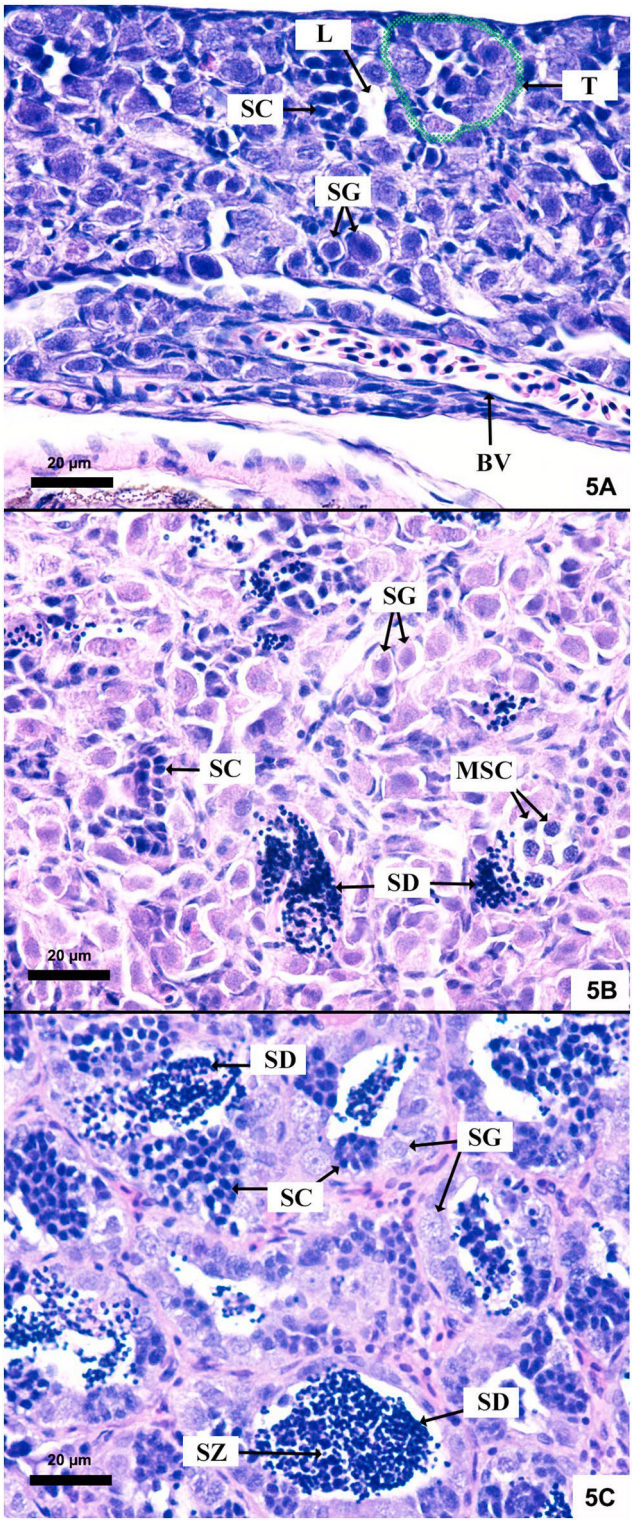

Figure A.5. 5A) Photomicrograph of testis in 69 dph male green sunfish. Clusters of spermatogonia (SG) and primary spermatocytes (SC) are present. Primary spermatocytes and spermatogonia border presumptive lumina (L). Seminiferous epithelium can be seen bordering early formations of tubules (T – border of tubule outlined in green). Blood vessels (BV) increased in size in developing testes. 5B) shows spermatocytes undergoing meiosis (MSC) and haploid sper- matids (SD) first seen in 99 dph males. 5C) By 322 dph the tubules are well defined and separated by seminiferous epithelium and the lumina are filled with mature spermatozoa (SZ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

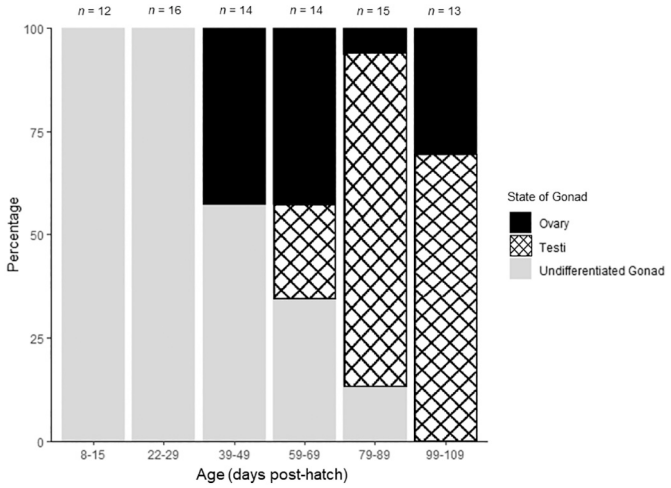

Figure A.6. Gonadal differentiation in green sunfish given rearing methods and growth rates in the present study. Ovaries begin to differentiate by 39 dph and testicular differentiation was detected after 49 dph. By 99 dph all sampled gonads were clearly differentiated as either ovaries or testes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the females and smaller males to feed more freely and decreased the incidence of enclosure wall trauma. Nevertheless, it was not until we added upside down laundry baskets with holes cut out of the sides that we observed consistent spawns. These laundry baskets were often utilized by the females while the males were more often observed guarding an artificial nest. The importance of in-tank cover had not been discussed in any of the *Lepomis* aquaculture literature we reviewed (Smith, 1975; Dupree and Hunter, 1984; Bryan et al., 1994; Mischke and Morris, 1997; Brunson and Morris, 2000). Cover and tank features, such as substrate, have been shown to increase aquaculture performance in other species (Barnes et al., 2005; Krebs et al., 2017). Aquaculturists have used submerged boxes with one side open to permit more spawns to occur in largemouth bass *M. salmoides* broodstock tanks (Breder Jr., 1936). Providing structure and in-tank cover for green sunfish broodstock appears essential due to the male's aggressive courtship behavior. Our observations also suggest a stocking ratio of 2:3 male to female can help alleviate conspecific aggression and reduce stress when attempting to induce spawning.

Despite unsuccessful spawning attempts for the first ten months, we chose not to try the use of hormone injections to cue spawning (Cuevas-Uribe et al., 2009). Since other researchers had reported consistent volitional spawns with sunfishes in captivity without the use of hormone injections (Smith, 1975; Bryan et al., 1994) we were confident that under the proper conditions (photoperiod, sex ratio, density, etc) we would obtain similar results. We observed spawns year-round after broodstock were exposed to a 4-week artificial winter and then maintained in an 18-h photoperiod with water temperatures between 23 and 27 °C the remainder of the year. It is unclear if the green sunfish would have consistently spawned without the artificial winter treatment if the upside down laundry baskets were added to the broodstock tanks first. The ability of the green sunfish to spawn continuously under a static photoperiod and temperature mimics findings by Smith (1975) where he was able to obtain volitional spawns from longear sunfish *Lepomis megalotis* maintained in a 16-h photoperiod in 25 °C water. The green sunfish's ability to continuously spawn in the laboratory is a beneficial trait for any aquacultured species (Stieglitz et al., 2017) or research animal (Gale and Buynak, 1982; Segner, 2009).

The growth rates of the lab-reared green sunfish varied widely within and among tanks. These results are validated by existing literature on green sunfish growth rates. By 30 dph we observed a mean TL of 11.9 mm which is slightly larger than the TL reported by Meyer (1970) at the

same age. However, Smith (1975) was able to rear green sunfish to a TL of 30 mm by 30 dph. Outliers in our study did exhibit similar growth rates as those reported by Smith (1975), but these fish were routinely culled due to their cannibalistic behavior. Smith's (1975) observations were likely the result of a relatively low stocking density of 50 embryos/60 L, whereas we cultured entire spawns in 37 L tanks until the fish were 30 dph before adjusting rearing densities. Smith (1975) also reported spawns by 16 weeks when the green sunfish had a mean TL of 100 mm. Based on our regression model, the mean TL of green sunfish in our rearing system was 32.87 mm at 16 weeks. Our observed growth rate and onset of sexual maturity in the offspring generation more closely matched results seen by Yun-Chang et al. (2008), who reported sexual maturity at 7–8 months at a TL of 87.50–94.50 mm. We observed sexually mature individuals by 213 dph when the mean TL was ~60 mm. In addition to starting with a lower stocking density, Smith (1975) fed brine shrimp to post larval green sunfish, whereas we only fed artificial diets after 37 dph. These two factors might have resulted in a faster growth rate than observed in our study. This variability in growth rates is another example of the plasticity of *Lepomis'* development and this genus's tolerance for overcrowding (Mittelbach, 1988; Aday et al., 2006).

As with most cultured species, green sunfish survival rates increased with size and age (Lorenzen, 1996). The survival rate of fish between 1 and 30 dph is likely overestimated considering green sunfish spawns can range from 2000 to 10,000 eggs (Yun-Chang et al., 2008). Green sunfish larvae stay benthic for at least 3 days, and we based stocking density estimates on counts of swim-up larvae from a sample of 500 mL of water when fish were 5 dph. It is possible that the water samples were not an accurate representation of the starting tank densities. Despite a continuous daily feeding regimen that ensured nauplii were always present in the tanks, growth variability likely resulted in significant reductions in survival due to concomitant cannibalism during the first 30 days of growout. Sorting and grading has been shown to reduce cannibalism in warmwater species (Kelly and Heikes, 2013). Routinely grading green sunfish during the first two months post hatch may increase survival rates and alleviate increasing growth disparities during growout.

A. hydrophila infections were another significant cause of mortality, especially during the weaning period from live nauplii to an artificial diet. *A. hydrophila* is a common bacterial infection in freshwater fishes that can cause severe disease and losses in cultured warmwater species (Cipriano et al., 1984; Swann and White, 1991). Uneaten diet would temporarily collect on the bottom of the larval rearing tanks when the juvenile green sunfish were transitioned from live nauplii to the artificial diets. Although there was no change in the tested water quality parameters during this time, the accumulation of uneaten food and waste, coupled with increased tank densities due to fish growth, may have facilitated *A. hydrophila* infections. Oxytetracycline treated diet cured infections and reduced mortalities, but prevention of these infections is obviously preferred. Bottom-draining tanks and increased filtration, especially UV filtration, during diet transitions might have helped prevent any changes to water quality conditions and helped reduce stress that facilitated infections (Swann and White, 1991). Continual feeding of live diets is labor intensive and makes administering treatments and medication via feeding challenging or impossible. With self-cleaning rearing tanks and more intensive filtration systems, the benefits to research and aquaculture operations of transferring the green sunfish onto an artificial diet during their development could outweigh any potential risk of disease outbreaks.

4.2. Gonadal differentiation of green sunfish

In this study we were able to describe the microscopic features of differentiation in green sunfish gonads using histology. We found that green sunfish are gonochoristic, with testes and ovaries differentiating from undifferentiated gonads. Ovarian differentiation preceded testicular differentiation by 30 days. By 99 dph all sampled gonads showed clear morphological sex differentiation (Fig. 6). We noted a heavily male-skewed sex ratio in fish sampled between 79 and 322 dph (71.67% male). We did not detect any transitions from ovaries to testes in the form of intersex gonadal tissue in our sampled individuals. In many fish species, increased temperatures or high stocking densities have been shown to increase the proportion of males in a reared cohort (Baroiller et al., 1995; Roncarati et al., 1997; Ospina-Alvarez and Piferrer, 2008). Temperature effects on sex ratios have also been identified in the closely related bluegill (Shen et al., 2016). It is possible that temperature effects or rearing densities could have a resulted in male-skewed sex ratios in our sampled fish.

The transformation of primordial germ cells to oogonia and the subsequent development of chromatin-nucleolus stage oocytes were the first observed signs of ovarian differentiation. The development of oogonia and chromatin-nucleolus stage oocytes has been described by many researchers with regards to ovarian differentiation (Jensen and Shelton, 1983; van der Ven and Wester, 2021; Uguz, 2008; Gao et al., 2009), yet we found these cytological differences a challenging benchmark to identify and instead, the presence of perinucleolus stage oocytes seemed to be a more reliable and conspicuous indicator of ovarian differentiation. Yun-Chang et al. (2008) also remarked that at one-month of age, distinguishing between male and female gonads is difficult. As similarly expressed by Yun-Chang et al. (2008), the most notable feature in a developing ovary is the highly basophilic cytoplasm in previtellogenic oocytes. This feature was ubiquitous in ovaries older than 49 dph.

Ovaries between 59 and 159 dph grew with little histological change aside from an increase in number of primary oocytes and the development of a thin layer of vacuoles along the periphery of the oocyte nucleus. Yun-Chang et al. (2008) observed larger flat droplets and increased vitellogenesis in green sunfish 4–5 months old. We only observed that level of vitellogenesis in 322 dph ovaries, after fish were moved to larger growout tanks. The relative delay in vitellogenesis observed in our study could be due to the high stocking density the fish were kept in during the first 150 days of growout or due to differences in diet (Schreck et al., 2001). Ovaries of sexually mature females exhibited various levels of vitellogenesis, ranging from primary to fully mature. This variety of oocytes at different stages of development could explain the green sunfish's ability to continuously spawn under the lab's broodstock holding conditions.

The first signs of testicular differentiation were clusters of presumptive spermatogonia that were loosely organized into lobules and supported by stroma. During the first two months of development, the spermatogonia were indistinguishable from undifferentiated primordial germ cells, but as previously described in other papers, germ cell clusters are indicative of testicular differentiation (Yun-Chang et al., 2008; Gao et al., 2009; van der Ven and Wester, 2021). Yun-Chang et al. (2008) reported the formation of seminal lobules in 1-month old testes. We did not observe this until fish were 59 dph. Aside from this earlier detection of differentiation, the overall pattern and monthly timing of testicular development described by Yun-Chang et al. (2008) was similar to our own findings, except they do not report any meiotic activity in testes until fish are four months of age, yet we saw spermatocytes yielding spermatids in fish as young as 99 dph.

Many researchers have discussed the plasticity of the timing of sexual development in fishes (Billard et al., 1981; Eyeson, 1983; Blay, 1985; Gao et al., 2009). The onset of ovarian differentiation and age at first spawn of our green sunfish was similar to that observed by Yun-Chang et al. (2008), yet we did not observe vitellogenesis in ovaries sampled up to 150 dph. Yun-Chang et al. (2008) described advanced stages of vitellogenesis in fish 4–5 months of age and observed earlier testicular differentiation as well, but our green sunfish displayed earlier testicular maturation.

The timing and pattern of testicular differentiation of our green sunfish resemble that of bluegill (Gao et al., 2009), but we observed earlier oocyte development in green sunfish than bluegill. Despite sharing similar growth rates and undifferentiated gonad descriptions as

compared to bluegill, we observed 49 dph green sunfish ovaries that already contained perinucleolus oocytes whereas Gao et al. (2009) did not observe oocytes at this stage of development in bluegill until 90 dph. Gao et al. (2009) demonstrated the plasticity in the timing of sexual development in bluegill by comparing the timing of sexual differentiation between a slow-growing batch that was stocked at a high density and a fast-growing batch stocked at a relatively low density. They also noted that sexual differentiation was a function of length, not age. It is likely that green sunfish exhibit similar plasticity in the timing of their sexual development and that age is an index that can be used to estimate TL under specific rearing conditions (Fig. 2B). Nevertheless, there was no difference in the timing of gonadal differentiation in our low-density and high density larval-rearing tanks. Under our rearing conditions, we first saw cytological differentiation of ovaries by 39 dph. Although cytological testicular differentiation was first detected at 69 dph, some individuals still had undifferentiated gonads at 89 dph. No fish sampled after 99 dph had undifferentiated gonads. Under the concept of the labile period of fish sex determination (Hackmann and Reinboth, 1974; Piferrer, 2001), green sunfish under similar rearing and feeding conditions could display permanently altered sex differentiation if exposed to endocrine disruptors or exogenous sex hormones before 39 dph (TL = 16–34 mm) through 99 dph (30–35 mm). Unsurprisingly, our proposed labile period is similar to the labile period identified in bluegill (Gao et al., 2009). However, since sex differentiation begins with biochemical processes (Haffrey et al., 2009) and anatomical differentiation typically precedes cellular differentiation (Jensen and Shelton, 1983; Sacobie and Benfey, 2005), beginning the treatment period earlier than 39 dph may be necessary for complete sex reversal. Starting to wean juvenile green sunfish onto artificial diets by 25 dph, as we did in this study, could help facilitate the administration of exogenous steroids during a critical period for altering their sex differentiation (Yamamoto, 1963) and assist in the development of TSC carrying fish (Schill et al., 2016; Teem et al., 2020).

5. Conclusions

We were able to obtain year-round spawns of green sunfish, rear juveniles using artificial diets, and produce an F2 generation in captivity. The ability to be reared successfully and to spawn so frequently may lend the species to a wide variety of laboratory studies. A detailed description of gonadal differentiation of green sunfish that identified their labile period in sexual development was also obtained. The acquisition of both detailed aquaculture protocols and an understanding of the gonadal development of green sunfish may contribute to enhanced stocking programs or alternatively, assist with eradication of invasive populations via development of TSC broodstocks.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

William Stewart of the United States Bureau of Reclamation solicited and provided funding for this research (United States Bureau of Reclamation, grant number G18AC0061 and modification G19AC0039). Gabriella Buonavolonta, Kylee Rath, Suhailah Hussein, and Cora Thomas assisted with data collection and construction and maintenance of aquaculture systems. Nathan Berg with the Arizona Game and Fish Department assisted with green sunfish broodstock collection. Zach Nemec, Larissa Lee, Taylor Ulrich, Emily Freed, Chris Jenney, Joshua Grant, Steven Ingram, and Erin Tracy with the University of Arizona provided assistance with construction of the aquaculture system and provided research advice. Arun Dhar and Luis Fernando Arunguren with the University of Arizona Aquaculture Pathology Laboratory performed histopathology and PCR analysis on submitted carcasses. Liz Mamer with Idaho Department of Fish and Game provided assistance analyzing histology slides. We thank Han Liu and Dr. Yushun Chen with the Wuhan University and Chinese Academy of Sciences for translating the paper by Yun-Chang et al. (2008). There is no conflict of interest declared in this study.

References

Adams, M.A., Johnsen, P.B., 1986. A solid matrix bioassay for determining chemical feeding stimulants. The Progressive Fish Culturist 48, 147–149.

Aday, D.D., Philipp, D.P., Wahl, D.H., 2006. Sex-specific life history patterns in bluegill (*Lepomis macrochirus*): interacting mechanisms influence individual body size. Oecologia 147, 31–38.

Al-Ablani, S.A., 1997. Use of synthetic steroids to produce monosex populations of selected species of Sunfishes (Family: Centrarchidae). Doctoral dissertation. Auburn University, Auburn, Alabama.

Arezo, M.J., Alessandro, S.D., Papa, N., de S´a, R., Berois, N., 2007. Sex differentiation pattern in the annual fish *Austrolebias charrua* (Cyprinodontiformes: Rivulidae). Tissue Cell 39, 89–98.

Barnes, M.E., Miller, J., Durben, D.J., 2005. Partial overhead tank cover use during feral brown trout rearing. N. Am. J. Aquac. 67, 319–323.

Baroiller, J.F., Chourrout, D., Fostier, A., Jalabert, B., 1995. Temperature and sex chromosomes govern sex ratios of the mouthbrooding cichlid fish *Oreochromis niloticus*. J. Exp. Zool. 273, 216–223.

Billard, R., Bry, C., Gillet, C., 1981. Stress, Environment and Reproduction in Teleost Fish. Stress and Fish. Academic Press, 1981, 0-12-554550-9. ffhal-01600274f.

Blasius, H.B., 2002. Chemical removal of green sunfish (*Lepomis cyanellus*) from O'Donnell Creek, Arizona. In: Desert Fishes Council 2002 Annual Meeting, San Luis Potosi, SLP, Mexico.

Blay, J., 1985. Observations on the balance in fish populations in a small reservoir in Ghana. Fish. Res. 3, 1–11.

Breder Jr., C.M., 1936. The reproductive habits of the North American sunfishes (Family Centrarchidae). Zoologica 21, 1–48.

Brunson, M.W., Morris, J.E., 2000. Species profile – Sunfish. Southern Regional Aquaculture Center Publication Number 724.

Brunson, M.W., Robinnette, H.R., 1985. Supplemental winter feeding of hybrid sunfish in Missisippi. Proc. Ann. Conf. Southeastern Assoc.Fish Wildlife Agencies 36 (1982), 157–161.

Bryan, M.D., Morris, J.E., Atchison, G.J., 1994. Technical notes: method for culturing bluegill in the laboratory. Progressive Fish-Culturist 56, 217–221.

Carr, W.E.S., 1976. Chemoreception and feeding behavior in the pigfish, *Orthopristis chrysopterus*: characterization and identification of stimulatory substances in a shrimp extract. Compar. Bio-Chem. Physiol. A, Compar. Physiol. 55, 153–157.

Cipriano, R.C., Bullock, G.L., Pyle, S., 1984. *Aeromonas Hydrophila* and Motile Aeromonad Septicemias of Fish. United States Fish and Wildlife Service, Washington, DC.

Cotton, S., Wedekind, C., 2007. Introduction of Trojan sex chromosomes to boost population growth. J. Theor. Biol. 249, 153–161.

Cuevas-Uribe, R., Gomelsky, B., Mims, S.D., Pomper, K.W., 2009. Progress in studies on hormonal sex reversal and genetic sex control in black crappie. Rev. Fish. Sci. 17, 1–7.

Desprez, D., M´elard, C., Philippart, J.C., 1995. Production of a high percentage of male offspring with 17a-ethynylestradiol sex-reversed *Oreochromis aureus*: II. Comparative reproductive biology of females and F2 pseudofemales and large scale production of male progeny. Aquaculture 130, 35–41.

Dhont, J., Dierckens, K., Støttrup, J., Van Stappen, G., Wille, M., Sorgeloos, P., 2013. 5 - rotifers, Artemia and copepods as live feeds for fish larvae in aquaculture. In: Allan, G., Burnell, G. (Eds.), Advances in Aquaculture Hatchery Technology. England, Cambridge, pp. 157–202.

Dudley, R., Matter, W., 2000. Effects of small green sunfish (*Lepomis cyanellus*) on recruitment of Gila chub (*Gila intermedia*) in Sabino Creek, Arizona. Southwest. Nat. 45, 24–29.

Dupree, H., Hunter, J., 1984. Propagation of black bass, sunfishes, tilapias, eels, and hobby fishes. In: Dupree, H., Hunter, J. (Eds.), Third Report to the Fish Farmers. U.S. Fish and Wildlife Service, Washington, D.C., pp. 119–135

Etnier, D., Starnes, D., 2001. The Fishes of Tennessee. The University of Tennessee Press, Knoxville, pp. 410–411.

Eyeson, K.N., 1983. Stunting and reproduction in pond-reared *Sarotherodon melanotheron*. Aquaculture 31, 257–267.

Fuller, P., Cannister, M., Neilson, M., 2021. *Lepomis cyanellus* Rafinesque, 1819: Geological Survey. Nonindigenous Aquatic Species Database, Gainesville, FL. https://nas.er.usgs.gov/queries/FactSheet.aspx?speciesID=380.

Gale, W.F., Buynak, G.L., 1982. Fecundity and spawning frequency of the fathead minnow- a fractional spawner. Trans. Am. Fish. Soc. 111, 35–40.

Gao, Z., Wang, H., Rapp, D., O'Bryant, P., Wallat, G., Wang, W., MacDonald, R., 2009. Gonadal sex differentiation in the bluegill sunfish *Lepomis macrochirus* and its relation to fish size and age. Aquaculture 294, 138–146.

Gomelsky, B., Mims, S.D., Onders, R.J., Bean, W.B., 2002. Hormonal sex reversal and evidence of female homogamety in black crappie. N. Am. J. Aquac. 64, 66–69.

Gugliandolo, C., Irrera, G.P., Lentini, V., Maugeri, T.L., 2008. Pathogenic *Vibrio*, *Aeromonas* and *Arcobacter* spp. associated with copepods in the Straits of Messina (Italy). Mar. Pollut. Bull. 56, 600–606.

Gutierrez, J.B., Teem, J.L., 2006. A model describing the effect of sex-reversed YY fish in an established wild population: the use of a Trojan Y chromosome to cause extinction of an introduced exotic species. J. Theor. Biol. 241, 333–341.

Hackmann, E., Reinboth, R., 1974. Delimitation of the critical stage of hormone-influenced sex differentiation in *Hemihaplochromis multicolor* (Hilgendorf) (Cichlidae). Gen. Comp. Endocrinol. 22, 42–53.

Haffrey, P., Petit, V., Guiguen, Y., Quillet, E., Rault, P., Fostier, A., 2009. Successful production of monosex female brook trout Salvelinus fontinalis using gynogenetic sex reversed males by a combination of methyltestosterone immersion and oral treatments. Aquaculture 290, 47–52.

Halos, S., La Vina, A.G., Lim, M.S., Caleda, M.J., 2004. Developing the National Biosafety Framework for the Philippines. Page 12. Department of Environment and Natural Resources-Protected Areas and Wildlife Bureau, Quezon City, Philippines.

Hayes, M.P., Jennings, M.R., 1986. Decline of Ranid frog species in western North America: are bullfrogs (*Rana catesbeiana*) responsible? J. Herpetol. 20, 490–509.

Hunn, J.B., Schoettger, R.A., Whealdon, E.W., 1968. Observations on the handling and maintenance of bioassay fish. Progressive Fish-Culturist 30, 164–167.

Hunter, J.R., 1963. The reproductive behavior of the green sunfish, *Lepomis cyanellus*. Zoologica 48, 13–24.

Jensen, J.L., Shelton, W.L., 1983. Gonadal differentiation in relation to sex control of grass carp, *Ctenopharyngodon Idella* (Pisces: Cyprinidae). Copeia 3, 749–755.

Jones, D.A., Kamarudin, M.S., Vay, L.L., 1993. The potential for replacement of live feeds in larval culture. J. World Aquacult. Soc. 24, 199–210.

Kaya, C.M., Hasler, A.D., 1972. Photoperiod and temperature effects on the gonads of green sunfish, *Lepomis cyanellus*, during the quiescent, winter phase of its annual sexual cycles. Trans. Am. Fish. Soc. 101, 270–275.

Kelly, A.M., Heikes, D., 2013. Sorting and grading warmwater fish. Southern Regional Aquaculture Center. Publication Number 391.

Kolar, C.S., Courtenay Jr., W.R., Nico, L.G., 2010. Managing undesired and invading fishes. In: Hubert, W.A., Quist, M.C. (Eds.), Inland Fisheries Management in North America, 3rd edition. American Fisheries Society, Bethesda, Maryland, pp. 213–259.

Krebs, J., Krebs, E., Barnes, M.E., Crank, K.M., 2017. Use of bottom structure and tank cover during rainbow trout rearing in circular tanks. J. Fish. Livestock Prod. 5, 247. https://doi.org/10.4172/2332-2608.1000247.

Lawrence, C., 2011. Advances in zebrafish husbandry and management. Methods Cell Biol. 104, 429–451.

Lee, D.S., Gilbert, C.R., Hocutt, C.H., Jenkins, R.E., McAllister, D.E., Stauffer Jr., J.R., 1980. Atlas of North American Freshwater Fishes. North Carolina State Museum of Natural History. Raleigh, NC.

Lemly, A.D., 1985. Suppression of native fish populations by green sunfish in first-order streams of Piedmont North Carolina. Trans. Am. Fish. Soc. 114, 705–712.

Lewis, W.M., Heidinger, R., 1971. Supplemental feeding of hybrid sunfish populations. Trans. Am. Fish. Soc. 100, 619–623.

Lorenzen, K., 1996. The relationship between body weight and natural mortality in juvenile and adult fish: a comparison of natural ecosystems and aquaculture. J. Fish Biol. 49, 627–642.

Lorenzen, K., 2008. Understanding and managing enhancement fisheries systems. Rev. Fish. Sci. 16, 10–23.

Lowerre-Barbieri, S., Ganias, K., Saborido-Rey, F., Murua, H., Hunter, J., 2011. Reproductive timing in marine fishes: variability, temporal scales, and methods. Marine and Coastal Fisheries Dynamics Management and Ecosystem Science Management, pp. 71–91.

Malison, J.A., Kayes, T.B., Best, C.D., Amundson, C.H., Wentworth, B.C., 1986. Sexual differentiation and use of hormones to control sex in yellow perch (*Perca flavescens*). Can. J. Fish. Aquat. Sci. 43, 26–35.

Mazzoni, T.S., Quagio-Grassiotto, I., 2017. Ovary differentiation and activity in teleostei fish. In: Carreira, R.P. (Ed.), Theriogenology. InTech, London, UK, pp. 129–156.

McCormick, J.L., Schill, D.J., Meyer, K.A., 2021. Simulated use of YY male stocking and suppression for eradicating common carp populations. N. Am. J. Fish Manag. 41, 366–382.

McKechnie, R.J., Tharratt, R.C., 1966. Green sunfish. In: Calhoun, A. (Ed.), Inland Fisheries Management. California Department of Fish and Game, Sacramento, CA, pp. 399–401.

Meyer, F.A., 1970. Development of some larval Centrarchids. Progressive Fish-Culturist 32, 130–136.

Mischke, C.C., Morris, J.E., 1997. Out-of-season spawning of sunfish *Lepomis* spp. in the laboratory. Progressive Fish-Culturist 59, 297–302.

Mischke, C.C., Morris, J.E., Lane, R.L., 2007. Species Profile: Hybrid Sunfish. Southern Regional Aquaculture Center. Publication Number 7205.

Mittelbach, G.G., 1988. Competition among refuging sunfishes and effects of fish density on littoral zone invertebrates. Ecology 69, 614–623.

Moyle, P.B., 1976. Fish introductions in California: history and impact on native fishes. Biol. Conserv. 9, 101–118.

Nakamura, M., Kobayashi, T., Chang, X.T., Nagahama, Y., 1998. Gonadal sex differentiation in teleost fish. J. Exp. Biol. 281, 362–372.

Ogle, D.H., 2016. Introductory Fisheries Analyses with R. Chapman & Hall/CRC, Boca Raton, FL.

Ospina-Alvarez, 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, e2837.

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197, 229–281.

Reinthal, P.N., Blasius, H., Haberstitch, M., 2020. Long-term monitoring of a desert fish assemblage in Aravaipa Creek, Arizona. In: Propst, J.W., Bestgen, K., Hoagstrom, C. (Eds.), Standing between Life and Extinction: Ethics and Ecology of Conserving Aquatic Species in North American Deserts. Chicago, Illinois, pp. 225–237.

Roncarati, A., Melotti, P., Mordenti, O., Gennari, L., 1997. Influence of stocking density of European eel (*Anguilla anguilla*, L.) elvers on sex differentiation and zootechnical performances. J. Appl. Ichthyol. 13, 131–136.

Rosen, P.C., Schwalbe, C.R., Parizek Jr., D.A., Holm, P.A., Lowe, C.H., 1995. Introduced aquatic vertebrates in the Chiricahua region: effects on declining native Ranid frogs. In: DeBano, L.H., Folliott, P.H., Ortega-Rubio, A., Gottfried, G.J., Hamre, R.H., Edminster, C.B. (Eds.), Biodiversity and Management of the Madrean Archipelago: the Sky Islands of Southwestern United States and Northwestern Mexico. US Forest Service, Rocky Mountain Forest and Range Experiment Station, Fort Collins, CO, pp. 251–261.

Sacobie, C.F.D., Benfey, T.J., 2005. Sex differentiation and early gonadal development in brook trout. N. Am. J. Aquac. 67, 181–186.

Schill, D.J., Heindel, J.A., Campbell, M.R., Meyer, K.A., Mamer, E.R.J.M., 2016. Production of a YY male brook trout broodstock for potential eradication of undesired brook trout populations. N. Am. J. Aquac. 78, 72–83.

Schill, D.J., Meyer, K.A., Hansen, M.J., 2017. Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. N. Am. J. Fish Manag. 37, 1054–1066.

Schreck, C.B., Contreras-Sanchez, W., Fitzpatrick, M.S., 2001. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197, 3–24.

Segner, H., 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. Compar. Biochem. Physiol. C: Toxicol. Pharmacol. 149, 187–195.

Senior, A.M., Krkosek, M., Nakagawa, S., 2013. The practicality of Trojan sex chromosomes as a biological control: an agent based model of two highly invasive *Gambusia* species. Biol. Invasions 15, 1765–1782.

Shapiro, D.Y., Rasotto, M.B., 1993. Sex differentiation and gonadal development in the diandric, protogynous wrasse, *Thalassoma bifasciatum* (Pisces, Labridae). J. Zool. 230, 231–245.

Shen, Z.G., Wang, H.P., Yao, H., O'Bryant, P., Rapp, D., Zhu, K.Q., 2016. Sex determination in bluegill sunfish *Lepomis macrochirus*: effect of temperature on sex ratio of four geographic strains. Biol. Bull. 230, 197–208.

Smith, W.E., 1975. Breeding and culture of two Sunfish, *Lepomis cyanellus* and *L . megalotis*, in the laboratory. Progressive Fish-Culturist 37, 227–229.

Stieglitz, J.D., Hoenig, R., Kloeblen, S., Tudela, C.E., Grosell, M., Benetti, D.D., 2017. Capture, transport, prophylaxis, acclimation, and continuous spawning of mahi-mahi (*Coryphaena hippurus*) in captivity. Aquaculture 479, 1–6.

Støttrup, J.G., 2003. Production and nutritional value of copepods. In: Støttrup, J.G., McEvoy, L.A. (Eds.), Live Feeds in Marine Aquaculture. United Kingdom, Oxford, pp. 145–205.

Swann, L.D., White, M.R., 1991. Diagnosis and treatment of "*Aeromonas hydrophila*" infection of fish. Cooperative. Extension Service Purdue University Fact Sheet AS-461.

Teem, J., Alphey, L., Descamps, S., Edgington, M., Edwards, O., Gemmell, N., Roberts, A., 2020. Genetic biocontrol for invasive species. Front. Bioeng. Biotechnol. 8, 1–18.

Uguz, C., 2008. Histological evaluation of gonadal differentiation in fathead minnows (*Pimephales promelas)*. Tissue Cell 40, 299–306.

van der Ven, L., Wester, P., 2021. Histology and Histopathology Atlas of the Zebrafish V2.01. available: zfin.org/hh_atlas.

Wang, H.P., Gao, Z., Beres, B., Ottobre, J., Wallat, G., Tiu, L., Yao, H., 2008. Effects of estradiol-17 β on survival, growth performance, sex reversal and gonadal structure of bluegill sunfish *Lepomis macrochirus*. Aquaculture 285, 216–223.

Ward, D.L., O'Neill, M.W., Ka'apu-Lyons, C., 2015. Effectiveness of backpack electrofishing for removal of non-native fishes from a small warm-water stream. J. Ariz. Nev. Acad. Sci. 46, 37–41.

Welcomme, R.L., 1988. International introductions of inland aquatic species. FAO Fisheries Technical Paper No. 294. available: http://www.fao.org/3/X5628E/x5628e0b.htm#lepomis%20cyanellus%20rafinesque:%20centrarchidae.

Werner, E.E., Hall, D.J., 1977. Competition and habitat shift in two sunfishes (Centrarchidae). Ecology 58, 869–876.

Yamamoto, T.O., 1963. Induction of reversal in sex differentiation of YY zygotes in the medaka, *Oryzias latipes*. Genetics 48, 293–306.

Yun-Chang, C.A.O., Wen-Sheng, L.I., Wei, Y.E., Hao-Ran, L.I.N., 2008. Gonadal development in green sunfish, *Lepomis cyanellus*: a histological study. Chinese J. Zool. 43, 88–95.

APPENDIX B

SEX-SPECIFIC MARKERS UNDETECTED IN GREEN SUNFISH *LEPOMIS CYANELLUS* USING RESTRICTION-SITE ASSOCIATED DNA SEQUENCING

Received: 30 November 2021 | Accepted: 11 April 2022
DOI: 10.1111/jfb.15063

REGULAR PAPER

JOURNAL OF FISH BIOLOGY fsbi

Sex-specific markers undetected in green sunfish *Lepomis cyanellus* using restriction-site associated DNA sequencing

Chad N. Teal[1] | D. Katharine Coykendall[2,3] | Matthew R. Campbell[3] | Daniel L. Eardley[2,3] | Thomas A. Delomas[2,3] | James T. Shira[4] | Daniel J. Schill[5] | Scott A. Bonar[6] | Melanie Culver[6]

[1]Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, Tucson, Arizona, USA

[2]Pacific States Marine Fisheries Commission, Eagle Fish Genetics Lab, Eagle, Idaho, USA

[3]Idaho Department of Fish and Game, Eagle Fish Genetics Lab, Eagle, Idaho, USA

[4]University of Arizona Genetics Core, Tucson, Arizona, USA

[5]Fisheries Management Solutions, Inc., Boise, Idaho, USA

[6]US Geological Survey, Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2, Tucson, Arizona, USA

Correspondence
Chad N. Teal, Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2 3H-2, 1064 E. Lowell St, Tucson, AZ 85721, USA.
Email: chadteal@email.arizona.edu

Funding information
William Stewart of the United States Bureau of Reclamation solicited and provided funding for this research (United States Bureau of Reclamation, grant number G18AC0061 and modification G19AC0039).

Abstract

We used restriction-site associated DNA sequencing for SNP discovery and genotyping of known-sex green sunfish *Lepomis cyanellus* DNA samples to search for sex-diagnostic single nucleotide polymorphisms (SNPs) and restriction-site associated sequences present in one sex and absent in the other. The bioinformatic analyses discovered candidate SNPs and sex-specific restriction-site associated sequences that fit patterns of male or female heterogametic sex determination systems. However, when primers were developed and tested, no candidates reliably identified phenotypic sex. The top performing SNP candidate (ZW_218) correlated with phenotypic sex 63.0% of the time and the presence-absence loci universally amplified in both sexes. We recommend further investigations that interrogate a larger fraction of the *L. cyanellus* genome. Additionally, studies on the effect of temperature and rearing density on sex determination, as well as breeding of sex-reversed individuals, could provide more insights into the sex determination system of *L. cyanellus*.

KEYWORDS
centrarchid, markers, RAD-seq, sex determination, sex identification

1 | INTRODUCTION

Green sunfish *Lepomis cyanellus* is a North American centrarchid species that is considered highly invasive when established outside of its native range (Dudley & Matter, 2000; Fuller *et al.*, 2021; Lemly, 1985; Yun-Chang *et al.*, 2008). *L. cyanellus* often overpopulate inhabited systems and are associated with the suppression of other game fishes (McKechnie & Tharratt, 1966; Morris *et al.*, 2005; Werner & Hall, 1977) as well as species of conservation concern (Dudley & Matter, 2000; Hayes & Jennings, 1986; Moyle, 1976; Rosen *et al.*, 1995). The removal of invasive *L. cyanellus* populations is challenging and can require years of eradication efforts to be successful (Blasius, 2002; Reinthal *et al.*, 2020; Ward *et al.*, 2015). Innovative methods for controlling or eradicating other fish populations by skewing sex ratios towards 100% male *via* daughterless breeding systems (Bax & Thresher, 2009) or Trojan sex chromosome (TSC)

© 2022 Fisheries Society of the British Isles.

strategies (Gutierrez & Teem, 2006; Schill *et al*., 2017; Senior *et al*., 2013; Teem *et al*., 2014; Teem *et al*., 2020) are theoretically effective and are being employed experimentally in attempts to eradicate invasive brook trout *Salvelinus fontinalis* populations (Kennedy *et al*., 2018; Schill *et al*., 2016). The development of genetic sex identification (Sex-ID) markers facilitates investigations into the potential use of a TSC strategy to control nuisance populations of fishes by elucidating the species' sex determination (SD) system. The two most common SD systems are XY, where males contain morphologically different sex chromosomes (heterogametic), and ZW, where females are the heterogametic sex (Furman *et al*., 2020). Sex-ID markers also simplify the production of TSC carrying fish and are useful in the management of aquaculture broodstock (Schill *et al*., 2016; Vu *et al*., 2019).

In addition to facilitating the development of TSC carrying fish, Sex-ID markers are a prerequisite for understanding the evolution of sex chromosomes and can aid in the discovery of SD genes (Fowler & Buonaccorsi, 2016; Lamatsch *et al*., 2015). The SD systems within the Centrarchidae family vary based on species and even population (Du *et al*., 2021; Gomelsky *et al*., 2002; Shen *et al*., 2016; Wang *et al*., 2018). These SD systems range from seemingly simple and chromosomal, such as the XX/XY SD system of largemouth bass *Micropterus salmoides* (Du *et al*., 2021), to more complicated systems, such as those in bluegill *Lepomis macrochirus*, where increased water temperatures can lead to male-skewed populations (Shen *et al*., 2016). Although the morphologies of *L. cyanellus* chromosomes have been described (Ohno, 1970; Roberts, 1964), sex chromosomes were not identified, the SD system of *L. cyanellus* is still unknown and no Sex-ID markers have been developed for this species. One commonly used method to develop Sex-ID markers is restriction-site associated DNA sequencing (RAD-seq) (Brown *et al*., 2016; Fowler & Buonaccorsi, 2016; Gamble, 2016; Gamble & Zarkower, 2014; Vu *et al*., 2019). Restriction-site associated DNA sequencing allows for the investigation and comparison of reduced representations of genomes by sequencing DNA adjacent to restriction enzyme cut sites (Miller *et al*., 2007). This method does not require a reference genome and is a cost-effective approach for single nucleotide polymorphism (SNP) discovery and genotyping of multiple individuals (Peterson *et al*., 2012). Since no reference genome exists for *L. cyanellus*, and sex differences in the *L. cyanellus* genome have not been explored, we used RAD-seq to search for a Sex-ID marker for this species.

The objectives of this study were to identify, develop and test potential Sex-ID markers for *L. cyanellus*. Research on Sex-ID markers in *L. cyanellus* could provide more insights into the complicated SD systems of centrarchids as well as help future efforts aimed at controlling nuisance *L. cyanellus* populations.

2 | MATERIALS AND METHODS

2.1 | DNA extraction and RAD-seq library construction

To determine how many individuals to include in our final RAD-seq library, we used *L. cyanellus* genome size estimates from the Animal Genome Size Database [9.78e^{9}–1.0e^{10} base pairs (bp), https://www.genomesize.com/search.php] and assumed a 40.7% GC content, based on the only genomic resource available in the National Center for Biotechnology Information's Genbank database from a centrarchid species at the time of this study, the *Micropterus floridanus* mitochondrial genome. We chose the restriction enzyme, PstI (New England Biolabs, Ipswich, MA, USA) with a 6 bp recognition site that should result in approximately 300,000 cut sites across the genome. We aimed for 40*X* coverage per locus, necessitating 16 samples per sex per library. The final, sequenced library contained 10 male and 10 female *L. cyanellus*, as well as 12 red shiner *Cyprinella lutrensis* individuals used in another project.

We extracted DNA using a Nexttec extraction kit with Proteinase K (Nexttec Biotechnology GmbH, Hilgershausen, Germany) from 10 phenotypic males and 10 phenotypic females collected from Parker Canyon Lake, Arizona (GPS coordinates 31°25'37.0"N, 110°27'25.0"W) during the spring and summer of 2018 and 2019. Phenotypic sex for all individuals was determined by either dissection and inspection of the gonads or by the expression of mature gametes. The RAD-seq library was constructed at Idaho Department of Fish and Game's Eagle Fish Genetics Lab in Eagle, Idaho. We constructed the RAD-seq library following methods described by Ali *et al*. (2016) and Vu *et al*. (2019). We quantified extracted DNA using a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) and Qubit 2.0 fluorometer, then normalized the DNA to 10 ng μl^{-1} and used 10 μl per sample. We conducted single-digest RAD-seq using the PstI restriction enzyme which has a 6 bp recognition sequence. The library was sequenced at Eagle Fish Genetics Lab using a NextSeq 500/550, 300 cycle High Output Kit on an Illumina NextSeq 500 (Illumina, San Diego, CA, USA) to yield 150 bp paired-end reads.

2.2 | Bioinformatics

2.2.1 | Sequence data processing and *de novo* assembly of loci

The sequencing data were analysed using the Stacks v2.4 pipeline (Catchen *et al*., 2013) and custom scripts written in Python 3.0. Stacks allows for SNP discovery by building catalogues of loci and comparing sequencing reads among and within individuals. The following Stacks programs and associated filter parameters were used to assemble catalogues of loci and discover SNPs before the custom Python scripts were applied to the sequence data for candidate sex-ID marker discovery. The Stacks program *process_radtags* was run with flags *q* and *r-bestrad* to remove low-quality reads, distinguish barcodes and demultiplex the RAD-seq library. The *clone_filter* program was used to remove potential PCR duplicates from the library. Since no *L. cyanellus* reference genome was available, we used the Stacks *de novo* pipeline to identify and genotype loci. The *ustacks* program was run with a required minimum depth of read coverage (*m*) of five to confidently call SNPs (Catchen *et al*., 2013). In *ustacks* we set the maximum distance in nucleotides allowed between reads within an individual at a locus (*M*) to be 2, 3, 4, 5, 6, 7 and 8. An *M* of 2 means that if there is

more than one nucleotide difference between reads, then these reads will be called as two different loci. In the *cstacks* program we specified the maximum number of nucleotide mismatches among individuals (*n*) at a locus to be *M*, *M* + 1 and *M* − 1 for each previously listed *M* value. Greater *M* and *n* parameters allow for more nucleotide mismatches within individuals and among individuals per locus, which leads to few lower loci identified. We used a variety of different parameter values for *M* in *ustacks* and *n* in *cstacks* to maximize the chance of detecting candidate sex-linked loci (Mussmann *et al*., 2021). Although using a wide range of parameter values can increase the rate of false-positive candidate sex-linked loci detected, we prioritized finding as many potentially sex-linked loci as possible and used validation and further testing to discover these false positives. We ran the *sstacks* and *tsv2bam* programs with default parameters for each *M* and *n* value. The *gstacks* program, which identifies SNPs within the metapopulation for each locus, was set with default parameters for each *M* and *n* value. We set the minimum percentage of individuals in a population required to process a locus for that population (*r*) to be 0.2 in the *populations* program.

2.2.2 | Sequence data analysis

To find evidence for which sex is heterogametic, we compared mean coverages over all loci and the total number of loci detected between the male and female populations used in our libraries by conducting two-sample *t*-tests. We visually inspected the data for normality using Q–Q plots and we tested for normality using Shapiro–Wilk normality tests. We also conducted Fisher's *F* tests to check for equal variances in mean coverages over all loci and the mean total number of loci between males and females. We removed one male sample (LCM_16) as an outlier when running comparisons due to this individual's high mean coverage (51.35*x*) and low number of loci (five). Mean coverages, number of loci and total SNPs detected were retrieved from the Stacks output using *M* = 4 and *n* = 4 parameters.

2.2.3 | Sex-ID marker discovery

We used several custom Python scripts (https://github.com/delomast/stacks_to_sex_marker) on the filtered and processed sequence data to identify two different types of possible sex identification markers: biallelic SNP markers (SNP candidates) and restriction-site associated sequences present in only one sex and absent in the other (pres-abs candidates). The SNP candidate discovery script (vcf_sex_markers.py) uses a sample list identifying the phenotypic sex of each individual used in the RAD-seq library in conjunction with the Stacks *populations* program's SNPs VCF file generated for each *M* and *n* value and finds SNPs heterozygous in all heterogametic individuals (males if XY SD, female if ZW SD) for the same two alleles and homozygous for a shared allele in the homogametic sex. We used another Python script to select and compile SNP candidates genotyped in at least 50% of the individuals in the RAD-seq library using the XY and ZW text files that were generated from the vcf_sex_markers.py script. We conducted a chi-square test to detect if the number of candidate ZW/ZZ and XX/XY SNPs was different from a 1:1 split as expected based on randomly sampled autosomal SNPs between males and females.

For pres-abs candidates, we were not interested in finding a single SNP, but instead a larger locus. A locus that is present in one sex and absent in the other would have been filtered out by the end of the Stacks pipeline, so it is necessary to investigate Stacks output from the middle of the pipeline. The pres-abs candidates python script (presence_absence_sex_markers.py) also uses a sample list identifying the phenotypic sex of each individual used in the RAD-seq library, combs through the Stacks *cstacks* and *sstacks* TSV output files of each *M* and *n* value and pulls consensus sequences present in at least 7/10 individuals in one sex and absent in the other sex. We conducted a chi-square test to detect if the number of candidate ZW/ZZ and XX/XY pres-abs loci was different from a 1:1 split as expected based on randomly sampled autosomal loci between males and females.

2.3 | Sex-ID marker development

The SNPs were chosen for TaqMan assay development if they genotyped in at least 12/20 individuals and if the SNP was called in at least two sets of conditions (*M* or *n* values). We chose SNP markers that were positioned away from the ends of the candidate loci, leaving at least 18–30 bp on either end of the SNP to allow for the design of forward and reverse primers. We also chose candidate SNP markers that had no more than three adjacent flanking polymorphisms to ensure primers and probes would bind and amplify effectively. We used the Thermo Fisher Custom TaqMan Assay Design Tool (https://www.thermofisher.com/order/custom-genomic-products/tools/genotyping/) with default parameters to design primers and fluorescently labelled hydrolysis probes.

Candidate presence-absence loci (pres-abs candidates) were selected for primer development based on if the loci were observed in at least four of eight *M* values. Forward and reverse primers for the pres-abs candidates were designed using Primer3web V 4.1.0 (Untergasser *et al*., 2012) with default design parameters and the primers were produced by Sigma-Aldrich (Sigma-Aldrich Corp, St Louis, MO, USA).

2.4 | Sex-ID marker testing

TaqMan assays for SNP candidates and end-point PCR with subsequent gel electrophoresis for pres-abs candidates were conducted at the University of Arizona Genetics Core (UAGC). For the SNP candidates, TaqMan Fast Advance Master mix was used and reactions were run on an Applied Biosystems 7900 HT Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using the following thermocycling profile: (1) 95°C for 5 min, (2) 95°C for 15 s, (3) 58°C for 1 min, with steps 2 and 3 repeated 40 times for annealing and extending. Each TaqMan SNP assay was a 15 μl reaction comprising

7.5 µl of TaqMan Fast Advance Master Mix, 0.375 µl of 40x TaqMan Genotyping assay (SNP candidate), 5.125 µl of PCR water and 2.0 µl of DNA.

The first two SNP candidates we developed were tested on four individuals that were genotyped for the SNPs from the RAD-seq library for validation and 20 additional individuals of known phenotypic sex to determine how well the markers worked in identifying phenotypic sex. The subsequent SNP markers we developed were tested on more individuals of unknown genotype after we acquired additional *L. cyanellus* DNA from unrelated individuals. We also increased the number of individuals used to validate the bioinformatic discovery after seeing failed validation in the first two SNP candidates. These later tests were conducted on eight individuals that were genotyped from the RAD-seq library and 27 individuals of known phenotypic sex.

The pres-abs candidates were initially tested for phenotype concordance on one male and one female used in the RAD-seq library to validate our bioinformatic discovery. In addition to the pres-abs candidate's forward and reverse primers, MiFish primers (Miya *et al.*, 2015) purchased from Integrated DNA Technologies (Coralville, IA, USA), were added to the PCR master mix. The MiFish primers are universal PCR primers that amplify a region of the 12SrRNA gene (163–185 bp) in most fish species (Miya *et al.*, 2015). The MiFish primers were used as a control by ensuring that an individual was not inaccurately marked as 'absent' for the pres-abs candidate because the PCR failed. The UAGC initially used the Terra PCR Direct Polymerase PCR kit (TaKaRa Bio USA Inc., Mountain View, CA, USA) on an ABI Applied Biosystems 7900 HT Fast Real-Time PCR System with the following thermoprofile: (1) 98°C for 3 min, (2) 98°C for 15 s, (3) 68°C for 20 s (−0.5°C/cycle), (4) 72°C for 1 min, (5) 98°C for 15 s, (6) 60°C for 20 s, (7) 72°C for 1 min, (8) 72°C for 2 min, (9) 4°C for 10 min and (10) 10°C indefinitely. Steps 2–4 were repeated 16 times for touchdown PCR followed by steps 5–7 repeated 23 times for final annealing. Each pres-abs PCR was a 15 µl reaction comprising 7.5 µl of 2x Terra Direct PCR buffer, 0.75 µl of MiFish(+) forward primer, 0.75 µl of MiFish(+) reverse primer, 0.75 µl of pres-abs forward primer, 0.75 µl of reverse primer, 3.0 µl of PCR water, 0.5 µl of Terra PCR Direct Polymerase Mix and 1.0 µl of sample DNA. This protocol is based on the Terra PCR Direct Polymerase Mix User Manual (https://www.takarabio.com).

We adjusted PCR conditions after observing amplification of both sexes in all but one pres-abs marker (ZW_4) during the validation step. First, we selected the most genotyped pres-abs candidate found during the bioinformatics (ZW_1) to compare the performance of the polymerase kit originally used (Terra PCR Direct) against the Platinum Hot Start PCR Master Mix (Life Technologies-Thermo Fisher, Waltham, MA). Next, we attempted PCR reactions with both polymerase kits with and without the addition of the MiFish primers. After seeing clearer and tighter bands with the Platinum Hot Start PCR Master Mix we performed PCR reactions with this kit at different annealing temperatures (59, 60.5, 61.5, 62.9, 64.4, 65.7, 66.7 and 68°C). All these reactions were conducted with DNA from one male and one female who were genotyped as absent and present, respectively, for this ZW pres-abs candidate in the bioinformatics.

After seeing unexpected universal amplification in samples using our pres-abs candidate primers despite varied PCR conditions, we conducted high-resolution melting (HRM) assays using each pres-abs candidate primer. HRM assays allow for screening of bp deviations and copy number differences among samples (Borun *et al.*, 2014). The HRM assays detect PCR amplicon quantity differences resulting from one sex containing one copy of the target sequence template (heterozygous) and the other sex containing two copies of the target sequence template (homozygous). This technique can be used to detect sex-linked sequences that are homozygous in one sex and heterozygous in the other, as well as SNPs associated with sex (England *et al.*, 2021; Matsunaga *et al.*, 2014; Perez-Enriquez *et al.*, 2020). In addition, since HRM assays are useful in the detection of amplicon sequence differences, they can detect primer sequence homology and PCR fidelity (Aralar *et al.*, 2020). We ran the HRM assays on DNA from all individuals used in the RAD-seq library as well as 10 phenotypic males and 11 phenotypic females.

For the HRM assays sample DNA was normalized to 1 ng µl^{-1}, plated into a 384-well qPCR reaction and then dried. The total reaction template was 1 ng per well and reactions were run in duplicate. The qPCR reaction master-mix was a 15 µl reaction comprising 7.5 µl of SYBR Select Master Mix (Applied Biosystems, Waltham, MA), 0.75 µl of forward pres-abs candidate primer, 0.75 µl of reverse primer and 6 µl of PCR water. Reactions were conducted on the Roche LightCycler 480 with the following thermocycling protocol: (1) 45°C for 15 min to resolubilize DNA after loading, (2) 95°C for 5 min for Hot Start Taq activation, (3) 95°C for 15 s, (4) 60°C for 1 min, (5) 60°C for 1 min to complete PCR extension prior to melting, (6) 50°C to 90°C with a 1°C increase per minute for melt analysis, then steps 3 and 4 repeat 40 times.

The presence or absence of an amplicon from the HRM assays was used as the initial detection of concordance with phenotypic sex. The XY pres-abs candidates were considered concordant with phenotypic sex if they produced an amplicon in males and failed to produce a PCR product in females, and the ZW pres-abs candidates were considered concordant with phenotypic sex if they produced an amplicon in females and failed to produce a PCR product in males.

In addition, threshold cycle (C_T) data were used from the HRM assays to detect amplicon polymorphisms and copy number differences between males and females for each pres-abs candidate. We calculated the mean C_T of each duplicate reaction so that each sample had one C_T value which was the mean C_T from the two reactions. We visually inspected the C_T data for normality using Q–Q plots and we tested for normality using Shapiro–Wilk normality tests. We used the Wilcoxon rank sum test to detect differences in mean C_T between males and females for each pres-abs candidate HRM assay.

Sanger sequencing was conducted on amplicons from one pres-abs candidate marker (ZW_3) due to males having significantly higher mean C_T than females during the HRM assay. The Sanger sequencing was conducted on ZW_3 amplicons from two males with relatively high C_T, two males with relatively low C_T, two females with relatively high C_T and two females with relatively low C_T. In addition, end-point PCR with varying amplification cycles (22, 27, 32 and 40) and

Table B.1. *Lepomis cyanellus* Sex-ID SNP candidates' performances at correlating with phenotypic sex

Sex-ID SNP candidate	Phenotype	Samples from RAD-seq library validation (*N*)	Validation phenotype concordance score (%)	Randomly sampled (*N*)	Randomly sampled phenotype concordance score (%)	Total undetermined genotype	Total score (%)
ZW_218	Male	4	100.0	13	84.6	0	88.2
	Female	4	100.0	14	21.4	2	38.9
	Total	8	100.0	27	51.9	2	63.0
XY_285	Male	4	100.0	13	53.8	0	64.7
	Female	4	100.0	14	50.0	2	66.7
	Total	8	100.0	27	51.9	2	62.9
ZW_204	Male	4	100.0	13	69.2	0	76.5
	Female	4	50.0	14	42.9	2	44.4
	Total	8	75.0	27	55.6	2	60.0
ZW_224	Male	2	50.0	10	80.0	0	75.0
	Female	2	50.0	10	40.0	0	41.7
	Total	4	50.0	20	60.0	0	58.3
XY_261	Male	2	50.0	10	70.0	0	67.0
	Female	2	50.0	10	50.0	1	33.0
	Total	4	50.0	20	60.0	1	58.3
XY_271-3	Male	4	50.0	13	30.8	1	29.4
	Female	4	75.0	14	42.9	2	50.0
	Total	8	62.5	27	37.0	3	42.9
ZW_228	Male	4	0	13	0	17	0
	Female	4	0	14	0	18	0
	Total	8	0	27	0	35	0
ZW_293	Male	4	0	13	0	17	0
	Female	4	0	14	0	18	0
	Total	8	0	27	0	35	0

Note: Samples used in the RAD-seq library construction were used for the validation step. Randomly sampled, wild-caught, *L. cyanellus* were used to further test SNP concordance with phenotypic sex.

subsequent gel electrophoresis using these eight samples were inspected for band intensity differences between the males and females, which may indicate copy number differences of the template sequence (Pan *et al.*, 2015).

2.5 | Ethical statement

The care and use of experimental animals complied with the United States' animal welfare laws, guidelines and policies as approved by the University of Arizona's Institutional Animal Care and Use Committee.

3 | RESULTS

3.1 | Sequencing and Sex-ID candidate selection

We generated 865,345,120 raw reads and retained 77.7% of them after removing low-quality reads, reads with ambiguous restriction enzyme cut sites or barcodes, and PCR duplicates. The mean coverage of the library was 27.77*X* (standard deviation = 6.74*X*). The mean coverages for both the male and female populations used in our RAD-seq library were normally distributed (Shapiro–Wilk normality test, $W > 0.95$, P value >0.82) and had equal variances (Fisher's F test, $F_{9/8} = 1.3634$, P value = 0.6729). The mean total numbers of loci in the male and female populations were normally distributed (Shapiro–Wilk normality test, $W > 0.93$, P value >0.53) and had equal variances (Fisher's F test, $F_{9/8} = 0.85$, P value = 0.8069). The male and female populations had similar mean coverages (male = 29.20*X*, female = 26.34*X*; two-sample t-test, $t_{17} = -0.20$, P value = 0.8439) and similar mean numbers of loci (male = 297,693.89, female = 312,583.70; two-sample t-test, $t_{17} = 0.20$, P value = 0.8423). Population heterozygosity for males and females was similar (~0.27).

We detected 1,379,619 SNPs in our RAD-seq library. When using 10 out of 20 as the minimum number of individuals genotyped for the SNP, we found 60 XX/XY SNP candidates with one allele fixed in females and only heterozygous genotypes in males, as well as 66 ZW/ZZ SNP candidates with one allele fixed in males and only

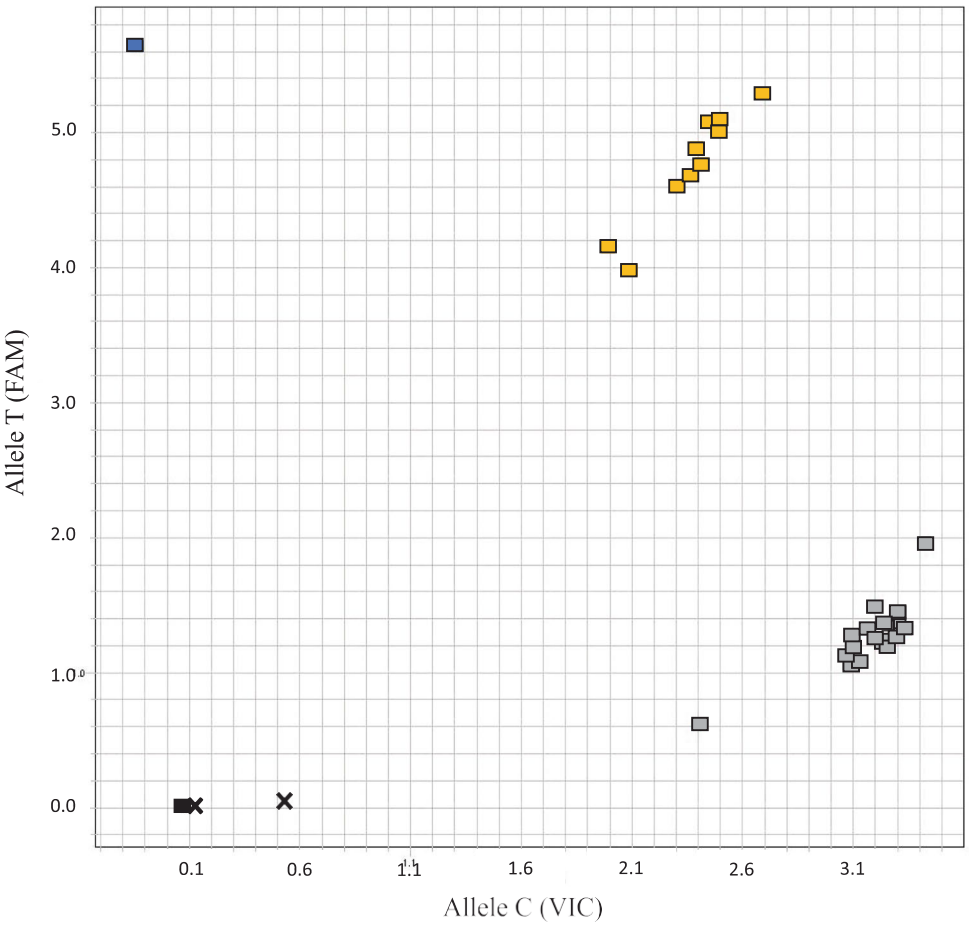

Figure B.1. An allelic discrimination plot for the top-scoring Sex-ID SNP candidate (ZW_218) for *Lepomis cyanellus*. The C/C (grey squares) should be males and the C/T (orange squares) should be females according to the bioinformatic discovery. One female was homozygous for the T allele (blue square (■) C/C; (■) C/T; (■) T/T;(✘) undetermined; (■) NTC

heterozygous genotypes in females. The number of SNPs that fit one pattern of SD versus another were not different from a 1:1 split as expected based on randomly sampled autosomal SNPs between males and females (chi-square test, d.f. = 1, *P* value = 0.592983). Five ZW SNP candidates and three XY SNP candidates met our selection criteria for primer development and testing (Table 1 and Supporting Information Table S1).

When using seven out of 10 as the minimum number of individuals genotyped for a pres-abs marker, we found 36 ZW/ZZ pres-abs candidates where the sequence was present in females and absent in males and five XX/XY pres-abs candidates where the sequence was present in males and absent in females. When tested against a 1:1 split as expected based on a random number of autosomal loci between the sexes, we saw that *L. cyanellus* exhibited a larger number of pres-abs candidates that fit the ZW/ZZ pattern than the XX/XY pattern (chi-square test, d.f. = 1, *P* value < 0.00001). Six ZW pres-abs candidates and two XY pres-abs candidates met our selection criteria for primer development and testing (Supporting Information Table S1).

No SNP candidates were genotyped in more than 14/20 individuals and no pres-abs candidates were genotyped in more than 8/10 individuals. One male (LCM_22) was not genotyped for any of the candidate Sex-ID loci and had a relatively low mean depth of coverage ($\bar{X}$= 20.7*X*) as compared to the mean depth of coverage for the entire library ($\bar{X}$= 27.7*X*) and a relatively low number of loci genotyped (21,492.00) as compared to the mean number of loci per sample (290,254.35).

3.2 | Sex-ID marker testing

The top-performing SNP candidate (ZW_218) had a combined Sex-ID concordance of 63.0% with males and females (Table 1 and Figure 1) and a relatively high concordance with males (88.2%), but a low concordance with females (38.9%). All ZW SNP candidates had a higher male concordance than female concordance. The XY SNP candidates did not share this same pattern in correlating with one sex more consistently than the other. Two ZW SNP candidates' (ZW_228 and ZW_293) TaqMan assays results were ambiguous and genotypes from these assays could not confidently be called. All but one SNP marker (ZW_224) genotyped some individuals as homozygous for the alternate allele in the sex being tested as heterogametic (*i.e.*, genotyped YY males or WW females).

Only pres-abs candidate ZW_4 passed the initial validation step. All other pres-abs candidates did not pass the validation step due to amplification in both sexes. Attempts to increase the specificity of pres-abs candidates *via* changes to PCR conditions and the removal of the MiFish primers did not resolve the issue of amplification in

Table B.2. Phenotypic sex concordance scores for the pres-abs candidate markers tested *via* PCR amplification with *Lepomis cyanellus* samples

Pres-abs candidate	Phenotype	Samples from RAD-seq library validation (*N*)	Validation phenotype concordance score (%)	Randomly sampled (*N*)	Randomly sampled phenotype concordance score (%)	Total score (%)
ZW_1	Male	10	0.00	10	0.00	0.00
	Female	10	100.00	11	100.00	100.00
	Total	20	50.00	21	52.38	51.22
ZW_2	Male	10	0.0	10	10.0	5.00
	Female	10	100.0	11	100.0	100.0
	Total	20	50.0	21	57.14	53.66
ZW_3	Male	10	30.00	10	10.00	10.00
	Female	10	100.00	11	72.72	90.00
	Total	20	65.00	21	42.86	53.66
ZW_4	Male	10	50.00	10	10.00	30.00
	Female	10	80.00	11	63.63	71.43
	Total	20	65.00	21	38.10	51.22
ZW_5	Male	10	0.00	10	10.00	5.00
	Female	10	100.00	11	100.00	100.00
	Total	20	50.00	21	57.14	53.66
ZW_6	Male	10	0.00	10	10.00	5.00
	Female	10	100.00	11	100.00	100.00
	Total	20	50.00	21	57.14	53.65
XY_2	Male	10	0.00	10	20.00	10.00
	Female	10	100.00	11	100.00	100.00
	Total	20	50.00	21	61.90	56.10

samples that were genotyped as absent of the target locus in the bioinformatic discovery.

When using amplification (amplicon presence or absence) as the marker for males or females in the pres-abs candidate HRM assays, the top scoring marker (XY_2) had a phenotype and genotype concordance score of 56.10% (Table 2). All pres-abs candidates amplified in most samples despite phenotypic sex or whether or not the locus was absent in the sample during the bioinformatic discovery.

For pres-abs candidate ZW_3, the mean C_T between males (C_T = 29.54) and females (C_T = 27.14) was significantly different (Wilcoxon rank sum test, W = 70, P value = 0.006471). The C_T range for ZW_3 was 23.81–36.45, with none of the female reactions having a higher C_T than 30 and none of the male reactions having a lower C_T than 26. Sanger sequencing the ZW_3 amplicons of two males with high C_T (LCM71 C_T = 30.51, LCM4 C_T = 32.34), two males with lower C_T (LCM72 C_T = 26.98, LCM2 C_T = 26.97), two females with high C_T (LCF21 C_T = 28.74, LCF26 C_T = 29.67) and two females with low C_T (LCF12 C_T = 26.44, LCF65 C_T = 26.17) did not reveal any polymorphisms between the male and female amplicon sequences. In addition, end-point PCR with varying amplification cycles (22, 27, 32 and 40) and subsequent gel electrophoresis using these eight samples resulted in the expected reduced band brightness in the high C_T samples, but there was no concordance with band brightness depending on phenotypic sex (Figure 2).

The remaining pres-abs candidates showed no difference in C_T between males and females (Wilcoxon rank sum test, W > 113, P value >0.1672). Primer XY 1 failed PCR condition optimization, resulting in primarily "undetected" reaction results, and was therefore left out of C_T analysis and further presence–absence PCR testing.

4 | DISCUSSION

In some cases, RAD-seq methods have been effective for producing Sex-ID markers and elucidating the SD systems in species across various classes (Fowler & Buonaccorsi, 2016; Gamble *et al.*, 2015; Gamble & Zarkower, 2014; Jeffries *et al.*, 2018; Vu *et al.*, 2019). Even without finding functional SNP or pres-abs markers, it is possible to infer SD systems based on differences in depths of coverage and SNP densities between the sexes (Palmer *et al.*, 2019). The heterogametic sex will have higher heterozygosity, higher SNP density and, in species with highly divergent sex chromosomes, lower depth of coverage associated with the nonrecombining loci (Palmer *et al.*, 2019). Similarly, when using an appropriate minimum depth of coverage for the mean coverage of the RAD-seq library, as we did in our study (m = 5X coverage), we may observe more loci for the heterogametic sex (Palmer *et al.*, 2019). In our RAD-seq library, the overall differences in heterozygosity, SNP densities, number of loci and depths of coverage

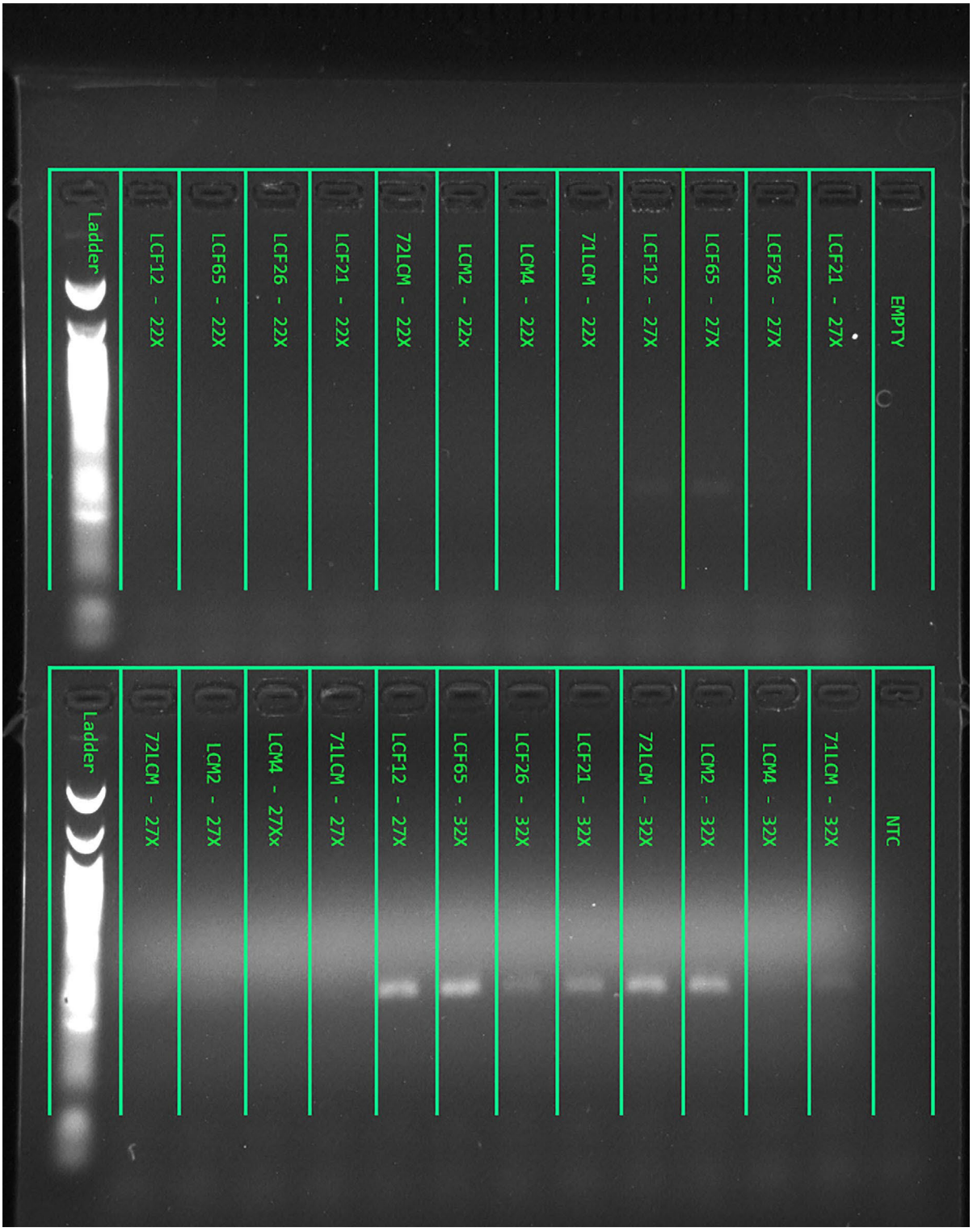

Figure B.2. A gel from PCR using various numbers of amplification cycles (22*X*, 27*X* and 32*X*) for with pres-abs candidate ZW_3. LCM, male samples; LCF, female samples

between males and females were slight. Therefore, no inferences could be made using this data on which sex, if either, is heterogametic. Having more information on potential SD regions would allow us to do a more targeted and powerful comparison in heterozygosity, SNP densities, number of loci and coverages between the sexes instead of making comparisons of these metrics based on a reduced representation of the entire genome.

When using genotype success in 10/20 individuals as a minimum threshold for consideration, we saw that there were six more potential SNP candidates that fit the ZW/ZZ pattern than the XX/XY

pattern. Overall, the numbers of SNP candidates that fit the ZW/ZZ or the XX/XY pattern were similar. The ZW SNP candidates were often homozygous, resulting in a relatively high concordance with males with a low concordance with females. However, some males were genotyped as heterozygous for the ZW SNP candidates and these markers showed they were not sex-linked within our sampled population. Both of the ZW and XY SNP candidate assays produced results that varied (Table 1) and proved to be ineffective as a genetic Sex-ID tool.

With our bioinformatic selection threshold for pres-abs candidates we saw more ZW/ZZ candidates than XX/XY candidates. The larger number of ZW/ZZ pres-abs candidates discovered with our selection criteria could be a clue to the underlying sex chromosome system of *L. cyanellus*. Bluegill, *Lepomis macrochirus*, a closely related species to *L. cyanellus*, are speculated to have an underlying ZW/ZZ sex determination system with increased temperatures skewing sex ratios towards male in some populations (Shen *et al.*, 2016; Wang *et al.*, 2018). In addition, both *L. macrochirus* and *L. cyanellus* exhibit heavily male-skewed sex ratios when hybridized with each and other *Lepomis* species (Childers & Bennett, 1961), suggesting that females may be the heterogametic sex, according to Haldane's rule (Haldane 1922). In a separate study, we saw heavily male-skewed sex ratios (82.61%, 95% confidence interval = 64.10%–100.00%) of F1 progeny in cohorts of *L. cyanellus* reared in temperatures of 27–30°C (unpublished data). Evidence from these F1 sex ratios, the male-skewed sex ratios in *L. cyanellus* hybrid progeny and the high number of pres-abs candidates that fit the ZW/ZZ suggests that *L. cyanellus* may have an underlying ZW/ZZ SD system with a temperature influence. However, if temperature has a significant effect on SD in the population or environment our samples were drawn from, then identifying an accurate genetic sex marker based on observations of phenotypic sex would be impossible.

All the tested ZW and XY pres-abs candidates resulted in PCR product and amplification in both sexes during HRM assays. This universal amplification was unexpected considering the pres-abs candidates we developed were from sequences that were observed only in one sex and were extracted with stringent to permissive parameter tolerances in the bioinformatics discovery. The ubiquitous amplification from the pres-abs candidate PCR reactions resulted in a high concordance of the respective sex that was putatively present for the target sequence and a very low, often zero, concordance with the sex that was absent of the target sequence during bioinformatic discovery. This pattern resulted in an overall pres-abs candidate marker phenotype concordance ranging from 51.22%–56.10% (Table 2).

The PCR amplicons we produced with the pres-abs primers could be due to homology in the other sex's genome or to a lack of specificity and fidelity involving the developed primers and PCR conditions (Cha & Thilly, 1993; Lebedev *et al.*, 2008). Even though the use of Hot Start PCR conditions and Hot Start DNA polymerases increases the specificity, yield and fidelity of PCR reactions (D'Aquila *et al.* 1991, Lebedev *et al.*, 2008), none of our reaction conditions prevented amplification from occurring during validation. Similarly, Gamble *et al.* (2015) found that most Sex-ID candidates identified during bioinformatics failed PCR validation and amplified in both sexes. Other studies aimed at creating Sex-ID markers using RAD-seq have shown that PCR validation may fail if the loci used to build the pres-abs candidates are flanked by sequences conserved between the sexes (Fowler & Buonaccorsi, 2016; Gamble, 2016). Fowler and Buonaccorsi (2016) used a reference genome in their study to map their pres-abs candidates and found that a post-PCR restriction enzyme digest allowed them to rescue the utility of their pres-abs markers by cleaving the pres-abs marker amplicon, resulting in two bands in males and one band in females. Our SNP candidate discovery would have detected this difference in our pres-abs candidates. However, obtaining a reference genome for green sunfish would allow us to detect any homologous sequences in males and females flanking our pres-abs candidates, thus allowing us to redesign our pres-abs candidate forward and reverse primers so that we could achieve sex-specific amplification.

The C_T data analysis from the HRM assays were inconclusive at detecting polymorphisms and copy number differences between male and female pres-abs candidate amplicons. Although we saw no evidence of this occurring in the examined RAD-seq literature, it is theoretically possible that the 'Y-linked' or 'W-linked' pres-abs candidates we found were instead located on the X or Z chromosome, respectively. Since the homogametic sex has two copies of the X-linked or Z-linked sequences, while the heterogametic sex only has one, sex-linked loci from the homogametic sex are more likely to be sequenced by RAD-seq methods (Palmer *et al.*, 2019) and Y-linked or W-linked loci maybe missed during sequencing or bioinformatically filtered out due to low coverage. The PCR validation of these 'Y' or 'W' pres-abs candidates will then result in amplification in both sexes, as we saw in our results. However, with the mean depth of coverage in our RAD-seq library being ~27*X*, and using our pres-abs candidate selection criteria, we should have detected the X-linked or Z-linked sequences in the heterogametic sex. The pres-abs candidate ZW_3 HRM assays did result in significantly higher mean C_T values in males than females. Further analysis of the male and female ZW_3 amplicons *via* Sanger sequencing and reduced cycle end-point PCR with subsequent gel electrophoresis concluded that the differences observed in male and female C_T values were likely not due to sequence polymorphisms nor copy number differences. This significant difference in C_T values could be due to DNA degradation of the template sequence in males or due to reagent preparation (Lee *et al.*, 2011). Additional qPCR methods such as developing fluorescently labelled probes within the amplicon could further clarify if copy number differences were the reason for the higher C_T in many of the males.

Searching for sex markers *via* RAD-seq allows for relatively inexpensive and simple genetic comparisons in many samples with deep sequencing coverage without the need for a reference genome (Rochette & Catchen, 2017; Willing *et al.*, 2011). However, an issue with using RAD-seq is that it produces a reduced representation of the investigated genomes (Baird *et al.*, 2008) by targeting small sections of the genome adjacent to restriction enzyme cut sites. In species lacking sex chromosomes or species with little divergence in their sex chromosomes, the sex-specific regions of the genome can be

missed and therefore absent from the RAD-seq library (Palmer *et al*., 2019). By switching to a restriction enzyme with a 4 bp cut site, such as MluCI, instead of a 6 bp cutter, such as PstI, sampling more of the genome is possible, increasing the odds of finding sex-specific loci (Gamble, 2016). The downside to using a restriction enzyme that cuts more frequently is that to maintain adequate coverage, it is necessary to either reduce the number of samples used in the library or increase the capacity, and associated costs, of the sequencing run. Another method for finding Sex-ID markers with RAD-seq is using linkage maps derived from sequencing parents and offspring (Baxter *et al*., 2011; Brown *et al*., 2016; Wilson *et al*., 2014). With linkage maps, sex chromosomes and their associated loci are discovered by identifying regions of the genome where there is no recombination in males and females (Palmer *et al*., 2019). Linkage maps allow for the discovery of loci in homomorphic sex chromosomes through the direct measurement of recombination rates instead of through SNPs associated with sex in unrelated individuals (Palmer *et al*., 2019).

All Sex-ID candidates we tested did not correlate with phenotypic sex, a result obtained by others studying the SD system of the closely related *L. macrochirus* (Gao *et al*., 2010). Gao *et al*. (2010) were unable to find sex-specific markers in *L. macrochirus* using AFLPs and Wang *et al*. (2018) concluded that 'The sex determination system in bluegill is very complicated.' Al-Ablani (1997) stated that the SD mechanism in *L. macrochirus* is probably polygenic, and that sex differentiation is dictated by the sum of influences from various genes on autosomes. Wang *et al*. (2018) reported that at least some *L. macrochirus* populations exhibit a ZZ/ZW sex determination system and that temperature influences sex differentiation in some geographic populations. Perhaps *L. cyanellus* also exhibit a complicated SD system that may produce challenges in the development of Sex-ID markers for this species, such as environmental drivers of sex, several sex determining loci acting epistatically or a very small sex determining region.

Nevertheless, many more methods could be attempted to find Sex-ID markers in *L. cyanellus* and elucidate their SD system. An expression-based approach, such as using transcriptomes from gonadal tissue, has been effective at producing Sex-ID markers (Lamatsch *et al*., 2015; Lu *et al*., 2014) and could provide a more targeted approach for identifying genetic differences between males and females (Palmer *et al*., 2019). If there is access to multiple generations, then a segregation analysis approach or linkage mapping could be attempted to track how SNPs are inherited by male and female progeny from their parents (Palmer *et al*., 2019). Sequencing a reference genome would allow for mapping putative sex-specific loci acquired in this study onto the genome. The reference genome could then be used to discover if homologous sequences in males and females are flanking our pres-abs candidates, causing amplification in both sexes during PCR testing. Test crosses with sex-reversed individuals and wild-type individuals have been used to elucidate fishes' SD systems by assessing the sex ratios of the progeny (Desprez *et al*., 1995; Gomelsky *et al*., 2002). However, before test crosses are attempted with *L. cyanellus*, rearing trials at various temperatures (Shen *et al*., 2016) and rearing densities (Roncarati *et al*., 1997) should be executed to see how these environmental factors might affect sex differentiation. Once the effects of temperature and rearing density on sex ratios has been determined, deviations from those sex ratios in progeny from crosses involving sex-reversed individuals could be used as evidence for the underlying sex chromosome system in *L. cyanellus*. It is possible that the wild population we sampled to build our RAD-seq library features environmental influence on sex differentiation. This would complicate the discovery of Sex-ID markers since the underlying SD system may have been masked by environmental effects that resulted in a different sexual phenotype. The discovery of effective Sex-ID markers would be facilitated by sampling from families that are known to have a balanced 1:1 sex ratio.

This is the first study attempting to find Sex-ID markers in *L. cyanellus*. Our findings suggest that genetic differences between males and females may be limited and that there may be little divergence between sex chromosomes or sex-coding autosomes. Even though the methods we implemented did not result in a functional Sex-ID marker, there are still many options available for future investigation of the SD system of *L. cyanellus*. First, any further efforts at developing Sex-ID markers in *L. cyanellus* should be done by sampling from either a wild or captive population that has a known 1:1 sex ratio. In addition, understanding what, if any, environmental factors influence sex differentiation in *L. cyanellus* could ensure further genetic investigations into this species' SD mechanisms are not complicated by environmentally influenced sex differentiation. Once environmental influences on sex ratio are uncovered they can be experimentally controlled. This will allow the underlying genetic SD system to be investigated with conventional means, such as assessing the sex ratios of progeny derived from gynogenesis, androgenesis or crosses involving sex-reversed individuals (Glennon *et al*., 2012; Gomelsky *et al*., 2002; Li *et al*., 2018). Future genomic research into the SD system of *L. cyanellus* may benefit from using a restriction enzyme with a 4 bp cut site that will sample more of the genome. Using a restriction enzyme that cuts more frequently, in combination with linkage maps derived from parents and offspring with a 1:1 sex ratio, would offer higher resolution in how loci and SNPs sexually segregate and are inherited, providing a more targeted discovery of Sex-ID candidates. Employing an expression-based, RNA-seq approach may also provide a more targeted investigation into genetic differences between males and females. Sampling gonadal tissue from sexually differentiating individuals for transcriptomes would allow for the potential discovery of sex-related genes or sex-specific markers (Lamatsch *et al*., 2015).

An initial assessment of the feasibility of using the TSC strategy for *L. cyanellus* requires identifying what genetic and environmental factors might impact the SD system of this species. Our research represents the nascent attempts at these efforts. The results we present offer future researchers the opportunity to attempt different methods that may provide finer resolution for testing markers, investigation of more of the genome and discovery of the underlying sex chromosome system of *L. cyanellus* with sex ratios, thus providing more insights into the evolution of SD systems in the *Lepomis* genus and a possible management tool for invasive *L. cyanellus* populations.

ACKNOWLEDGEMENTS

William Stewart of the United States Bureau of Reclamation solicited and provided funding for this research (United States Bureau of Reclamation, grant number G18AC0061 and modification G19AC0039). Alex Erwin, Ellie Wisely, Natalie Mercer and Karla Vargas with the University of Arizona Conservation Genetics Laboratory provided advice on project design, methods, and results interpretation. Nathan Berg with the Arizona Game and Fish Department and Colby Roberts with the University of Arizona assisted with *L. cyanellus* tissue collection.

CONFLICT OF INTEREST

There is no conflict of interest declared in this study.

AUTHOR CONTRIBUTIONS

C.N.T. worked on the study design, prepared the RAD-seq libraries, ran bioinformatics analysis, analysed data and wrote the first draft of the manuscript. D.K.C. worked on the study design, wrote bioinformatic scripts for marker selection, ran bioinformatics analysis, analysed data and consulted with C.N.T. on bioinformatic methods. M.R.C. worked on study design, secured resources for the study and consulted with C.N.T. on methods and results. D.L.E. conducted and supervised the RAD-seq library preparation, and managed RAD-seq library data. T.A.D. wrote bioinformatic scripts for marker selection and consulted with C.N.T. on methods and results. J.T.S. conducted all wet-lab methods, helped formulate alternative PCR conditions for primer testing, designed HRM assays, edited manuscript drafts and consulted on interpretation of results. D.J.S. worked on the study design, consulted with C.N.T. on methods and results, and secured resources for the study. S.A.B. secured funding and resources for the study, assisted with *L. cyanellus* sample collections and consulted with C.N.T. on results. M.C. worked on the study design and consulted with C.N.T. on the laboratory methods and results.

ORCID

Chad N. Teal https://orcid.org/0000-0002-7847-0841

REFERENCES

Ali, O. A., O'Rourke, S. M., Amish, S. J., Meek, M. H., Luikart, G., Jeffres, C., & Miller, M. R. (2016). RAD capture (rapture): Flexible and efficient sequence-based genotyping. *Genetics*, *202*, 389–400.

Al-Ablani, S. A. (1997). *Use of synthetic steroids to produce monosex populations of selected species of Sunfishes (Family: Centrarchidae)* (Doctoral dissertation. Auburn University, Auburn, Alabama, USA).

Aralar, A., Yuan, Y., Chen, K., Geng, Y., Ortiz Velez, D., Sinha, M., … Fraley, S. I. (2020). Improving quantitative power in digital PCR through digital high-resolution melting. *Journal of Clinical Microbiology*, *58*, e00325–e00320.

Baird, N. A., Etter, P. D., Atwood, T. S., Currey, M. C., Shiver, A. L., Lewis, Z. A., … Johnson, E. A. (2008). Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS One*, *3*, e3376. https://doi.org/10.1371/journal.pone.0003376.

Bax, N. J., & Thresher, R. E. (2009). Ecological, behavioral, and genetic factors influencing the recombinant control of invasive pests. *Ecological Applications*, *19*, 873–888.

Baxter, S. W., Davey, J. W., Johnston, J. S., Shelton, A. M., Heckel, D. G., Jiggins, C. D., & Blaxter, M. L. (2011). Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. *PLoS One*, *6*, e19315. https://doi.org/10.1371/journal.pone.0019315.

Blasius, H.B. (2002). Chemical removal of green sunfish (*Lepomis cyanellus*) from O'Donnell Creek, Arizona. In: *Desert fishes council 2002 annual meeting*. San Luis Potosi, SLP.

Borun, P., Kubaszewski, L., Banasiewicz, T., Walkowiak, J., Skrzypczak-Zielinska, M., Kaczmarek-Rys, M., & Plawski, A. (2014). Comparative-high resolution melting: A novel method of simultaneous screening for small mutations and copy number variations. *Human Genetics*, *133*, 535–545.

Brown, J. K., Taggart, J. B., Bekaert, M., Wehner, S., Palaiokostas, C., Setiawan, A. N., … Penman, D. J. (2016). Mapping the sex determination locus in the h-apuku (*Polyprion oxygeneios*) using ddRAD sequencing. *BMC Genomics*, *17*, 448. https://doi.org/10.1186/s12864-016-2773-4.

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: An analysis tool set for population genomics. *Molecular Ecology*, *22*, 3124–3140.

Cha, R. S., & Thilly, W. G. (1993). Specificity, efficiency, and fidelity of PCR. *Genome Research*, *3*, 18–29.

Childers, W. F., & Bennett, G. W. (1961). Hybridization between three species of sunfish (Lepomis). *State of Illinois Natural History Survey Division, Biological Notes*, *46*, 1–15.

D'Aquila, R. T., Bechtel, L. J., Videler, J. A., Eron, J. J., Gorczyca, P., & Kaplan, J. C. (1991). Maximizing sensitivity and specificity of PCR by pre-amplification heating. *Nucleic Acids Research, 19*, 3749.

Desprez, D., Mélard, C., & Philippart, J. C. (1995). Production of a high percentage of male offspring with 17a-ethynylestradiol sex-reversed *Oreochromis aureus*: II. Comparative reproductive biology of females and F2 pseudofemales and large scale production of male progeny. *Aquaculture*, *130*, 35–41.

Du, J., Zhou, J., Li, S., Shao, J., Jiang, P., Dong, C., & Bai, J. (2021). A PCR-based method for genetic sex identification and evidence of the XX/-XY sex determination system in largemouth bass (*Micropterus salmoides L.*). *Aquaculture*, *545*, 737220, ISSN 0044-8486. https://doi.org/10.1016/j.aquaculture.2021.737220.

Dudley, R., & Matter, W. (2000). Effects of small green sunfish (*Lepomis cyanellus*) on recruitment of Gila chub (*Gila intermedia*) in Sabino Creek, Arizona. *The Southwestern Naturalist*, *45*, 24–29.

England, A. D., Kheravii, S. K., Musigwa, S., Kumar, A., Daneshmand, A., Sharma, N. K., … Wu, S. B. (2021). Sexing chickens (*Gallus gallus domesticus*) with high-resolution melting analysis using feather crude DNA. *Poultry Science*, *100*, 100924.

Fowler, B. L. S., & Buonaccorsi, V. P. (2016). Genomic characterization of sex-identification markers in *Sebastes carnatus* and *Sebastes chrysomelas* rockfishes. *Molecular Ecology*, *25*, 2165–2175.

Fuller, P., Cannister, M., & Neilson, M. (2021). *Lepomis cyanellus Rafinesque, 1819: Geological survey*. Gainesville, FL: Nonindigenous Aquatic Species Database. https://nas.er.usgs.gov/queries/FactSheet.aspx?speciesID=380.

Furman, B. L. S., Metzger, D. C. H., Darolti, I., Wright, A. E., Sandkam, B. A., Almeida, P., … Mank, J. E. (2020). Sex chromosome evolution: So many exceptions to the rules. *Genome Biology and Evolution*, *12*, 750–763.

Gamble, T. (2016). Using RAD-seq to recognize sex-specific markers and sex chromosome systems. *Molecular Ecology*, *25*, 2114–2116.

Gamble, T., Coryell, J., Ezaz, T., Lynch, J., Scantlebury, D. P., & Zarkower, D. (2015). Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Molecular Biology and Evolution*, *32*, 1296–1309.

Gamble, T., & Zarkower, D. (2014). Identification of sex-specific molecular markers using restriction site-associated DNA sequencing. *Molecular Ecology Resources*, *14*, 902–913.

Gao, Z. X., Wang, H. P., Yao, H., Tiu, L., & Wang, W. M. (2010). No sex-specific markers detected in bluegill sunfish *Lepomis macrochirus* by AFLP. *Journal of Fish Biology*, *76*, 408–414.

Glennon, R. P., Gomelsky, B., Schneider, K. J., Kelly, A. M., & Haukenes, A. (2012). Evidence of female heterogamety in largemouth bass, based on sex ratio of gynogenetic progeny. *North American Journal of Aquaculture*, *74*, 537–540.

Gomelsky, B., Mims, S. D., Onders, R. J., & Bean, W. B. (2002). Hormonal sex reversal and evidence of female homogamety in black crappie. *North American Journal of Aquaculture*, *64*, 66–69.

Gutierrez, J. B., & Teem, J. L. (2006). A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y chromosome to cause extinction of an introduced exotic species. *Journal of Theoretical Biology*, *241*, 333–341.

Haldane, J. B. (1922). Sex ratio and unisexual sterility in hybrid animals. *Journal of genetics*, *12*, 101–109.

Hayes, M. P., & Jennings, M. R. (1986). Decline of Ranid frog species in western North America: Are bullfrogs (*Rana catesbeiana*) responsible? *Journal of Herpetology*, *20*, 490–509.

Jeffries, D. L., Lavanchy, G., Sermier, R., Sredl, M. J., Miura, I., Borzée, A., … Perrin, N. (2018). A rapid rate of sex-chromosome turnover and non-random transitions in true frogs. *Nature Communications*, *9*, 4088. https://doi.org/10.1038/s41467-018-06517-2.

Kennedy, P. A., Meyer, K. A., Schill, D. J., Campbell, M. R., & Vu, N. V. (2018). Survival and reproductive success of hatchery YY male brook trout stocked in Idaho streams. *Transactions of the American Fisheries Society*, *147*, 419–430.

Lamatsch, D. K., Adolfsson, S., Senior, A. M., Christiansen, G., Pichler, M., Ozaki, Y., … Nakagawa, S. (2015). A transcriptome derived female-specific marker from the invasive western mosquitofish (*Gambusia affinis*). *PLoS One*, *10*, e0118214. https://doi.org/10.1371/journal.pone.0118214.

Lebedev, A. V., Paul, N., Yee, J., Timoshchuk, V. A., Shum, J., Miyagi, K., … Zon, G. (2008). Hot start PCR with heat-activatable primers: A novel approach for improved PCR performance. *Nucleic Acids Research*, *36*, e131. https://doi.org/10.1093/nar/gkn575.

Lee, H. K., Lee, C. K., Loh, T. P., Tang, J. W. T., Tambyah, P. A., & Koay, E. S. C. (2011). High-resolution melting approach to efficient identification and quantification of H275Y mutant influenza H1N1/2009 virus in mixed-virus-population samples. *Journal of Clinical Microbiology*, *49*, 3555–3559.

Lemly, A. D. (1985). Suppression of native fish populations by green sunfish in first-order streams of Piedmont North Carolina. *Transactions of the American Fisheries Society*, *114*, 705–712.

Li, X. Y., Liu, X. L., Zhu, Y. J., Zhang, J., Ding, M., Wang, M. T., … Gui, J. F. (2018). Origin and transition of sex determination mechanisms in a gynogenetic hexaploid fish. *Heredity*, *121*, 64–74.

Lu, J., Luan, P., Zhang, X., Xue, S., Peng, L., Mahbooband, S., & Sun, X. (2014). Gonadal transcriptomic analysis of yellow catfish (*Pelteobagrus fulvidraco*): Identification of sex-related genes and genetic markers. *Physiological Genomics*, *46*, 798–807.

Matsunaga, T., Ieda, R., Hosoya, S., Kuroyanagi, M., Suzuki, S., Suetake, H., … Kikuchi, K. (2014). An efficient molecular technique for sexing tiger pufferfish (fugu) and the occurrence of sex reversal in a hatchery population. *Fisheries Science*, *80*, 933–942.

McKechnie, R. J., & Tharratt, R. C. (1966). Green sunfish. In A. Calhoun (Ed.), *Inland fisheries management* (pp. 399–401). Sacramento, CA: California Department of Fish and Game.

Miller, M. R., Dunham, J. P., Amores, A., Cresko, W. A., & Johnson, E. A. (2007). Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers. *Genome Research*, *17*, 240–248.

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., … Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *Royal Society Open Science*, *2*(150088), 150088. https://doi.org/10.1098/rsos.150088.

Morris, J.E., Mischke, C.C., & Lane, R.L. (2005). Culture and propagation of sunfish and their hybrids. *Natural Resource Ecology and Management Conference Papers, Posters and Presentations*, 26.

Moyle, P. B. (1976). Fish introductions in California: History and impact on native fishes. *Biological Conservation*, *9*, 101–118.

Mussmann, S. M., Saltzgiver, M. J., Delomas, T. A., Campbell, M. R., Diver, T. A., & Wilson, W. D. (2021). Rapid molecular determination of sex for western north American chub (*Gila* spp.). *North American Journal of Fisheries Management*, *42*, 73–84. https://doi.org/10.1002/nafm.10717.

Ohno, S. (1970). The enormous diversity in genome sizes of fish as a reflection of nature's extensive experiments with gene duplication. *Transactions of the American Fisheries Society*, *99*, 120–130.

Palmer, D. H., Rogers, T. F., Dean, R., & Wright, A. E. (2019). How to identify sex chromosomes and their turnover. *Molecular Ecology*, *28*, 4709–4724.

Pan, Z. J., Li, X. Y., Zhou, F. J., Qiang, X. G., & Gui, J. F. (2015). Identification of sex-specific markers reveals male heterogametic sex determination in *Pseudobagrus ussuriensis*. *Marine Biotechnology*, *17*, 441–451.

Perez-Enriquez, R., Llera-Herrera, R., Torres, P. G., & Avila, S. (2020). A fast sex detection method for the Whiteleg shrimp *Litopenaeus vannamei* by post-PCR high resolution melting (HRM). *Aquaculture*, *520*, 734784.

Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., & Hoekstra, H. E. (2012). Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS One*, *7*, e37135. https://doi.org/10.1371/journal.pone.0037135.

Reinthal, P. N., Blasius, H., & Haberstitch, M. (2020). Long-term monitoring of a desert fish assemblage in Aravaipa Creek, Arizona. In D. Propst, J. Williams, K. Bestgen, & C. Hoagstrom (Eds.), *Standing between life and extinction: Ethics and ecology of conserving aquatic species in north American deserts* (pp. 225–237). Chicago, IL: The University of Chicago Press.

Roberts, F. L. (1964). A chromosome study of twenty species of Centrarchidae. *Journal of Morphology*, *76*, 408–414.

Rochette, N., & Catchen, J. (2017). Deriving genotypes from RAD-seq short-read data using stacks. *Nature Protocols*, *12*, 2640–2659.

Roncarati, A., Melotti, P., Mordenti, O., & Gennari, L. (1997). Influence of stocking density of European eel (*Anguilla Anguilla*, *L.*) elvers on sex differentiation and zootechnical performances. *Journal of Applied Ichthyology*, *13*, 131–136.

Rosen, P. C., Schwalbe, C. R., Parizek, D. A., Jr., Holm, P. A., & Lowe, C. H. (1995). Introduced aquatic vertebrates in the Chiricahua region: Effects on declining native Ranid frogs. In L. H. DeBano, P. H. Folliott, A. Ortega-Rubio, G. J. Gottfried, R. H. Hamre, & C. B. Edminster (Eds.), *Biodiversity and management of the Madrean Archipelago: The sky islands of southwestern United States and northwestern Mexico* (pp. 251–261). Fort Collins, CO: US Forest Service, Rocky Mountain Forest and Range Experiment Station.

Schill, D. J., Heindel, J. A., Campbell, M. R., Meyer, K. A., & Mamer, E. R. J. M. (2016). Production of a YY male brook trout broodstock for potential eradication of undesired brook trout populations. *North American Journal of Aquaculture*, *78*, 72–83.

Schill, D. J., Meyer, K. A., & Hansen, M. J. (2017). Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. *North American Journal of Fisheries Management*, *37*, 1054–1066.

Senior, A. M., Krkosek, M., & Nakagawa, S. (2013). The practicality of Trojan sex chromosomes as a biological control: An agent based model of two highly invasive *Gambusia* species. *Biological Invasions*, *15*, 1765–1782.

Shen, Z. G., Wang, H. P., Yao, H., O'Bryant, P., Rapp, D., & Zhu, K. Q. (2016). Sex determination in bluegill sunfish *Lepomis macrochirus*: Effect of temperature on sex ratio of four geographic strains. *Biological Bulletin*, *230*, 197–208.

Teem, J., Alphey, L., Descamps, S., Edgington, M., Edwards, O., Gemmell, N., … Roberts, A. (2020). Genetic biocontrol for invasive species. *Frontiers in Bioengineering and Biotechnology*, *8*, 1–18.

Teem, J. L., Gutierrez, J. B., & Parshad, R. D. (2014). A comparison of the Trojan Y chromosome and daughterless carp eradication strategies. *Biological Invasions*, *16*, 1217–1230.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3 – new capabilities and interfaces. *Nucleic Acids Research*, *40*, e115. https://doi.org/10.1093/nar/gks596.

Vu, N. V., Eardley, D. L., Delomas, T. A., & Campbell, M. R. (2019). Identification of sex-specific SNPS in burbot *Lota lota* using RAD sequencing: Conservation and management applications. *Fisheries And Aquatic Sciences*, *22*, 18.

Wang, H. P., Zhi-Gang, S., Ze-Xia, G., Hong, Y., Rapp, D., & O'Bryant, P. (2018). Sex determination, differentiation, and control in bluegill. In H.-P. Wang, F. Piferrer, S.-L. Chen, & Z.-G. Shen (Eds.), *Sex Control in Aquaculture* (pp. 361–384). Hoboken, NJ: Wiley Blackwell.

Ward, D. L., O'Neill, M. W., & Ka'apu-Lyons, C. (2015). Effectiveness of backpack electrofishing for removal of non-native fishes from a small warm-water stream. *Journal of the Arizona-Nevada Academy of Science*, *46*, 37–41.

Werner, E. E., & Hall, D. J. (1977). Competition and habitat shift in two sunfishes (Centrarchidae). *Ecology*, *58*, 869–876.

Willing, E. M., Hoffmann, M., Klein, J. D., Weigel, D., & Dreyer, C. (2011). Paired-end RAD-seq for de novo assembly and marker design without available reference. *Bioinformatics*, *27*, 2187–2193.

Wilson, C. A., High, S. K., McCluskey, B. M., et al. (2014). Wild sex in zebrafish: Loss of the natural sex determinant in domesticated strains. *Genetics*, *198*, 1291–1308.

Yun-Chang, C. A. O., Wen-Sheng, L. I., Wei, Y. E., & Hao-Ran, L. I. N. (2008). Gonadal development in green sunfish, *Lepomis cyanellus*: A histological study. *Chinese Journal of Zoology*, *43*, 88–95.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Teal, C. N., Coykendall, D. K., Campbell, M. R., Eardley, D. L., Delomas, T. A., Shira, J. T., Schill, D. J., Bonar, S. A., & Culver, M. (2022). Sex-specific markers undetected in green sunfish *Lepomis cyanellus* using restriction-site associated DNA sequencing. *Journal of Fish Biology*, *100*(6), 1528–1540. https://doi.org/10.1111/jfb.15063

APPENDIX C

THE EFFECTS OF ESTRADIOL-17β ON THE SEX REVERSAL, SURVIVAL, AND GROWTH OF GREEN SUNFISH *LEPOMIS CYANELLUS*

Aquaculture 562 (2023) 738853

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

The effects of estradiol-17β on the sex reversal, survival, and growth of green sunfish Lepomis cyanellus

Chad N. Teal [a,*], Daniel J. Schill [b], Susan B. Fogelson [c], Colby M. Roberts [d], Kevin Fitzsimmons [e], Javan M. Bauder [f], William T. Stewart [g], Scott A. Bonar [f]

[a] Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2 Rm N315, 1064 E. Lowell St., Tucson, AZ 85721, USA
[b] Fisheries Management Solutions, Inc., 9950 W. Prairie Road, Boise, ID 83714, USA
[c] Fishhead Labs, LLC, 5658 SE Pine Ave, Stuart, FL 34997, USA
[d] University of Arizona, School of Natural Resources and the Environment, 1064 E Lowell St., Tucson, AZ 85721, USA
[e] University of Arizona, Department of Environmental Science, Forbes 306, 1140 E. South Campus Drive, Tucson, AZ 85719, USA
[f] U.S. Geological Survey, Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2, 1064 E. Lowell St., Tucson, AZ 85721, USA
[g] U.S. Bureau of Reclamation, Phoenix Area Office, 6150 W. Thunderbird Road, Glendale, AZ 85306, USA

ARTICLE INFO

Keywords:
Neofemales
Exogenous steroids
Feminization

ABSTRACT

The feminization of green sunfish *Lepomis cyanellus* could expand their utility as a game fish or aquacultured species by preventing overcrowding and precocious reproduction in stocked systems. Feminization of green sunfish could also help elucidate information on their sex determination system. We report the feminization of green sunfish cohorts via oral administration of estradiol-17β (E2) during early development. A low-dose (100 E2 mg per kg of diet) and a high-dose (150 E2 mg per kg of diet) experimental E2 treatment were fed to juvenile green sunfish from 30 to 90 days post-hatch. Fish were subsequently evaluated for any treatment effect on gonadal development, survival, and growth. Both E2 treatments resulted in 100% feminization, with no morphological or histological differences detected between E2 treated ovaries and those from a control group. The control group was composed mostly of males (82.61%). Overall, there was no effect of E2 on survival ($P = 0.310$) and growth rate data suggested no statistical differences ($P = 0.0805$). However, the growth rate of the high-dose group increased slightly higher after the treatment ended than the other treatments ($P = 0.042$), suggesting that E2 might suppress growth in green sunfish. In addition, the control group did not exhibit a higher survival rate after the treatment period ended ($P = 0.266$), whereas both E2 treated groups did ($P = 0.0003$–0.0050). We found that the low-dose, 100 E2 mg per kg of diet, was sufficient for fully feminizing green sunfish if administered during development from 30 to 90 days post-hatch and E2 dosages may result in deleterious effects on green sunfish's health and growth.

1. Introduction

Green sunfish *Lepomis cyanellus* is a widespread North American Centrarchid species that has been introduced to exotic locales around the world (Lemly, 1985; Dudley and Matter, 2000; Yun-Chang et al., 2008; Fuller et al., 2021). This species belongs to one of the most economically important teleost families, Centrarchidae, which has value in both commercial aquaculture and sport fisheries (Brunson and Robinette, 1986; Wang et al., 2008; Morris and Clayton, 2009; Quinn and Paukert, 2009). However, management of Centrarchids in small water bodies can be difficult due to their proclivity for precocious reproduction resulting in overcrowding and stunting (Goodson Jr., 1966; Hackney, 1975; Wang et al., 2008). Green sunfish specifically have a propensity to overpopulate their habitats leading to the suppression of sport fishes and threatened native species (McKechnie and Tharratt, 1966; Moyle, 1976; Werner and Hall, 1977; Dudley and Matter, 2000; Morris et al., 2005). For example, male green sunfish are especially aggressive due to their courtship and nest guarding behaviors (Brunson

* Corresponding author.
E-mail addresses: chadteal@arizona.edu (C.N. Teal), dschill@fishmgmtsolns.com (D.J. Schill), susan.fogelson@fishheadlabs.com (S.B. Fogelson), kevfitz@ag.arizona.edu (K. Fitzsimmons), jbauder@arizona.edu (J.M. Bauder), wstewart@usbr.gov (W.T. Stewart), sbonar@ag.arizona.edu (S.A. Bonar).

https://doi.org/10.1016/j.aquaculture.2022.738853
Received 21 January 2022; Received in revised form 14 September 2022; Accepted 15 September 2022
Available online 19 September 2022
0044-8486/© 2022 Elsevier B.V. All rights reserved.

and Morris, 2000; Teal et al., 2022a) potentially leading to displacement and stunting of more desirable gamefish such as bluegill *Lepomis macrochirus* (Werner and Hall, 1977). The production and stocking of monosex green sunfish via hormonal sex reversal may facilitate stocking green sunfish as sportfish or for commercial aquaculture purposes where reproduction is undesired (Al-Ablani, 1997) and thereby could reduce the problem of overcrowding and assist population management.

Sex reversal methods are useful in aquaculture because they facilitate faster growth curves and the growout of the larger sex (Al-Ablani, 1997; Wang et al., 2008), thus increasing production and profitability. Aquaculture methods for members of the *Lepomis* family are relatively sparse and more research needs to be conducted on the production and economic feasibility of culturing these species (Brunson and Morris, 2000). Since male green sunfish are larger than females (Hunter, 1963), the production of males for aquaculture purposes could increase profitability. Feminization of males through the administration of estrogen during their sexual development can allow for indirect production of all-male cohorts of fishes (Piferrer, 2001). Feminization is performed by feminizing genetic males to the extent of developing functional ovaries and then selectively spawning these sex-reversed males (neofemales) with wild type males (Piferrer, 2001; Wang et al., 2008). If the fish have a ZZ-male/ZW-female sex determination system then the resulting spawn from a neofemale would be 100% male (Senior et al., 2013), barring any non-chromosomal effects on sex determination (Piferrer, 2001; Shen et al., 2016). If the fish have an XY-male/XX-female sex determination system then YY males from the resulting spawn are selected as broodstock and crossed with wild type females to produce 100% male cohorts (Mair et al., 1997; Piferrer, 2001). The indirect method of producing all-male cohorts is preferential to the hormonal masculinization of cohorts, because stocked or commercially sold fish are never exposed to the exogenous steroid treatment and the possibility of incomplete sex reversal is eliminated (Piferrer, 2001; Wang et al., 2008).

Evaluating feminization methods for green sunfish could be crucial in elucidating their sex determination system (Desprez et al., 1995; Gomelsky et al., 2002). The mechanisms of sex determination and differentiation in green sunfish are unknown. Roberts (1964) did not identify sex chromosomes in green sunfish through karyotyping. Other green sunfish studies found evidence of female genetic markers using amplified fragment length polymorphism (Lo´pez-Fern´andez and Bolnick, 2007) and restriction-site associated DNA sequencing (Teal et al., 2022b). However, these studies either did not test their markers on larger sample sizes (Lo´pez-Ferna´ndez and Bolnick, 2007) or were unable to develop a reliable marker (Teal et al., 2022b). While these previous studies suggest that females maybe the heterogametic sex, these female specific loci may have been false positives as markers for the sex chromosome due to the small sample sizes and loci discovery methods implemented in their methods. Effective sex reversal treatments could validate the presence of sex chromosomes because sex ratios of progeny from neofemales crossed with wild-type males will be 3:1 male to female or 100% male depending on if the female is the homogametic sex or the heterogametic sex, respectively (Desprez et al., 1995; Gomelsky et al., 2002). This evidence would validate or dispute the preexisting evidence that female green sunfish are heterogametic for the sex determining region or regions of the genome. Uncovering of the sex determination system in green sunfish could provide more insight into the complicated evolution of sex determination systems in Centrarchids (Gamble et al., 2015; Nelson, 2018; Wang et al., 2018).

If sex chromosomes exist in green sunfish, then effective sex reversal treatments could facilitate efforts at controlling invasive populations. Green sunfish are ecologically destructive when introduced outside of their native range (McKechnie and Tharratt, 1966; Lemly, 1985; Dudley and Matter, 2000). Novel approaches at suppressing and eradicating invasive fish populations, such as the release of Trojan sex chromosome (TSC) carriers, are theoretically effective (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2017; McCormick et al., 2021) and are already undergoing field trials with brook trout *Salvelinus fontinalis* (Kennedy et al., 2018; Teem et al., 2020). Green sunfish's persistence and fast generation time makes it a desirable candidate for the use of a TSC eradication strategy. The development of TSC carriers requires an effective sex reversal treatment and subsequent selective spawning to develop a broodstock capable of producing large numbers of either YY individuals or ZZ females (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2016). These TSC carriers would then be released into a nuisance population where they could spawn with wild-type females and shift the sex ratio towards all male, theoretically eradicating the population (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2017; Teem et al., 2020; McCormick et al., 2021). The development of an effective sex reversal treatment would allow for initial investigations into the capability of using a TSC eradication strategy for green sunfish and feminization methods could be useful in uncovering if the basic reproductive biology of this species is conducive to this type of eradication strategy. In a species that is either male or female heterogametic, the first step in producing TSC carriers is the feminization of genetic males (Senior et al., 2013; Schill et al., 2016).

Green sunfish, as with all studied Centrarchids (Arslan, 2018), are gonochoristic, with ovaries and testes differentiating directly from undifferentiated gonads (Teal et al., 2022a). Fish are most susceptible to permanent sex reversal via exogenous hormone treatments if the hormone treatments are administered prior to gonadal differentiation and end when gonadal differentiation is first observable through histology (Hackmann and Reinboth, 1974; Piferrer, 2001). This period of gonadal plasticity is referred to as the "labile period" (Piferrer, 2001), the growth period under certain rearing conditions where exposure to endocrine disruptors or exogenous sex hormones can result in permanently altered sex differentiation (Hackmann and Reinboth, 1974; Piferrer, 2001). Although the gonadal development of green sunfish has been investigated (Yun-Chang et al., 2008), the timing of the labile period is still generally unknown. We found in a previous study that the labile period is 39 dph up to 99 dph under our rearing conditions (Teal et al., 2022a). However, this information was unavailable to us when designing the featured sex reversal treatments and our onset, duration, and hormone dosages in this study were based on effective male to female sex reversal trials conducted on bluegill (Wang et al., 2008).

Estradiol-17β (E2) is a natural estrogen commonly used in the feminization of male fish. However, E2 treatments have varied in their effectiveness at feminizing certain species. The range for effective E2 dosages for feminization is from 1 mg E2 per kg of diet up to 750 mg E2 per kg of diet depending on the species treated and the duration of the treatment (Piferrer, 2001). Further, E2 treatments can negatively impact the survival and growth rates of fish if an exposure threshold is surpassed (Hunter et al., 1986; George and Pandian, 1996; Piferrer, 2001; Wang et al., 2008). The objective of this study was to examine the effects of two doses of E2 administered via diet on the sex reversal, survival, and growth rates of green sunfish.

2. Methods

2.1. Larval production

Spawns for the sex reversal treatments were obtained from four 473-L broodstock tanks stocked with two adult males and three adult females. The adult broodstock ($\bar{x}_{\text{total length}}$ = 153.6 mm, SD = 47.2 mm) were collected from Parker Canyon Lake, Arizona, USA (GPS coordinates 31°25′37.0" N, 110°27'25.0" W) during the Spring and Summer of 2018 and 2019. Green sunfish rearing methods and feed transitions followed protocols designed by Teal et al. (2022a). Briefly, eggs from each broodstock spawn were given a 30 min 100-ppm formalin treatment before being stocked in 37.9-L plastic tubs each outfitted with a 50-W Jager EHEIM drop in heater (EHEIM GmbH & Co, Deizisau, Germany), air stone, 10 g of activated carbon, and QANVEE Bio Sponge filter (Taian Qanvee Aquarium Equipment Co., Ltd.,

Shandong, China). Once eggs hatched, larvae were reared in the same tanks with the following water quality parameters: temperature 27–30 °C, ammonia <0.25 ppm, nitrite <1.0 ppm, and pH 8.0–8.4. Upon swim-up stage (3–4 days post-hatch [dph]) larvae were fed with <24-h old brine shrimp nauplii four times per day at a rate of ~125 nauplii/l (estimate based on weight of unhatched cysts and ~ 90% hatching rate). At 25 dph we continued to feed the green sunfish nauplii four times a day and began feeding Otohime B1 diet (B1: 200–360 μm, 51% crude protein, 11% crude fat) (Pentair Aquatic Eco-Systems, North Carolina, U.S.A.) twice a day. When fish were 30 dph, we fed them nauplii once a day and started feeding B1 diet six times a day using an EHEIM automated fish feeder.

2.2. Experimental design and E2 treatments

At 30 dph, when fish were 7.5 mm to 21.0 mm in total length (TL), 50 juveniles from each larval tank, that were progeny from one of four brood stock tanks, were randomly assigned to a treatment tank to create a randomized block design. In our usage of this design, the broodstock tank the juveniles originated from determined their "block". Therefore, each treatment tank was a replicate and contained progeny from one of four broodstock tanks, with a total of four replicates for each treatment. To avoid pseudoreplication, each treatment tank was considered a study unit with each treatment (control, low-dose, high-dose) having four replicates for a total of 600 fish involved in the study. The E2 treatment groups were fed either a 100 mg E2 per kg of diet (low-dose) or a 150 mg E2 per kg of diet (high-dose) from 30 to 90 dph.

Following methods from Wang et al. (2008), treated diets were prepared by dissolving 100 mg E2 or 150 mg of E2 into 400 ml of ethanol. The estradiol-17β was purchased from Sigma-Aldrich (Sigma-Aldrich, Massachusetts, U.S.A). One hundred milliliters of this solution was mixed with 250 g of the B1 diet in a stand mixer to achieve the 100 mg E2 per kg of diet and the 150 mg E2 per kg of diet concentrations. The treated diet was then spread across a large baking sheet and placed in a fume-hood overnight. The control diet was prepared the same way except without the addition of E2. The tanks used during the treatments had identical configurations as the larval rearing tanks and water quality parameters of these treatment tanks were maintained at: temperature 15–24 °C, ammonia <0.25 ppm, nitrite <1.0 ppm, and pH 8.0–8.4. Each treatment tank was self-contained with its own individual filter and no water was shared between treatment tanks.

Subsets of 10–22 of these 50 randomly selected fish assigned to each treatment tank were measured for TL (mm). Until 37 dph, six daily feedings of E2 treated diet or control diet were supplemented with one daily feeding of nauplii to assist with weaning fish off a live diet. At 37 dph we stopped feeding nauplii and only fed B1 treated diets six times a day. During the treatment period the fish in each tank were fed 5.97–11.24% body weight per day. This feed rate converts to 55.00–75.60 mg of diet fed to each tank daily. The total amount of E2 distributed to each treatment tank during the treatment period was 0.33–0.45 mg. At 91 dph the fish were switched onto an untreated diet and all the fish were measured for TL (mm) and weight (g). At 91 dph a 50% water change was performed to expedite the clearing of any re- sidual hormone from the treatments. Mortalities were recorded daily from the start of the feeding trial at 30 dph to the study conclusion at 495 dph. The treatment tanks were siphoned daily and a 10% water change was performed weekly.

At 285 dph, all surviving fish from each larval rearing tank were measured for TL (mm) and weight (g) before being transferred to one of twelve 757 L round fiberglass tanks that were part of a recirculating aquaculture system (RAS). The RAS was composed of thirty 757 L round fiberglass tanks connected to a filtration system featuring a Lifegard ¾ hp. in-line pump, an Emperor 750 W UV sterilizer (Pentair Aquatic Eco-Systems), a DF-6 Polygeyser bead filter (Aquaculture Systems Technologies, Baton Rouge, Louisiana), and a Dayton ½ hp. in-line pump (Dayton Electric Mfg. Co., Niles, Illinois 60,714 U.S.A.). Aeration was provided to each tank by a blower (WW80 Whitewater, Pentair Aquatic Eco-Systems).

From December 12, 2020 – December 18, 2020, 5–14 green sunfish between 437 and 495 dph were removed from each treatment group replicate and euthanized by immersion for 10 min in 100 ppm of MS 222 (Pentair Aquatic Eco-Systems, North Carolina, U.S.A) buffered with 150 ppm sodium bicarbonate. Fish from each replicate were all the same age, but age varied among treatment replicates. We chose this age range for sampling (437–495 dph) because we knew green sunfish could reach sexual maturity by seven months (Yun-Chang et al., 2008; Teal et al., 2022a) and we wanted to ensure that all individuals were reproductively mature. The fish were measured for TL (mm) and weight (g). Both gonads were removed from the fish and weighed (g). The sex ratio of each replicate tank was evaluated based on macroscopic inspection of gonads and conducting the gonad squash method on one gonad (Guerrero and Shelton, 1974). The other gonad from 20 green sunfish from each E2 treatment group and the other gonad from 15 green sunfish from the control group were submitted to Fishhead Labs (Stuart, Florida) for routine histological processing and hematoxylin and eosin staining. One histology slide was prepared per submitted fish with two sections of sagitally bisected ovary mounted to each slide. The histology slides were inspected to verify sex ratios obtained from the gonad squash method and to detect intersex individuals. General oocyte developmental stages and structure of the ovaries were compared among the treatment groups, as well as to relevant fish gonad literature (Yun-Chang et al., 2008; Teal et al., 2022a; van der Ven and Wester, 2022) to check for any deviation from normal development. We investigated differences in oocyte development by using an AmScope 40×-2000 × 3 W LED Seidentopf trinocular compound microscope and AmScope 14MP camera (United Scope, LLC, California, U.S.A.) to count previtellogenic, vitellogenic, and atretic oocytes in a randomly selected 1.2 mm^2 section of ovary for all histology samples. Slides were inspected at 100× magnification. Due to the overall uniformity of oocytes seen among the treatment groups, oocyte developmental stages were classified as previtellogenic, vitellogenic, and atretic. Vitellogenic oocytes were defined as any oocytes with conspicuous yolk granule ("oil droplet") development. We noted numbers of atretic oocytes because exposure to exogenous E2 has been shown to increase atresia and inhibit maturation of oocytes in zebrafish *Danio rerio* (van der Ven and Wester, 2022).

2.3. Data analysis

Data analysis was conducted using Microsoft Excel V 2102 and Program R V 3.6.1 (R Core Team, 2013). We used proportional binomial generalized linear models (GLMs) to compare the mean proportion of fish that were females in the E2 treated groups with the mean proportion of fish that were females in the control group. We used generalized linear mixed models (GLMMs) with a Gaussian error distribution to model the effects of age (dph) and tank treatment (low-dose, high-dose, or control) on the number of previtellogenic oocytes, vitellogenic oocytes, and atretic oocytes. We used random intercepts by 'tank' to control for pseudoreplication among fish from the same tank (Gillies et al., 2006; Bolker et al., 2009; Zuur et al., 2009). We then conducted a Tukey post hoc analysis with the GLMMs using the Kenward-Roger method for calculating degrees of freedom to compare mean number of previtellogenic oocytes, vitellogenic oocytes, and atretic oocytes among the various treatment groups. To isolate the effect that the differences in ages (i.e., days post-hatch) among the replicates might have had on the number of previtellogenic, vitellogenic oocytes, and atretic oocytes we used a GLMM with Gaussian error distribution to test the relationship of age with number of previtellogenic, vitellogenic, and atretic oocytes. We grouped together all sampled fish from the control group to conduct a chi-square test and assess if the sex ratio was significantly divergent from a 1:1 sex ratio. We used $\alpha = 0.05$ for all statistical tests.

We used a beta generalized linear model (BGLM) to compare the mean proportion of fish that survived among the treatment during the

treatment period (30–90 dph) and during the post-treatment period (91–285 dph). We then fit additional BGLMs to conduct a post hoc analysis comparing the survival rates for each treatment group during the treatment period (30–90 dph) with their survival rates during the post-treatment period (91–285 dph) and used a Holm-Bonferroni (Holm, 1979) correction to adjust *P* values for experiment-wise error.

We tested for differences in TL, weight, and gonadosomatic index among treatment groups using generalized linear mixed models (GLMMs) with Gaussian error distributions and random intercepts by 'tank'. We then conducted a Tukey post hoc analysis with the GLMMs using the Kenward-Roger method for calculating degrees of freedom to compare means among the various treatment groups. One control replicate's mean weight was an outlier that was over one standard deviation (SD) larger than the next largest mean weight. The removal of this one control replicate's mean weight did not change the *P* value enough to affect the significance of the differences among mean weights of the treatment groups so we included this replicate in our analysis. We used a GLMM with a Gaussian error distribution to model the effects of age (dph) and tank treatment (low-dose, high-dose, or control) on TL to test for differences in overall growth rates between the treatment groups during the first 285 dph.

We calculated absolute growth rates (AGRs) to compare growth rates of the different tank treatments during the treatment period (Wang et al., 2008), as well as 195 days after the treatment period ended. AGRs were calculated using the formula $AGR = (TL_2 - TL_1)/T \times 100$. Where TL_1 and TL_2 are the mean fish total lengths at the start and end of the growth period for each of the treatment tanks, and T is the time between measurements (Teal et al., 2022a). We used a one-way ANOVA to test for differences in AGR among the treatment groups at the end of the treatment period and 195 days after the end of the treatment. We then used paired *t*-tests with a Holm-Bonferroni correction to compare differences in mean AGR between the treatment period and post-treatment period for each treatment group.

3. Results

Based on the gonadal squash method and histology results, 100% of fish sampled from the E2 treatment groups were feminized to the extent of developing ovaries absent of spermatogenesis (Table 1). We observed no morphological or histological differences between ovaries in the E2 treatment groups and ovaries in the control group. Oocyte maturation in the E2 treated groups appeared normal when compared to ovaries in the control group and the relevant histology literature (Fig. 1). The mean number of previtellogenic, vitellogenic, and atretic oocytes in the treatment groups did not differ significantly (GLMM, $t_{9.38} < 0.830$, *P* value >0.6951; Table 2). We did not observe buildup of eosinophilic staining plasma or evidence of inhibition of ovary maturation that could have resulted from the E2 treatments (van der Ven and Wester, 2022). The number of oocytes at various stages of development were not a significant function of age (GLMM, $t_{6.956} < -0.943$, *P* value >0.370).

The mean percentage of green sunfish that were sampled in the control group that were female was 17.39% (SD = 16.64%). The percentages of fish sampled that were male from each control group replicate were 100% (6/6), 83.33% (5/6), 83.33% (5/6), and 60.00% (3/5). The sex ratio of the control group was significantly divergent from a 1:1 sex ratio (Chi-Square Test, df = 1, *P* value <0.005). The percentages of green sunfish that were phenotypic females in the E2 treatment groups were significantly greater than the percentage of females in the control group (GLM, $Z > 2.83$, *P* value <0.005).

The mean female GSI of the high-dose group ($\bar{x}$ = 1.62, 95% CI = 1.44–1.79) was higher than the mean female GSI of the low-dose group ($\bar{x}$ = 1.34, 95% CI = 1.13–1.55) and the control group ($\bar{x}$ = 1.22, 95% CI = 0.88–1.55), but the differences in mean GSI among the treatment groups were variable and suggest no statistical significance (GLMM, $t_{36.42} = 2.226$, *P* value = 0.0802).

Differences in mean survival rates to the end of the treatment (91 dph) were small and not statistically significant among the treatment groups (BGLM, $Z = 1.015$, *P* value = 0.310). There was large variability of survival rates among replicates across the treatment groups (Table 3). Although not statistically significant, E2 did appear to have a deleterious effect on mean survival during the treatment period (Table 1; Fig. 2). The control group had a slightly higher survival rate to 91 dph ($\bar{x}$ = 47.50% survived, 95% CI = 23.00–72.00%) than the low-dose treatment group ($\bar{x}$ = 40.00% survived, 95% CI = 22.84–57.16%) and the low-dose treatment group had a slightly higher survival rate than the high-dose treatment group ($\bar{x}$ = 36.00% survived, 95% CI = 21.07–50.93%). The differences in mean survival rates from 91 dph to 285 dph (195 days after end of treatment) among the treatment groups were not significant (BGLM, Z = 0.462, *P* value = 0.644). Mean survival rates increased for all treatment groups during the post-treatment period (Fig. 2). This increase in survival rate was significant in the low-dose treatment group (BGLM, $Z = 3.045$, *P* value = 0.004660) and high-dose treatment group (BGLM, $Z = 3.866$, *P* value = 0.000333). The control group did not show a significant increase in mean survival rate during the post-treatment period (BGLM, $Z = 1.113$, *P* value = 0.266).

At the beginning of the treatment period (30 dph) there were no statistical differences (GLMM, $t_{8.94} < 0.986$, *P* value >0.605) in mean TLs among the control group ($\bar{x}$ = 12.10 mm, 95% CI = 10.09–14.10 mm) and the E2 treatment groups (low-dose treatment $\bar{x}$ = 10.90 mm, 95% CI = 8.86–12.90; high-dose treatment $\bar{x}$ = 11.80 mm, 95% CI = 9.76–13.80). The control group had a slightly longer mean TL ($\bar{x}$ = 26.49 mm, 95% CI = 24.37–28.60 mm) than the low-dose treatment ($\bar{x}$ = 23.57 mm, 95% CI = 22.29–24.85 mm) and the high-dose treatment ($\bar{x}$ = 23.49 mm, 95% CI = 21.80–25.19 mm) at the end of the treatment period (91 dph), but the differences in mean TLs (mm) were not suggestive of being statistically significant (GLMM, $t_{8.85} = 2.492$, *P* value = 0.0805). The differences in mean weights (g) among treatment groups at the end of the treatment period were not significant (GLMM, $t_{9.72} < 1.845$, *P* value >0.2065). Overall growth rates (Fig. 3), based on mean TLs (mm), did not differ significantly among the treatment groups from the start of the treatment (30 dph) to 285 dph (195 days after end of treatment) (GLMM, $t_{8.506}$ *control* $\beta = 1.645$, *P* value = 0.136).

Mean AGR among the treatment groups did not differ significantly during the treatment period (One-way ANOVA, $F_{2,9} = 1.916$, *P* value = 0.203), and mean AGR among the treatment groups did not differ during the 195 days after the treatment ended (One-way ANOVA, $F_{2,9} = 0.074$, *P* value = 0.929). Although mean AGR increased for both the low-dose group and the control group, only the high-dose treatment group showed an increase in mean AGR between the treatment period (30–90 dph) and post-treatment period (91–285 dph) that suggested statistical

Table C.1.
Total fish survived treatment and mean percent female of each estradiol-17β treatment group and control group (0 mg estradiol-17β per kg of diet) of green sunfish.

Treatment Dose (E2 mg/kg of diet)	*N*	Initial Number of Fish Per Treatment	Total Number of Fish Survived to end of Treatment	Total Number of Fish Sampled from Each Treatment Group for Gonad Assessment	Treatment Duration (dph)	Mean % Female (SD)	95% CI
0	4	200	95	23	30–90	17.39% (16.64%)	0%-35.90%
100	4	200	83	32	30–90	100% (0%)	0%
150	4	200	72	24	30–90	100% (0%)	0%

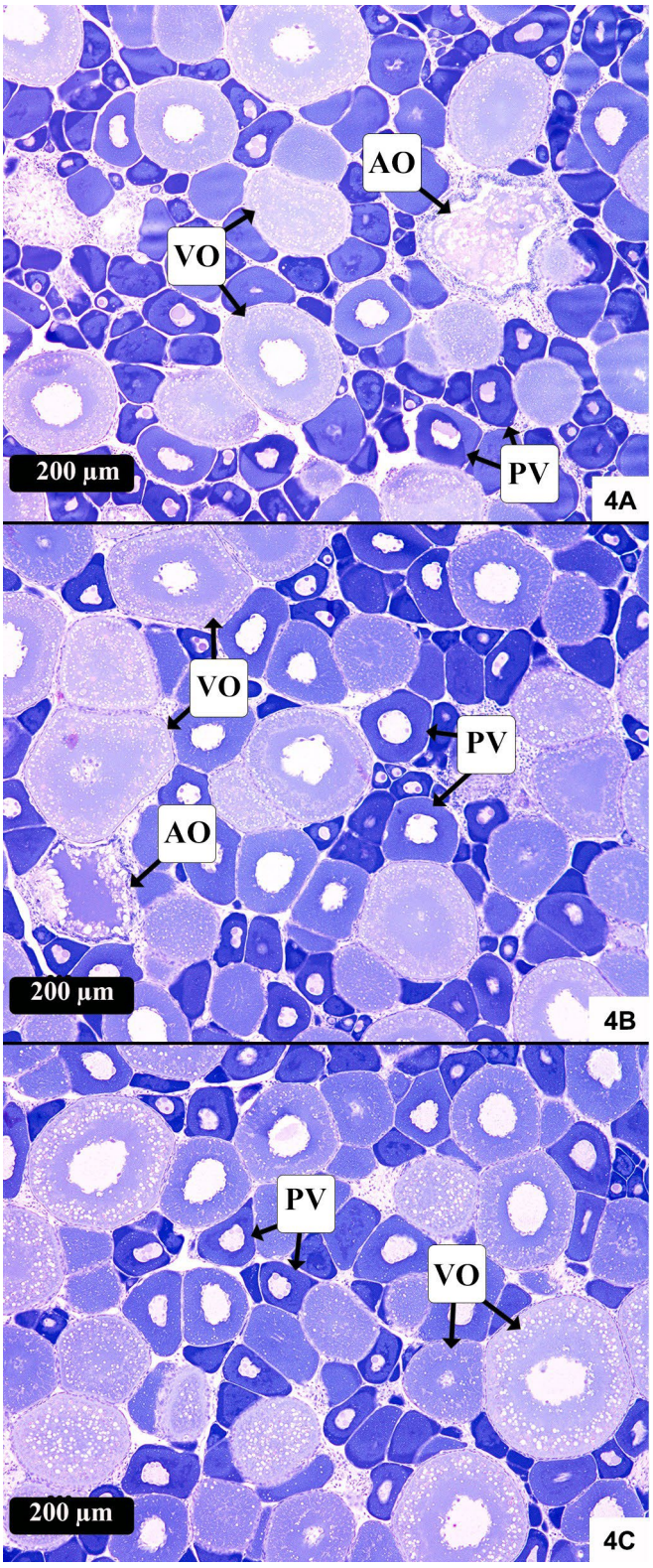

Figure C.1. Ovaries from 431 to 480 dph green sunfish exposed to 100 mg estradiol- 17β per kg of diet (1B) or 150 mg estradiol-17β per kg of diet (1C) exhibited normal development and contained oocytes at various levels of maturation (PV = previtellogenic oocyte, VO = vitellogenic oocyte, AO = atretic oocyte), similar to the ovary in this 437 dph green sunfish female from the control group (1A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table C.2.
Mean number of oocytes at various stages of maturation among the green sunfish treatment groups.

Oocyte Stage	Treatment Groups (E2 mg/kg of diet)	Mean Number of Oocytes	SD	95% CI
Previtellogenic	0	91.0	20.8	52.9–129.0
	100	95.5	18.80	74.2–117.0
	150	93.7	30.67	71.9–116.0
Vitellogenic	0	16.2	3.51	4.05–28.3
	100	21.2	6.77	14.06–28.3
	150	20.7	8.13	13.34–28.0
Atretic	0	0.53	1.15	0.00–1.61
	100	0.43	0.94	0.00–0.96
	150	0.46	0.61	0.0–1.00

significance (Paired *t*-test, $t_3 = 3.401$, *P* value = 0.0424; Fig. 4), with the mean AGR during the treatment period being 18.86 (95% CI = 15.08–22.64) and the mean AGR post-treatment being 24.74 (95% CI = 22.83–26.66).

4. Discussion

The treatment duration and E2 dosages we used were highly effective at feminizing green sunfish. We could not discern any morphological or histological differences between the E2 treated groups and the control group. Wang et al. (2008) observed one intersex individual out of 20 bluegill (*L. macrochirus*) sampled from their 30–90 dph treatment fed a 100 E2 mg per kg of diet. We did not observe any evidence of incomplete sex reversal in either the low-dose or high-dose treatment. Wang et al. (2008) conducted their treatments in a flow-through system, whereas we used self-contained tanks with filters. Even though we added 10 g of activated carbon to each tank to adsorb any E2 leeching out from the diet, our treated fish may have had some immersion exposure to the E2 since we did not use a flow-through system (Hulak et al., 2008; McGree et al., 2010). Using our treatment tank configurations, it may be possible to fully feminize green sunfish if given a lower E2 dosage than 100 mg E2 per kg of diet from 30 to 90 dph. Based on the complete cohort feminization we observed, and the 39–99 dph labile period reported by Teal et al. (2022a), we believe the E2 treatment onset and duration were appropriate for this species. However, alternative E2 exposure methods have been attempted and had varied success at feminization in other species (Piferrer, 2001). For example, hormone baths while roughly half of the eggs have hatched from a spawn have proven successful at feminizing cohorts of some Salmonids (Feist et al., 1996). Therefore, alternate methods for administering E2 and shorter treatment durations may also be effective at feminizing green sunfish. Using the lowest possible E2 dosage and shortest treatment duration is preferential since our results and previous work show that E2 can have negative impacts on fish health and growth (Hunter et al., 1986; George and Pandian, 1996; Piferrer, 2001; Peterson and Davis, 2012). More studies should be conducted with green sunfish to identify the lowest effective E2 treatment for complete feminization.

Multiple studies have shown that exogenous E2 exposure can cause inhibition in the progression of oocytes through vitellogenesis which in severe cases can result in sexual sterility (van der Ven and Wester, 2022; Komen et al., 1989). Furthermore, other studies on hormonal sex reversal treatments in other fish species often exhibited highly conspicuous effects of E2 on fish gonads such as mixed sex ratios and intersex tissue in gonads of E2 treated fish (Yamazaki, 1983; Komen et al., 1989; Wang et al., 2008; Carvalho et al., 2014). Although infertility due to duct deformities are typically associated with exogenous androgen exposure (Johnstone et al., 1979; Piferrer, 2001), male to female sex reversals from exogenous estrogen exposure or other endocrine disruptors can result in the development of aberrant gonadal ducts (Jobling et al., 2002). We did not investigate occlusions of gonadal ducts or genital pores that could result in sexual dysfunction of our fish, but

Table C.3.
Summary statistics for each green sunfish treatment tank (replicate). Empty parenthesis for standard deviation (SD) parenthesis in the GSI column are because there was only one female in these replicates. * Denotes that a mean GSI could not be calculated due to there being less than two females sampled from this tank.

Treatment Tank (Replicate)	Treatment Dose (E2 mg/kg of diet)	Number of Fish Survived to End of Treatment	Mean GSI at maturity* (SD)	Mean TL (mm) at End of Treatment (SD)	Mean Weight (g) at End of Treatment (SD)
L24	0	18	NA	25.17 (5.00)	0.23 (0.14)
L28	0	21	1.32 ()	29.71 (5.12)	0.46 (0.24)
L30	0	42	1.09 ()	25.57 (3.89)	0.23 (0.10)
L31	0	14	1.23 (0.12)	25.50 (4.77)	0.24 (0.12)
L19	100	22	1.34 (0.11)	24.00 (4.33)	0.19 (0.12)
L21	100	18	1.19 (0.37)	24.11 (2.61)	0.19 (0.07)
L25	100	32	1.33 (0.29)	24.53 (3.19)	0.24 (0.10)
L27	100	11	1.39 (0.14)	21.64 (4.43)	0.14 (0.10)
L22	150	17	1.51 (0.40)	21.24 (3.40)	0.15 (0.08)
L23	150	14	1.88 (0.64)	24.79 (3.07)	0.20 (0.08)
L26	150	12	1.35 (0.30)	24.92 (1.38)	0.24 (0.04)
L29	150	29	1.66 (0.28)	23.03 (2.63)	0.21 (0.10)

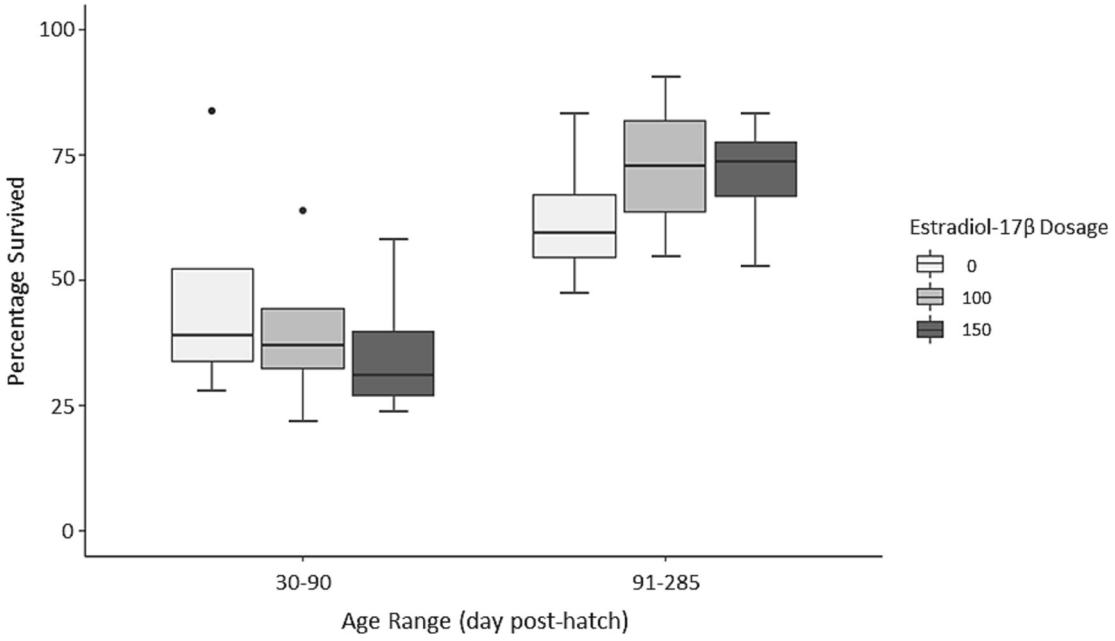

Figure C.2. Mean percentage of green sunfish survival rate across two groups treated with increased estradiol-17β dosages (100 = 100 mg estradiol-17β per kg of diet; 150 = 150 mg estradiol-17β per kg of diet) and a control group (0 mg estradiol-17β per kg of diet) during the treatment period (30–90 days post-hatch) and during a post-treatment period (91–285 days post-hatch).

the overall macroscopic similarity of the ovaries coupled with the absence of any abnormal oocyte development provides strong evidence that the E2 treated fish we examined were sexually viable. We also saw no differences in the number oocytes at various stages of maturation and viability among the treatment groups and no inhibition of vitellogenesis. We therefore have no reason to believe our E2 treated fish are infertile nor sexually dysfunctional (Iwamatsu, 1999). It is possible the E2 treated groups contained larger vitellogenic oocytes than the control group which may explain the marginally significant (P value = 0.0802) increase in GSI in the E2 treated groups. However, additional experiments would be needed to explicitly test this hypothesis. In contrast to the trends observed in our study, exogenous estrogens, such as E2, may reduce ovary size, and thus the fish's GSI (Komen et al., 1989; Piferrer, 2001). We did sample fish from December 12–18, which is temporally distant from the typical green sunfish spawning season in southeast Arizona. So. we are uncertain how this slight, and possibly not significant, increase in GSI observed during the winter may translate to GSI or fecundity in the spring spawning season.

The highly male-skewed sex ratio (82.61% male) seen in the control group could be evidence of an environmental influence on the sex determination system of green sunfish. It is well known that rearing environment can influence sex ratios in many fish species (Piferrer, 2001; Baroiller et al., 2009; Shen et al., 2016). Stress, high temperatures, and high rearing densities during development can result in male-skewed sex ratios in many fish species (Baroiller et al., 1995; Roncarati et al., 1997; Ospina-A Lvarez and Piferrer, 2008; Mankiewicz et al., 2013; Hattori et al., 2020). In the closely related bluegill, which in at least some populations are speculated of having an underlying ZW/WW sex determination system, increased temperatures during sexual development can skew sex ratios towards all-male (Wang et al., 2018). Since we saw a sex ratio that is highly divergent from a 1:1 male:female ratio, it is possible that environmental conditions may influence the sex determination or differentiation of green sunfish. Without understanding how various rearing temperatures, rearing densities, and other stressors impact sex ratios, it will be difficult to use sex ratios of progeny from the crosses of neofemales with wild-type males to elucidate if green sunfish have a chromosome-based sex determination system. A chromosome-based sex determination system is necessary for the production of TSC carrying individuals that can be used to control nuisance populations (Senior et al., 2013). Therefore, uncovering the mechanisms that direct sex determination and differentiation is vital in assessing the candidacy of a species for a TSC eradication strategy.

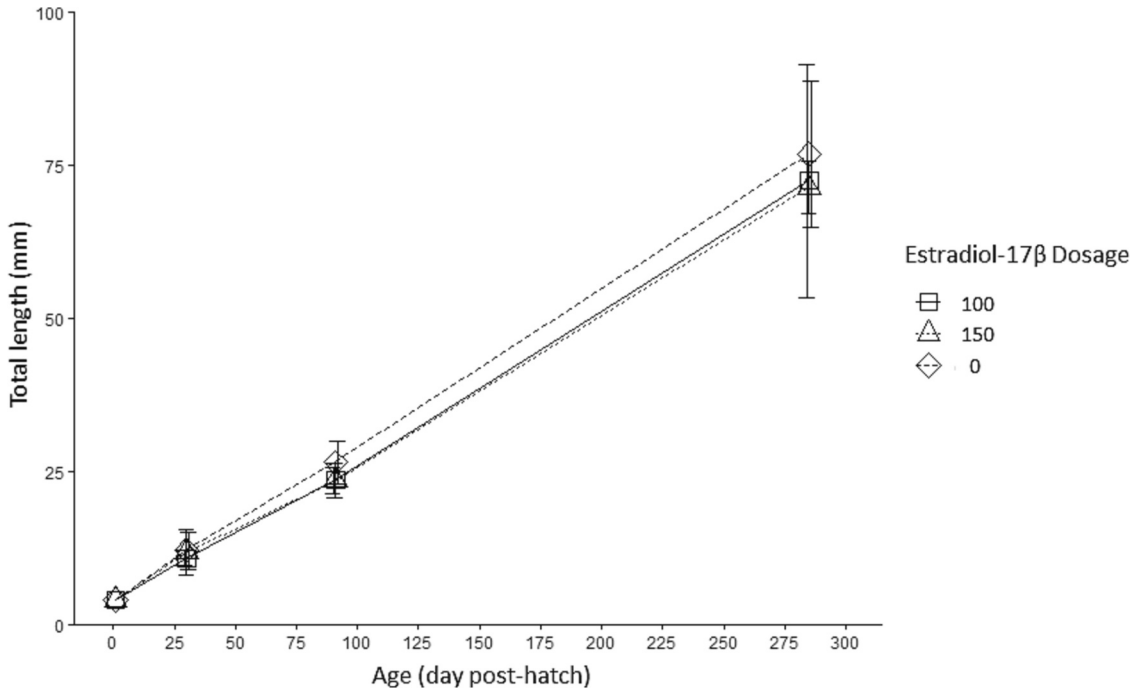

Figure C.3. The mean total lengths (mm) among the estradiol-17β treatment groups (100 = 100 mg estradiol-17β per kg of diet, 150 = 150 mg estradiol-17β per kg of diet) and the control group (0 = 0 mg estradiol-17β per kg of diet) of green sunfish up to 285 days post-hatch (error bars represent 95% confidence intervals).

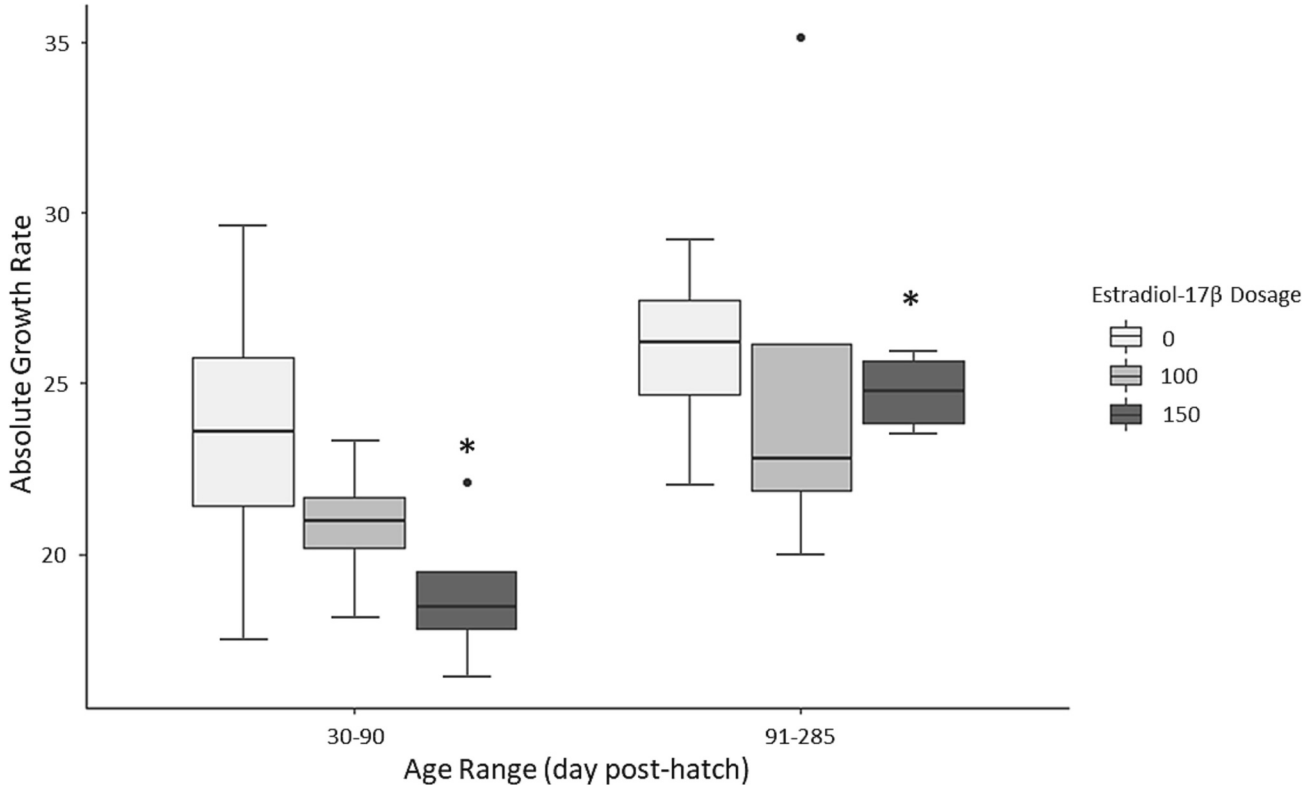

Figure C.4. Comparison of absolute growth rates (AGR) during the treatment period (30–90 days post-hatch) versus after the treatment period (91–285 days post-hatch) for green sunfish across two estradiol-17β treatment groups (100 = 100 mg estradiol-17β per kg of diet; 150 = 150 mg estradiol-17β per kg of diet) and a control group (0 mg estradiol-17β per kg of diet). Significant differences between treatment periods are denoted with asterisks.

Our heavily male-skewed control group suggests that producing all male cohorts without manipulating a chromosomal-based sex determination system and without the use of exogenous steroids could theoretically be possible for green sunfish (Piferrer, 2001; Angienda et al., 2010). The water temperature we reared our treatment cohorts in was 27–30 °C which is within the suitable temperature range for bluegill reproduction (20–30 °C; Mischke and Morris, 1997) and well below the lethal temperature threshold of 41.2 °C green sunfish (Carveth et al.,

2006). Therefore, higher rearing temperatures or higher rearing densities could be attempted to consistently produce all or mostly male green sunfish cohorts that can be stocked for fisheries or aquaculture practices. Environmental manipulation of green sunfish sex determination may also allow aquaculturists to produce high proportions of the larger sex without the need to selectively breed neofemales for indirect masculinization methods, thus avoiding regulatory oversight in the U.S. by the Food and Drug Administration. However, utilizing increased temperatures for producing male-skewed cohorts have been shown to reduce survival and growth rates in tilapia (Baras et al., 2001). Treatments attempting various rearing temperatures and densities with green sunfish should be conducted with a consideration of how these treatments may impact the fish's health.

Overall, the effects of the E2 treatment on the survival of green sunfish were slight. In a concurrent study, *Aeromonas hydrophila* infections were prevalent when green sunfish were being weaned from live nauplii to an artificial diet (Teal et al., 2022a). The increase in infection during this time was likely due to increased organic matter in the form of uneaten artificial diet and concomitant reduction in water quality in the tanks. Other studies have reported a reduced capacity of E2 treated fish to activate their immune response, decreasing their survival rate when challenged with pathogens (Yamaguchi et al., 2001; Wang and Belosevic, 1994; Casanova-Nakayama et al., 2011; Wenger et al., 2011). Additional investigations are needed to test the hypothesis that *Aeromonas hydrophila* infection rates are higher in E2 treated groups than the control group and that this contributed to slightly lower survival in the E2 groups. Exogenous E2 can also cause severe liver and kidney damage which can result in organ failure and be lethal (Zaroogian et al., 2001; Costa et al., 2010). The lethal E2 dose varies by species due to fishes' broad range in sensitivity to estrogens (Costa et al., 2010). In the current study it appears we did not cross a lethal threshold with our E2 dosages. However, lower E2 dosages should still be attempted in green sunfish to mitigate possible damage or increased infection risk derived from E2 induced sex reversals.

The effects of E2 on the growth rate of green sunfish was small and not statistically significant. It is possible that the E2 did cause a slight reduction in mean TL at the end of the treatment period, but other factors such as varying survival and concomitant rearing densities among the treatment replicates might have confounded these effects. Previous research has reported compensatory growth in bluegill (Wang et al., 2008) and brook trout (Schill et al., 2016) after E2 treatments ended that may be attributed to a suppression of growth during the E2 treatments. We observed an increase in mean AGR for all treatment groups during the growth interval after the E2 treatment period, but only in the high-dose group was the increase statistically significant. The increase in AGR of the high-dose treatment group after the treatment period may be evidence of growth suppression caused by E2, but since we also observed a slight increase in AGR in the control group after the treatment period, other factors such as rearing densities may have contributed to this difference.

Fishes often react to exogenous steroids as either growth-promoting agents or as growth suppressors that may cause increased mortality (Pandian and Sheela, 1995; Piferrer, 2001). The deleterious effects of E2 typically only occur if a particular threshold of E2 treatment dosage or duration is surpassed (Hunter et al., 1986; George and Pandian, 1996; Piferrer, 2001; Wang et al., 2008). Although the reduction in survival and AGR we noted among the treatment groups were small and deemed not statistically significant, the high-dose group did exhibit the poorest survival rate and lowest AGR during the treatment period. We also observed that the E2 treated groups exhibited significantly higher survival rates during the post-treatment period than the treatment period while the control group did not show a significant increase in survival rate. Although fish typically exhibit an increased likelihood of survival up to a certain age (Lorenzen, 1996), only the control group did not differ significantly in survival rates between the treatment period and the post-treatment period which may be due to the E2 treatments increasing mortalities during the treatment period. Overall, the variability in survival and growth rates were high, but our results suggest that exogenous E2 does not act as a growth-promoter in green sunfish and may increase mortality at high-doses.

The marginal differences we observed in survival and growth rates of our E2 treated fish further suggest that it is possible to produce and use TSC carrying green sunfish for managing green sunfish populations if sex chromosomes are present in the species. Gutierrez and Teem's (2006) model demonstrated that 3% of the annual reproductive stock of a wild population must be YY females (TSC carrier) in order to eradicate a nuisance population over a matter of decades. The proportion of the wild population that needs to be a TSC carrier to eradicate a population only increases if using YY males instead of YY females or if a faster timeframe for eradication is desired (Schill et al., 2017). Therefore, reliable and efficient production of the TSC carrier broodstock and the TSC carriers is integral to the TSC eradication strategy since continual reintroductions of TSC carriers is necessary for female extirpation (Gutierrez and Teem, 2006), especially if TSC carrier fitness is lower than wild-type fitness (Senior et al., 2013; Schill et al., 2017). If a chromosomal sex determination system is ever discovered within green sunfish, than their fast maturation time and their amenable nature to E2 treatments could alleviate potential TSC carrier production constraints.

5. Conclusions

We developed highly effective male to female sex reversal methods for green sunfish. Using our rearing methods, feeding juvenile green sunfish 100 E2 mg per kg of diet or 150 E2 mg per kg of diet from 30 to 90 dph resulted in 100% feminization of our treated cohorts with no gonadal abnormalities observed. Although the reductions in AGR and survival we saw for both the low-dose treatment and the high-dose treatment were small and not statistically significant during the treatment period when compared to the control group, we still recommend using a low-dose E2 treatment to prevent potential negative effects on the health and growth of this species. The information presented here could help expand the utility of this species as a game fish or aquacultured species, as well as help elucidate information on the sex determination system of green sunfish. We recommend additional studies evaluate possible environmental variables influencing sex ratios in this species.

CRediT authorship contribution statement

Chad N. Teal: Conceptualization, Methodology, Software, Data curation, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Daniel J. Schill:** Conceptualization, Methodology, Writing – review & editing. **Susan B. Fogelson:** Validation, Investigation, Visualization, Writing – review & editing. **Colby M. Roberts:** Methodology, Investigation, Data curation, Writing – review & editing. **Kevin Fitzsimmons:** Methodology, Resources, Writing – review & editing. **Javan M. Bauder:** Methodology, Software, Writing – review & editing. **William T. Stewart:** Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Scott A. Bonar:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We thank the U.S. Bureau of Reclamation for funding this research (United States Bureau of Reclamation, grant number G18AC0061 and modification G19AC0039). Gabriella Buonavolonta, Kylee Rath, Suhailah Hussein, Cora Thomas, Kim Smoot and Jesus Bustamante assisted with data collection and construction and maintenance of aquaculture systems. Nathan Berg with the Arizona Game and Fish Department assisted with green sunfish broodstock collection. Chris Jenney, Kaitlyn Gahl, Zach Nemec, Larissa Lee, Taylor Ulrich, Emily Freed, Joshua Grant, Steven Ingram, and Erin Tracy with the University of Arizona provided assistance with construction of the aquaculture system and provided research advice. Liz Mamer with Idaho Department of Fish and Game assisted in analyzing histology slides. We thank Han Liu and Dr. Yushun Chen with the Wuhan University and Chinese Academy of Sciences for translating the paper by Yun-Chang et al. (2008). There is no conflict of interest declared in this study. The comments of eight anonymous reviewers improved the quality of this manuscript.

References

Al-Ablani, S.A., 1997. Use of Synthetic Steroids to Produce Monosex Populations of Selected Species of Sunfishes (Family: Centrarchidae). Dissertation. Auburn University.

Angienda, P.O., Aketch, B.O., Waindi, E.N., 2010. Development of all-male fingerlings by heat treatment and the genetic mechanism of heat induced sex determination in Nile tilapia (*Oreochromis niloticus L.*). International Journal of Biological and Life Sciences 6, 38–43.

Arslan, T., 2018. Sex-determining mechanisms and control of sex differentiation in largemouth bass and crappies. In: Wang, H.P., Piferrer, F., Chen, S.L., Shen, Z.G. (Eds.), Sex Control in Aquaculture. Hoboken, New Jersey, pp. 385–403.

Baras, E., Jacobs, B., M´elard, C., 2001. Effect of water temperature on survival, growth and phenotypic sex of mixed (XX–XY) progenies of Nile tilapia *Oreochromis niloticus*. Aquaculture 192, 187–199.

Baroiller, J.F., Chourrout, D., Fostier, A., Jalabert, B., 1995. Temperature and sex chromosomes govern sex ratios of the mouthbrooding cichlid fish *Oreochromis niloticus*. J. Exp. Zool. 273, 216–223.

Baroiller, J.F., D'Cotta, H., Bezault, E., Wessels, S., Hoerstgen-Schwark, G., 2009. Tilapia sex determination: where temperature and genetics meet. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 153, 30–38.Bolker, B. M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H, White, J. S.S., 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol. Evol. 24, 127–135.

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H., White, J.S.S., 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol. Evol. 24 (3), 127–135.

Brunson, M.W., Morris, J.E., 2000. Species profile – sunfish. Southern Regional Aquaculture Center Publication Number 724.

Brunson, M.W., Robinette, H.R., 1986. Evaluation of male bluegill×female green sunfish hybrids for stocking in Mississippi farm ponds. N. Am. J. Fish Manag. 6, 156–167.

Carvalho, C.V.A.D., Passini, G., Costa, W.D.M., Vieira, B.N., Cerqueira, V.R., 2014. Effect of estradiol-17β on the sex ratio, growth and survival of juvenile common Snook (*Centropomus undecimalis*). Acta Scientiarum. Animal Sciences 36, 239–245.

Carveth, C.J., Widmer, A.M., Bonar, S.A., 2006. Comparison of upper thermal tolerances of native and nonnative fish species in Arizona. Trans. Am. Fish. Soc. 135 (6), 1433–1440.

Casanova-Nakayama, A., Wenger, M., Burki, R., Eppler, E., Krasnov, A., Segner, H., 2011. Endocrine disrupting compounds: can they target the immune system of fish? Mar. Pollut. Bull. 63, 412–416.

Costa, D.M., Neto, F.F., Costa, M.D.M., Morais, R.N., Garcia, J.R.E., Esquivel, B.M., Ribeiro, C.O., 2010. Vitellogenesis and other physiological responses induced by 17-β-estradiol in males of freshwater fish *Rhamdia quelen*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 151, 248–257.

Desprez, D., M´elard, C., Philippart, J.C., 1995. Production of a high percentage of male offspring with´17a-ethynylestradiol sex-reversed *Oreochromis aureus*: II. Comparative reproductive biology of females and F2 pseudofemales and large scale production of male progeny. Aquaculture 130, 35–41.

Dudley, R., Matter, W., 2000. Effects of small green sunfish (*Lepomis cyanellus*) on recruitment of Gila chub (*Gila intermedia*) in Sabino Creek, Arizona. Southwest. Nat. 45, 24–29.

Feist, G., Schreck, C.B., Gharrett, A.J., 1996. Controlling the sex of salmonids. Oregon Sea Grant, Ploidy/Sex Manipulation Work Group, Corvallis, Oregon, ORESU-H-96-001.

Fuller, P., Cannister, M., Neilson, M., 2021. *Lepomis cyanellus* Rafinesque, 1819: Geological Survey. Nonindigenous Aquatic Species Database, Gainesville, FL. https://nas.er.usgs.gov/queries/FactSheet.aspx?speciesID=380.

Gamble, T., Coryell, J., Ezaz, T., Lynch, J., Scantlebury, D.P., Zarkower, D., 2015. Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. Mol. Biol. Evol. 32, 1296–1309.

George, T., Pandian, T.J., 1996. Hormonal induction of sex reversal and progeny testing in the zebra cichlid *Cichlasoma nigrofasciatum*. J. Exp. Zool. 275, 374–382.

Gillies, C.S., Hebblewhite, M., Nielsen, S.E., Krawchuk, M.A., Aldridge, C.L., Frair, J.L., Jerde, C.L., 2006. Application of random effects to the study of resource selection by animals. J. Anim. Ecol. 75, 887–898.

Gomelsky, B., Mims, S.D., Onders, R.J., Bean, W.B., 2002. Hormonal sex reversal and evidence of female homogamety in black crappie. N. Am. J. Aquac. 64, 66–69.

Goodson Jr., L.F., 1966. Crappie. In: Calhoun, A. (Ed.), Inland Fisheries Management. California Department of Fish and Game, Sacramento, pp. 312–331.

Guerrero, R.D., Shelton, W.L., 1974. An aceto-carmine squash method for sexing juvenile fishes. The Progressive Fish-Culturist 36, 56.

Gutierrez, J.B., Teem, J.L., 2006. A model describing the effect of sex-reversed YY fish in an established wild population: the use of a Trojan Y chromosome to cause extinction of an introduced exotic species. J. Theor. Biol. 241, 333–341.

Hackmann, E., Reinboth, R., 1974. Delimitation of the critical stage of hormone-influenced sex differentiation in *Hemihaplochromis multicolor* (Hilgendorf) (Cichlidae). Gen. Comp. Endocrinol. 22, 42–53.

Hackney, P.A., 1975. Bass populations in ponds and community lakes. In: Clepper, H. (Ed.), Black Bass Biology and Management. Sport Fishery Institute, Washington, D. C., pp. 133–139

Hattori, R.S., Castan˜eda-Cort´es, D.C., Arias Padilla, L.F., Strobl-Mazzulla, P.H., Fernando, J.I., 2020. Activation of stress response axis as a key process in environment-induced sex plasticity in fish. Cell. Mol. Life Sci. 77, 4223–4236.

Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 6, 65–70.

Hulak, M., Paroulek, M., Simek, P., Kocour, M., Gela, D., Rodina, M., Linhart, O., 2008. Water polluted by 17α-methyltestosterone provides successful male sex inversion of common carp (*Cyprinus carpio L.*) from gynogenetic offspring. J. Appl. Ichthyol. 24, 707–710.

Hunter, J.R., 1963. The reproductive behavior of the green sunfish, *Lepomis cyanellus*. Zoologica 48, 13–24.

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, 295–302.

Iwamatsu, T., 1999. Convenient method for sex reversal in a freshwater teleost, the medaka. J. Exp. Zool. 283, 210–214.

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighty, G.C., Sumpter, J.P., Tyler, C.R., 2002. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. Biol. Reprod. 66, 272–281.

Johnstone, R., Simpson, T.H., Youngson, A.F., Whitehead, C., 1979. Sex reversal in salmonid culture: part II. The progeny of sex-reversed rainbow trout. Aquaculture 18, 13–19.

Kennedy, P.A., Meyer, K.A., Schill, D.J., Campbell, M.R., Vu, N.V., 2018. Survival and reproductive success of hatchery YY male brook trout stocked in Idaho streams. Trans. Am. Fish. Soc. 147, 419–430.

Komen, J., Lodder, P.A.J., Huskens, F., Richter, C.J.J., Huisman, E.A., 1989. Effects of oral administration of 17α-methyltestosterone and 17β-estradiol on gonadal development in common carp, *Cyprinus carpio L.* Aquaculture 78, 349–363.

Lemly, A.D., 1985. Suppression of native fish populations by green sunfish in first-order streams of Piedmont North Carolina. Trans. Am. Fish. Soc. 114, 705–712.

Lo´pez-Fern´andez, H., Bolnick, D.I., 2007. What causes partial F1 hybrid viability? Incomplete penetrance versus genetic variation. PLoS One 2, e1294.

Lorenzen, K., 1996. The relationship between body weight and natural mortality in juvenile and adult fish: a comparison of natural ecosystems and aquaculture. J. Fish Biol. 49 (4), 627–642.

Mair, G.C., Abucay, J.S., Abella, T.A., Beardmore, J.A., Skibinski, D.O.F., 1997. Genetic manipulation of sex ratio for the large-scale production of all-male tilapia *Oreochromis niloticus*. Can. J. Fish. Aquat. Sci. 54, 396–404.

Mankiewicz, J.L., Godwin, J., Holler, B.L., Turner, P.M., Murashige, R., Shamey, R., Borski, R.J., 2013. Masculinizing effect of background color and cortisol in a flatfish with environmental sex-determination. Integr. Comp. Biol. 53, 755–765.

McCormick, J.L., Schill, D.J., Meyer, K.A., 2021. Simulated use of YY male stocking and suppression for eradicating common carp populations. N. Am. J. Fish Manag. 41, 366–382.

McGree, M.M., Winkelman, D.L., Vieira, N.K.M., Vajda, A.M., 2010. Reproductive failure of the red shiner (*Cyprinella lutrensis*) after exposure to an exogenous estrogen. Can. J. Fish. Aquat. Sci. 67, 1730–1743.

McKechnie, R.J., Tharratt, R., 1966. Green sunfish. Inland fisheries management. California Department of Fish and Game. p, pp. 399–401.

Mischke, C.C., Morris, J.E., 1997. Out-of-season spawning of sunfish Lepomis spp. in the laboratory. The Progressive Fish-Culturist 59 (4), 297–302.

Morris, J.E., Clayton, R.D., 2009. Centrarchid aquaculture. In: Cooke, S., Phillip, D.P. (Eds.), Centrarchid Fishes: Diversity, Biology and Conservation. Hoboken, New Jersey, pp. 293–311.

Morris, J.E., Mischke, C.C., Lane, R.L., 2005. Culture and propagation of sunfish and their hybrids. Natural Resource Ecology and Management Conference Papers, Posters and Presentations. 26.

Moyle, P.B., 1976. Fish introductions in California: history and impact on native fishes. Biol. Conserv. 9, 101–118.

Nelson, E.J.H., 2018. Sex Determination and Population Genetics of Smallmouth Bass (*Micropterus dolomieu*) in Eastern Lake Ontario. M.S. thesis. Queen's University, Kingston, Ontario, Canada.

Ospina-A Lvarez, 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, e2837.

Pandian, T.J., Sheela, S.G., 1995. Hormonal induction of sex reversal in fish. Aquaculture 138, 1–22.

Peterson, B.C., Davis, K.B., 2012. Effects of gender and sex hormones on disease susceptibility of channel catfish to *Edwardsiella ictaluri*. J. World Aquacult. Soc. 43, 733–738.

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197, 229–281.

Quinn, S., Paukert, C., 2009. Centrarchid fisheries. In: Cooke, S., Phillip, D.P. (Eds.), Centrarchid Fishes: Diversity, Biology and Conservation. Hoboken, New Jersey, pp. 312–339.

R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.

Roberts, F.L., 1964. A chromosome study of twenty species of Centrarchidae. J. Morphol. 76, 408–414.

Roncarati, A., Melotti, P., Mordenti, O., Gennari, L., 1997. Influence of stocking density of European eel (*Anguilla anguilla*, L.) elvers on sex differentiation and zootechnical performances. J. Appl. Ichthyol. 13, 131–136.

Schill, D.J., Heindel, J.A., Campbell, M.R., Meyer, K.A., Mamer, E.R.J.M., 2016. Production of a YY male brook trout broodstock for potential eradication of undesired brook trout populations. N. Am. J. Aquac. 78, 72–83.

Schill, D.J., Meyer, K.A., Hansen, M.J., 2017. Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. N. Am. J. Fish Manag. 37, 1054–1066.

Senior, A.M., Krkosek, M., Nakagawa, S., 2013. The practicality of Trojan sex chromosomes as a biological control: an agent based model of two highly invasive *Gambusia* species. Biol. Invasions 15, 1765–1782.

Shen, Z.G., Wang, H.P., Yao, H., O'Bryant, P., Rapp, D., Zhu, K.Q., 2016. Sex determination in bluegill sunfish *Lepomis macrochirus*: effect of temperature on sex ratio of four geographic strains. Biol. Bull. 230, 197–208.

Teal, C.N., Schill, D.J., Fogelson, S.B., Roberts, C.M., Fitzsimmons, K., Bonar, S.A., 2022a. Development of aquaculture protocols and gonadal differentiation of green sunfish (*Lepomis cyanellus*), aquaculture, 547, 737515. ISSN 0044–8486. https://doi.org/10.1016/j.aquaculture.2021.737515.

Teal, C.N., Coykendall, D.K., Campbell, M.R., Eardley, D.L., Delomas, T.A., Shira, J.T., Culver, M., 2022b. Sex-specific markers undetected in green sunfish *Lepomis cyanellus* using restriction-site associated DNA sequencing. J. Fish Biol. https://doi.org/10.1111/jfb.15063.

Teem, J., Alphey, L., Descamps, S., Edgington, M., Edwards, O., Gemmell, N., Roberts, A., 2020. Genetic biocontrol for invasive species. Frontiers in Bioengineering and Biotechnology 8, 1–18.

van der Ven, L., Wester, P., 2022. Histology and Histopathology Atlas of the Zebrafish V2.01. available. http://zfin.org/hh_atlas.

Wang, R., Belosevic, M., 1994. Estradiol increases susceptibility of goldfish to *Trypanosoma danilewskyi*. Developmental & Comparative Immunology 18, 377–387.

Wang, H.P., Gao, Z., Beres, B., Ottobre, J., Wallat, G., Tiu, L., Yao, H., 2008. Effects of estradiol-17 β on survival, growth performance, sex reversal and gonadal structure of bluegill sunfish *Lepomis macrochirus*. Aquaculture 285, 216–223.

Wang, H.P., Zhi-Gang, S., Ze-Xia, G., Hong, Y., Rapp, D., O'Bryant, P., 2018. Sex determination, differentiation, and control in bluegill. In: Wang, H.-P., Piferrer, F., Chen, S.-L., Shen, Z.-G. (Eds.), Sex Control in Aquaculture. Hoboken, New Jersey, pp. 361–384.

Wenger, M., Sattler, U., Goldschmidt-Clermont, E., Segner, H., 2011. 17 Beta-estradiol affects the response of complement components and survival of rainbow trout (*Oncorhynchus mykiss*) challenged by bacterial infection. Fish & Shellfish Immunology 31, 90–97.

Werner, E.E., Hall, D.J., 1977. Competition and habitat shift in two sunfishes (Centrarchidae). Ecology 58, 869–876.

Yamaguchi, T., Watanuki, H., Sakai, M., 2001. Effects of estradiol, progesterone and testosterone on the function of carp, *Cyprinus carpio*, phagocytes in vitro. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 129, 49–55.

Yamazaki, F., 1983. Sex control and manipulation in fish. Aquaculture 33, 329–354.

Yun-Chang, C.A.O., Wen-Sheng, L.I., Wei, Y.E., Hao-Ran, L.I.N., 2008. Gonadal development in green sunfish, *Lepomis cyanellus*: a histological study. Chinese Journal of Zoology 43, 88–95.

Zaroogian, G., Gardner, G., Horowitz, D.B., Gutjahr-Gobell, R., Haebler, R., Mills, L., 2001. Effect of 17β-estradiol, o, p'-DDT, octylphenol and p, p'-DDE on gonadal development and liver and kidney pathology in juvenile male summer flounder (*Paralichthys dentatus*). Aquat. Toxicol. 54, 101–112.

Zuur, A.F., Leno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M., 2009. Mixed Effects Models and Extensions in Ecology with R. New York, USA, New York.

APPENDIX D

DEVELOPMENT OF AQUACULTURE PROTOCOLS AND GONADAL DIFFERENTIATION OF RED SHINER

North American Journal of Aquaculture 83:145–154, 2021
© 2021 American Fisheries Society
ISSN: 1522-2055 print / 1548-8454 online
DOI: 10.1002/naaq.10176

ARTICLE

Development of Aquaculture Protocols and Gonadal Differentiation of Red Shiner

Chad N. Teal*
Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2 Room N315, 1064 East Lowell Street, Tucson, Arizona 85721, USA

Daniel J. Schill
Fisheries Management Solutions, Inc., 9950 West Prairie Road, Boise, Idaho 83714, USA

Susan B. Fogelson
Fishhead Labs, LLC, 5658 Southeast Pine Avenue, Stuart, Florida 34997, USA

Scott A. Bonar
U.S. Geological Survey, Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2 Room N315, 1064 East Lowell Street, Tucson, Arizona 85721, USA

Abstract

Developing detailed rearing methods and describing the onset of gonadal differentiation in Red Shiners *Cyprinella lutrensis* could facilitate the development of novel techniques to control or enhance populations, enable toxicology studies, and help construct bioassays. In this study, we develop and report aquaculture practices for Red Shiner that ensure consistent year-round production in laboratory settings and evaluate the timing of sexual differentiation via histological gonad examinations. Our methods resulted in a mean of 56.00% (SD = 8.98%) survival through the larval stages of development, and we obtained spawns from captive-reared Red Shiners 138 d posthatch. Red Shiners are gonochoristic, and both ovaries and testes differentiate directly from undifferentiated gonads. Ovaries begin to differentiate in females 45 d posthatch, while testes begin differentiating in males 105 d posthatch. This study provides in-depth protocols for the closed-cycle aquaculture of Red Shiners and describes the gonadal differentiation and development of both sexes.

Red Shiner *Cyprinella lutrensis* is a minnow species native to the Great Plains region of central North America (Matthews et al. 1980). Red Shiners are ecologically destructive when introduced outside of their native range and can decimate native fishes (Douglas et al. 1994; Gido et al. 1999). Red Shiners displaced native Spikedace *Meda fulgida* in desert streams of Arizona and New Mexico (Rinne 1991), prevented the reestablishment of Woundfin *Plagopterus argentissimus* in the Virgin River (Deacon 1988), and are a predator of native larval fishes within the Colorado River system (Ruppert et al. 1993). Despite their ability to colonize a wide variety of habitats, some populations of Red Shiner struggle within their native range, and reestablishment of these populations can be difficult

*Corresponding author: chadteal@email.arizona.edu
Received August 28, 2020; accepted November 27, 2020

(Marsh-Matthews et al. 2011). The development of species-specific aquaculture techniques could aid in such restoration efforts.

Conversely, fisheries managers seek means to control many invasive Red Shiner populations (Mizzi 1996; Clarkson and Marsh 2010). One control approach that has been modeled in several species is the Trojan sex chromosome (TSC) eradication strategy (Gutierrez and Teem 2006; Senior et al. 2013; Schill et al. 2017). With this approach, nuisance fish populations are theoretically eradicated by shifting their sex ratios toward all male by introducing "YY males," which carry two copies of the Y sex chromosome instead of one Y and one X, or "ZZ females," which produce all male offspring when crossed with wild-type ZZ males (Senior et al. 2013; Schill et al. 2017). Though a largely unexplored approach to date, field experiments using YY male Brook Trout to control invasive Brook Trout populations are being conducted in four western U.S. states (Teem et al. 2020). Trojan sex chromosome carriers have also been suggested as a means to assist struggling populations (Cotton and Wedekind 2007) by skewing the sex ratio towards female (Wedekind 2002).

Understanding the sex determination system of a species is fundamental in implementing a TSC strategy, whether for enhancing a faltering native fish population (Cotton and Wedekind 2007) or attempting to eradicate an invasive species (Gutierrez and Teem 2006). Neither the sex determination system nor the timing of sex differentiation of Red Shiner has been investigated. Putative XX-female/XY-male sex determination systems and ZZ-male/ZW-female sex determination systems have been identified in fishes by assessing the sex ratios of progeny from crosses using sex-reversed individuals (Desprez et al. 1995; Gomelsky et al. 2002). The establishment of aquaculture methods and the ability to sex reverse fish during their gonadal development are requirements to produce TSC carriers (Gutierrez and Teem 2006; Cotton and Wedekind 2007) and can be used in the discovery of the sex determination system for a species (Piferrer 2001). Inducing sex reversal by administering exogenous steroids is most effective if steroid treatment begins before gonadal differentiation and ends when gonadal differentiation is observable through histology (Piferrer 2001). Systematic aquaculture methods and understanding the timing and pattern of gonadal differentiation for Red Shiner does not just facilitate the initial examination of a TSC strategy, but also helps construct cause and effects scenarios for toxicology studies (Uguz 2008) and the implementation of bioassays (Hunn et al. 1968; Carr 1976; Adams 1986).

Although Red Shiner were commonly used as baitfish (Hubbs 1954) and captive populations have been the subject of multiple studies (Saksena 1962; Koehn 1965; Matthews 1977; Gale 1986; Marsh-Matthews et al. 2007; McGree et al. 2010), aquaculture rearing methods are lacking. Only one study has reported rearing Red Shiner on artificial diets throughout their development (Islam 1972). The ability to rear fish on artificial diets facilitates the administration of exogenous steroids (Yamamoto 1963; Schill et al. 2016) and other drugs (Treves-Brown 2000).

Exposure to endocrine disruptors and effluent-derived xenobiotics can cause irreversible alterations in fish sex differentiation if exposure occurs during critical periods in their sexual development (Gimeno et al. 1996; Devlin and Nagahama 2002; Uguz et al. 2003; McGree et al. 2010). Before this study, no information existed on the gonadal development of Red Shiner, including the pattern and timing of sex differentiation. Understanding when and how Red Shiner gonads develop will not only facilitate the sex reversal of fish required to create TSC carriers, but will also aid in understanding how unintentional exposure to endocrine disruptors and exogenous steroids might alter sex ratios of wild populations. Histological examination of their developing gonads could provide more examples of sexual differentiation within the genus *Cyprinella* and identify timing of gonadal development in Red Shiner.

The specific objectives of this study were to (1) develop and report indoor rearing techniques for Red Shiners and (2) identify the pattern and timing of their gonadal differentiation. This research will help future efforts aimed at restoring native populations of Red Shiner, controlling invasive populations using TSC carriers, and propagating captive fish for toxicology studies and bioassay trials.

METHODS

Broodstock collection and stocking.— On June 23, 2018, 140 adult Red Shiners were collected from the Gila Box Riparian National Conservation Area in Arizona, USA (32°53'0.8872"N, 109°30'0.39489"W) using a seine net (3.0 × 1.2 m). Red Shiners were transported to the University of Arizona Fisheries Propagation Lab in a 142-L cooler filled with Gila River water. Portable aerators were used to keep dissolved oxygen above 5.0 ppm during transit. The fish were given a 1-h prophylactic immersion treatment using a combination of 0.05 ppm malachite green and 15 ppm formalin (ProForm C, Koi Care Kennel, South Jordan, Utah). The Red Shiners were then acclimated by removing half the water in the cooler and then refilling with aquarium water through aquarium airline tubing (4.8 mm inner diameter) to ensure a slow introduction of new water. Captured fish were used to establish a broodstock, and their mean TL was 58.9 mm (SD = 3.8 mm) and mean weight was 2.01 g (SD = 0.68 g).

One male and three females were stocked into each of four 37.9-L glass aquariums outfitted with an AquaClear

50 power filter (Hagen, Mansfield, Massachusetts) and an EHEIM Jager 100-W heater (EHEIM GmbH & Co., Deizisau, Germany). Broodstock tanks were kept on an 18-h light cycle to induce frequent spawning (Gale 1986). The water quality parameters were maintained within the following: water temperature 27–29°C, ammonia <0.25 ppm, nitrite <1.0 ppm, and pH 8.0–8.2. Broodstock were fed Zeigler Aquatox Fish Diet (Zeigler Bros., Gardners, Pennsylvania) and bloodworms ad libitum twice per day.

Spawning and hatching.— Spawning stacks were constructed using methods described by McGree et al. (2010). The spawning stacks were made from tan tiles (10.8 × 10.8 cm) that were stacked and separated 2–3 mm using two 6.35-mm stainless steel washers to provide the Red Shiner their preferred crevice width (Gale 1986). A hole was drilled in the center of each tile, and four tiles were used in each spawning stack. The tiles were held together with a 6.35-mm stainless steel bolt and 6.35-mm stainless steel nut. Three spawning stacks were placed in each broodstock tank. Spawning stacks were checked for eggs once per day for a year.

Spawning stacks with eggs were removed and egg counts were recorded from 17 randomly selected spawns. Eggs were counted by disassembling the spawning stacks and counting eggs on the surfaces of the tiles. Eggs in each spawning stack were counted by two researchers for verification. A spawning stack from each broodstock tank that contained over 100 eggs was then reassembled and given a 30 min 100-ppm formalin bath in an aerated 18.9-L bucket. After the formalin treatment, the spawning stacks were transferred to 37.9-L glass larval rearing tanks filled with dechlorinated tap water. Each larval rearing tank had an airstone, a QANVEE Bio Sponge filter (Taian Qanvee Aquarium Equipment Co., Ltd., Shandong, China), and an EHEIM Jager 100-W heater. Each of the four larval rearing tanks contained egg-bearing spawning stacks from one of the four broodstock tanks. Two more tanks with identical configurations were used to rear fish for the histology portion of the study (see "Gonadal development and histology" below).

Larval rearing and grow out.— Eggs hatched 3 d post fertilization in water 26.5–27.5°C. Five larvae from each of the four larval tanks were removed upon hatching and placed under a Zeiss Stemi 2000 stereo microscope (Carl Zeiss Ag, Oberkochen, Germany) to inspect for yolk sacs; none were observed. Upon hatching, total larvae were counted in each spawn and 100 Red Shiners were kept in each rearing tank to ensure that all tanks started at the same stocking density. All surplus larvae were euthanized by immersion in an aqueous solution of 75 ppm tricaine methanesulfonate (MS-222) and 150 ppm sodium bicarbonate.

Starting at one d posthatch (dph), larvae were fed Zeigler AP 100 larval diet (<50 μm particle size). A dissecting microscope was used to inspect five larvae from each of the four larval rearing tanks to look for the presence of food in the gut. Once food was observed in over 50% of the larvae (by 2 dph), an EHEIM automated fish feeder was added to each tank. The automated feeders dispensed 21.1 mg (SD = 2.52 mg; n = 20 measurements of diet dispersed) four times per day for the first 30 dph. Every 12 d, the next stage of Zeigler AP 100 larval diet was introduced into their feeding regimen. The diet particle sizes were <100, 100–150, 150–250, and 250–400 μm. In addition to the automated feedings, the larval tanks were fed ad libitum twice per day. The amount fed ad libitum was determined by how much the Red Shiners could eat in 2 min without the food set- tling on the bottom of the tank. Feeding stopped once food began to settle on the bottom of the tank. At 171 dph, fish were acclimated to Zeigler Aquatox Fish Diet flakes. Tanks were siphoned every day to remove unea- ten food and waste. Larval carcasses were fixed in 10% neutral buffered formalin and occasionally submitted to the University of Arizona's Aquaculture Pathology Lab for investigation. Water quality parameters were kept at temperature 26–27°C, ammonia <0.25 ppm, nitrite <1.0 ppm, and pH 8.0–8.4.

Total length measurements were taken from the F_1 generation throughout the first year of grow out. Fifteen larvae were measured at 1 dph (1 dph defined as within 24 h of hatching) for TL. All fish from each replicate were counted, weighed, and measured at 62, 125, and 262 dph. Measurements were taken from 10 randomly selected fish in each replicate at 387 dph. A Levene's test for equality of variances was performed on the TLs from the four larval rearing tanks grouped by age to assess if variation in TL changed during grow out.

At 125 dph, the Red Shiners were transferred to two 473-L aquariums with a screen partition in the center. Each of the 473-L tanks housed two of the replicates, and each replicate was randomly assigned to a tank and a side. Each tank had two 300-W EHEIM heaters and two AquaClear 110 power filters. In order to see if we could obtain spawns from the F_1 generation, spawning stacks were placed in grow-out tanks once male coloration started forming between 137–175 dph.

Gonadal development and histology.— We reared 122 larvae and 214 larvae under the conditions described above in two 37.9-L glass aquariums. Five fish were removed from one of the histology tanks once a week for the first 30 d and then every 10–15 d until the Red Shiners were 150 dph. Five spawning females and four spawning males were sacrificed for histology at 293 dph to ensure that we sampled gonads of fully mature fish. Fish were euthanized by immersion in an aqueous solution of 75 ppm MS-222 and 150 ppm sodium bicarbonate before being measured for TL and weight, and then they were

fixed in 10% neutral buffered formalin. Only abdominal sections were fixed in fish that were longer than 18 mm TL. Fixed samples were submitted to Animal Reference Pathology (Salt Lake City, Utah) for histology processing and hematoxylin and eosin staining. Samples under 100 dph were cut along the sagittal plane, and in samples older than 100 dph, three transverse cuts were made between the anus and anterior end of the pelvic fin. Histological sections were photomicrographed using an AmScope 40x-2000x 3W LED Seidentopf trinocular compound microscope and AmScope 14MP camera (United Scope, LLC, Irvine, California) or were scanned for digital review by Animal Reference Pathology.

Gonadal tissue was sexed and its development described using established literature (Jensen and Shelton 1983; van der Ven and Wester 1999–2003; Sacobie and Benfey 2005; Arezo et al. 2007; Uguz 2008; Gao et al 2009; Lowerre-Barbieri et al. 2011). Gonadal tissue and sex differentiation were identified and described by comparing Red Shiner histological sections with digital histological images and descriptions from the references listed above. Three small-bodied fish species (Fathead Minnow *Pimephales promelas*, Zebrafish *Danio rerio*, and *Austrolebias charrua*) were used as references to ensure accurate description of tissues and gonadal differentiation (van der Ven and Wester 1999–2003; Arezo et al. 2007; Uguz 2008).

RESULTS

Spawning and Hatching

Fish in the broodstock tanks spawned every 1 to 14 d over the entire year. The number of eggs produced in each spawn ranged from 5 to 613, with the mean 24-h clutch size being 226 eggs (SD = 162 eggs; $n = 17$). The mean egg count per mean female weight (g) per spawn was 35 eggs/g of female. The mean percentage of eggs that hatched in each clutch was 97.59% (SD = 6.39%; $n = 9$). First spawns from the F_1 generation began at 138 dph. Spawns began occurring in tanks when the largest fish reached approximately 27 mm TL.

Growth Rates

Red Shiner mean TL was 4.39 mm (SD = 0.16 mm, $n = 15$), 15.1 mm (SD = 3.8 mm; $n = 224$), 24.3 mm (SD = 4.1 mm; $n = 190$), 46.0 mm (SD = 6.6 mm; $n = 184$), and 49.5 mm (SD = 6.1 mm; $n = 40$) by 1, 62, 125, 262, and 387 dph, respectively (Figure 1A). Growth rate began to decrease by 262 dph (Figure 1B). Variability in Red Shiner length increased with age (Levene's test, $F_{4,\ 693} = 41.531$, $P < 0.0001$), and mean TLs were different among tanks after the first year of growth.

Survival Rates

Mortality decreased with age (Figure 2). Mean survival rate from 1 to 62 dph was 56.00% (SD = 8.98%), increasing to 85.76% (SD = 16.12%) from 62 to 125 dph. Mean survival rate from 125 to 262 dph approached unity at 95.82% (SD = 2.55%). No cannibalism was observed during grow out. No lesions, bacteria, fungi, oomycetes, protozoa, or virus could be identified in larval carcasses submitted to the University of Arizona's Aquaculture Pathology Lab.

Gonadal Development and Histology

All gonads in fish 21–35 dph were undifferentiated and contained primordial germ cells supported by mesenchymal stroma (Figure 3). Gonads did not begin to differentiate until after fish transition from the postlarval to juvenile stage. Evidence of ovarian differentiation was observed in fish sampled at 45 dph (Figure 4), with females being as small as 10 mm TL. We did not observe the transformation of primordial germ cells to oogonia, and we did not find any chromatin-nucleolus stage oocytes. Ovarian differentiation was characterized by the presence of large primary growth oocytes. Oocyte vitellogenesis was observed in females as young as 105 dph. By 293 dph, ovaries contained oocytes in all stages of development (Figure 5).

Definitive testicular differentiation was not observed in fish sampled prior to 105 dph (Figure 6) or smaller than 17 mm TL. Differentiation of male gonads was characterized by the clustering of presumptive spermatogonia (Figure 5). Clustered spermatogonia formed lobular structures and were organized into tubules by 135 dph (Figure 5). Both male and female gonads were clearly differentiated by 135 dph. By 293 dph, testes exhibited clusters with spermatocytes and spermatids as well as spermatozoa in the tubular lumina (Figure 5).

DISCUSSION

Basic biology and population dynamics of Red Shiner need to be more closely examined before it will be possible to determine the efficacy of using a TSC strategy. Developing aquaculture protocols and describing the gonadal sex differentiation in the species are necessary steps in the exploration of a TSC strategy. Even in well-researched species, there are still many uncertainties associated with TSC strategies, which is evident by the paucity of data from TSC field studies. Nevertheless, developing Red Shiner aquaculture protocols and describing their gonadal differentiation has important implications beyond its utility in TSC strategies.

Aquaculture of Red Shiners

In this study, we successfully used indoor aquaculture techniques to rear Red Shiners. We demonstrated the hardiness of the species and its potential for use in a variety

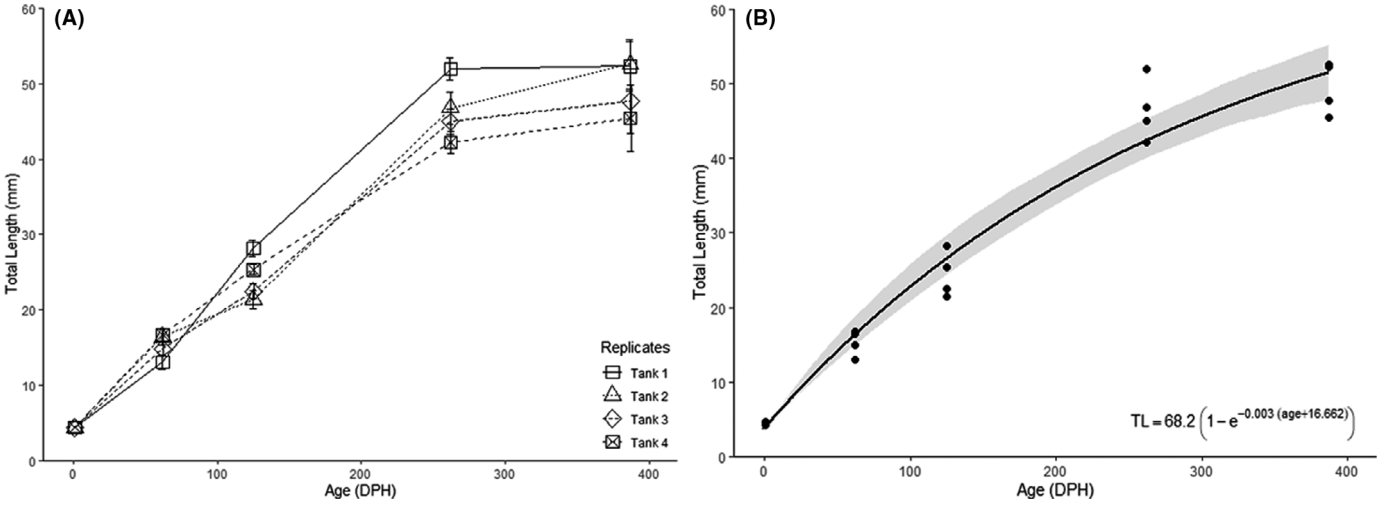

Figure D.1. **(A)** Growth plot of Red Shiner TL (mm) versus age (dph) separated by larval rearing tank. Variation in TL increased with age. Error bars are 95% confidence intervals. Growth rate decreased by 262 dph, as shown by the **(B)** Von Bertalanffy growth curve with 95% confidence bands.

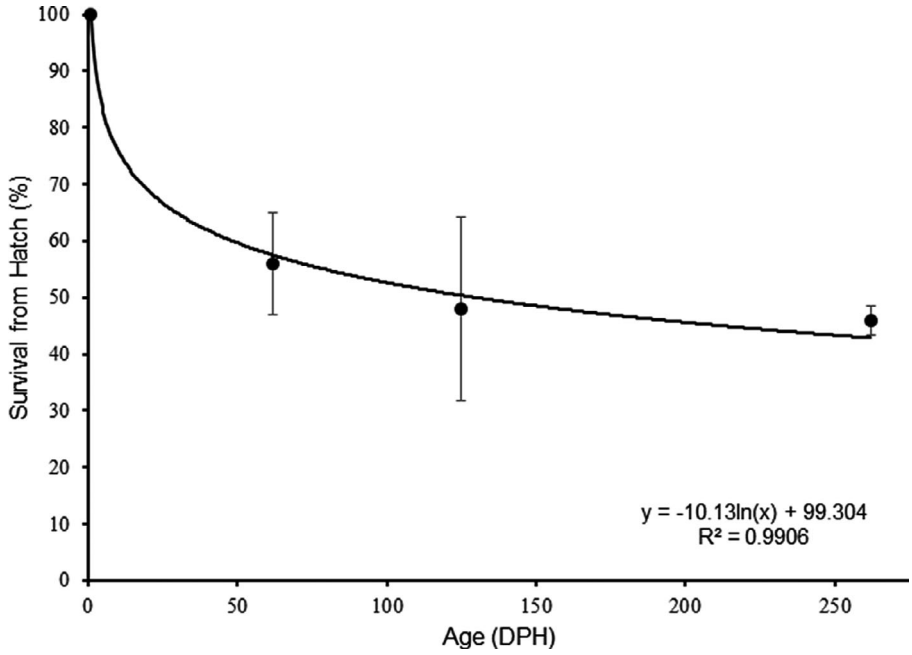

Figure D.2. Red Shiner survival (percentage of fish survived) as a function of age (dph). Error bars represent SD.

of studies with simple and inexpensive aquaria and filter systems. We successfully reared Red Shiners in static aquaria rather than a recirculating aquaculture system or flow-through system. The Red Shiner's ability to continuously spawn is a useful characteristic for use in research settings and is a shared trait with model species such as the Fathead Minnow (Gale and Buynak 1982) and Zebrafish (Segner 2009). Developing aquaculture methods for this species is imperative in assessing its feasibility as a candidate for a TSC eradication strategy. Previous studies have suggested a stocking rate of TSC carriers ranging from 3.2% to 50% of the wild population for the eradication of the nuisance population (Gutierrez and Teem 2006; Schill et al. 2017). Red Shiner broodstock exhibited high reproductive potential by producing consistent spawns year-round when maintained in 27–29°C water with an 18-h photoperiod. The ability to produce Red Shiner larvae throughout the year allows many opportunities to attempt different rearing techniques and ensures consistent production.

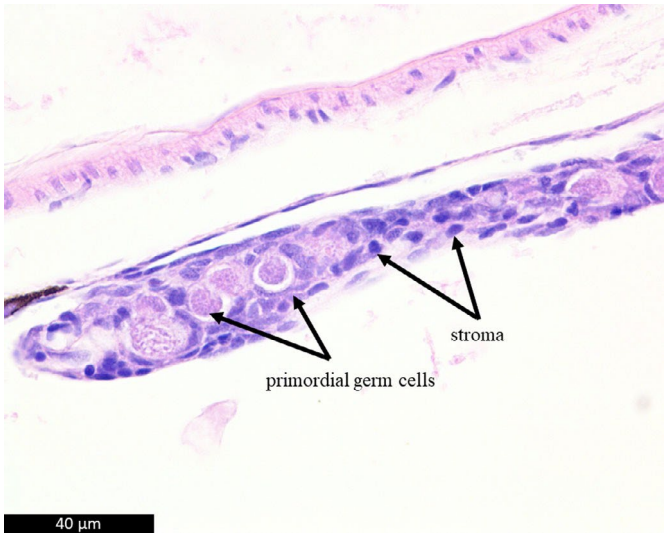

Figure D.3. Undifferentiated gonad in a 35-dph Red Shiner. The top of the image is the dorsal side of the body.

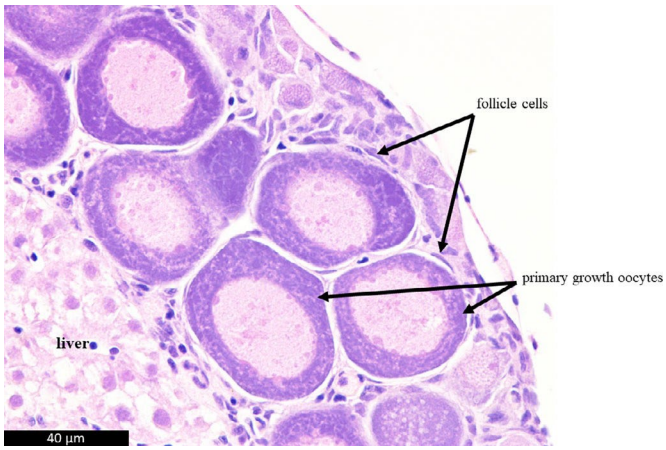

Figure D.4. Developing ovary in a 45-dph female Red Shiner. The top of the image is the dorsal side of the body.

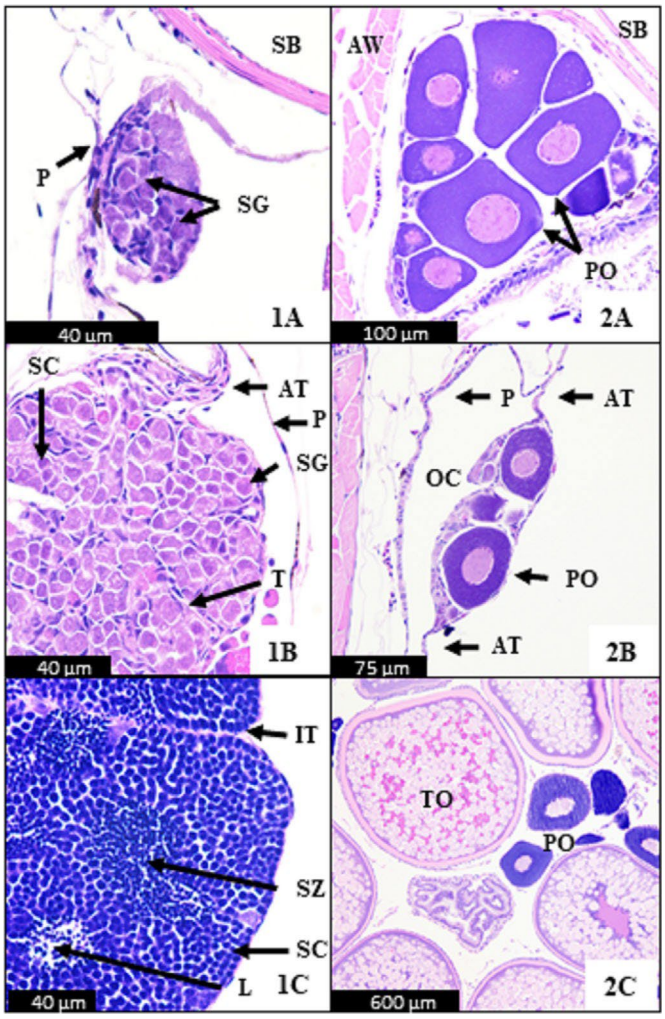

Figure D.5. A comparison of testes and ovaries in Red Shiners **(1A)** and **(2A)** at 105 dph, **(1B)** and **(2B)** at 135 dph, and **(1C)** and **(2C)** at 293 dph. Image 1A shows the formation and clustering of spermatogonia (SG) in early testicular differentiation and the position of testis along the peritoneum (P) by the swim bladder (SB). Image 2A shows large primary vitellogenic oocytes (PO) in maturing females and the ovary's position along the peritoneum (P) by the abdominal wall (AW) and swim bladder (SB). Image 1B shows that by 135 dph, the organization of tubules (T) is evident by fissures separating the proliferating spermatogonia. In image 1B the spermatogonia have proliferated and some have undergone meiotic processes to become primary spermatocytes (SC). Image 1B shows that a single attachment point (AT) to the peritoneum is characteristic of a testis, whereas ovaries (2B) exhibit two attachments to the peritoneum and an ovarian cavity (OC). Image 1C shows the lumina (L) are filled with spermatozoa (SZ) and the seminiferous tubules are separated by interstitial connective tissue (IT). Image 2C shows different stages of oocyte development in mature females (TO = tertiary vitellogenic oocytes).

We documented variable and indeterminate fecundity in Red Shiner broodstock, which is similar to observations by Gale (1986). However, we observed fewer eggs per spawn in 24-h periods than previous studies (Gale 1986; McGree et al. 2010), despite broodstock tanks containing one or two additional females per tank. We observed a mean of 226 eggs per spawn in a 24-h period, while McGree et al. (2010) observed a mean of roughly 800 eggs per spawn in a 24-h period, and Gale (1986) observed a mean clutch size of 585 eggs. Tank stocking density, nutrition, and environmental parameters may have been significant factors in determining egg number per spawn. Gale (1986) might have seen larger numbers of eggs per clutch due to their tanks being outdoors where they experienced relatively large temperature swings and a more

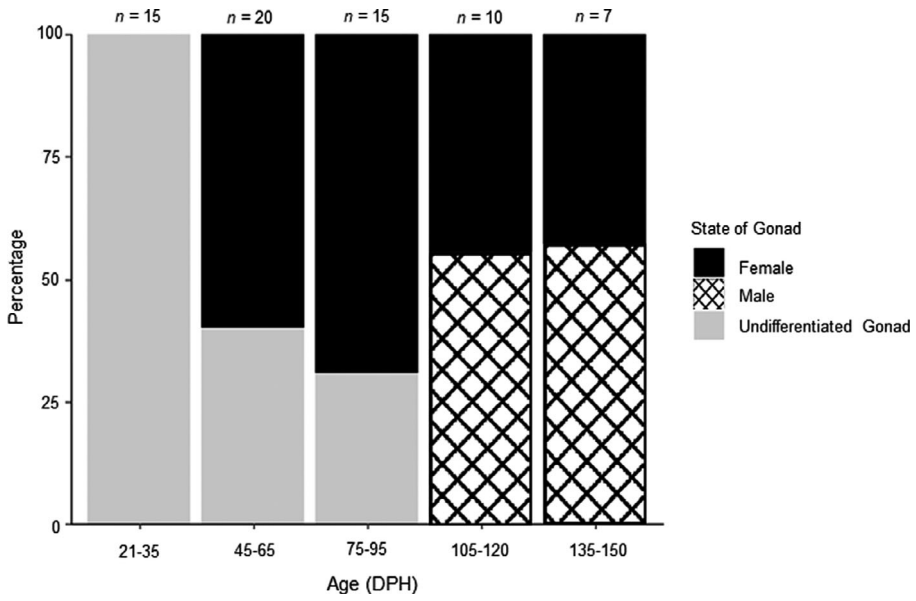

Figure D.6. Percentage of Red Shiners with differentiated gonads over time (dph). Ovaries began to differentiate after 35 dph, and testicular differentiation was not detected until 105 dph.

varied diet. Gale (1986) also noted that the largest spawns occurred after cool weather. However, we used the same spawning stack designs, same broodstock diet, and similar aquarium systems and water quality parameters as McGree et al. (2010). Thus, other undetermined mechanisms may have decreased the mean number of eggs per clutch. Gale (1986) reported that Red Shiners are fractional spawners with indeterminate fecundity and concluded that environmental cues, such as increased temperature and increased photoperiod, correlate with increased fecundity. These environmental conditions, along with food availability, spawning site availability, water depth, and intraspecific pheromones, are factors that can influence spawning behavior and alter fecundity in many fish species (Ali 1993; Brummet 1995; Kobayashi et al. 2002). To better control timing of Red Shiner spawning and fecundity, more research is needed to investigate how factors other than temperature and photoperiod could also play a role in the timing and size of spawns in a laboratory setting.

We found that the mean hatch rate of eggs was nearly 100%, which is higher than the 70–89% hatch rates reported in other Red Shiner studies (Islam 1972; McGree et al. 2010). We gave each spawn a prophylactic 100 ppm 10% neutral formalin bath. Neither Islam (1972) nor McGree et al. (2010) treated eggs with formalin before hatching. Formalin has been shown to reduce fungal growth on fish eggs and decrease egg mortality (Rach et al. 1997). Temperature can impact both the hatch rate and larval mortality of Red Shiners (Islam 1972). These findings suggest that a formalin treatment and rearing temperature of 26.5–27.5°C in aerated water leads to a high hatch rate of Red Shiner larvae.

Although environmental conditions were similar, growth rates varied among the four larval rearing tanks (Figure 1A). Study fish were stocked at the same starting density of 100 larvae per tank, but densities changed due to mortalities during grow out, and these differences in tank densities could have resulted in growth rate variation. Even though Red Shiners were fed ad libitum twice daily, tank density and conspecific competition might have resulted in unequal feed rates per fish, which could cause variation in growth rates (Ewa-Oboho and Enyenihi 2001). In all tanks, growth rates dropped drastically after Red Shiners reached 262 dph (Figure 1B). At 387 dph, the mean TL of the laboratory-reared Red Shiners was still almost 20% shorter than the original wild-caught broodstock. Nutrition has been shown to impact growth rates of many fishes (Lovell 1989; Kwiatkowski et al. 2008). The formulated diets we fed lab-reared Red Shiners could be a reason for their shorter TL. Also, Farringer et al. (1979) reported that Red Shiners live for 2 years in the wild. Despite the reduction in growth rates we saw after one year, it is possible that lab-reared Red Shiner would reach the wild-caught TL if given a second rearing year.

We observed spawns from the F_1 generation in fish as young as 138 dph. Lab-reared fish might have spawned earlier, but no spawning stacks were added to the maturation tanks until we noticed spawning colors starting to form on the males. Marsh-Matthews et al. (2007) reported that Red Shiner young of year can spawn, and they witnessed spawns from fish reared in outdoor experimental

ponds estimated to be as young as 119 dph and as small as 28 mm TL. Overall, the age of fish at sexual maturity and growth rates reported by Marsh-Matthews et al. (2007) are similar to our own findings. Islam (1972) reported rearing sexually mature Red Shiners in laboratory tanks in as few as 79 d with a TL of 43 mm, but this faster growth rate was in warmer water and at the expense of a lower survival rate. Islam (1972) also fed the developing Red Shiners a different diet, ground Purina Catfish and Trout Chow, while the present study used Zeigler AP 100 larval diet during larval and juvenile stages. A potential benefit of using a larval diet with a series of different particle sizes is that it allows for the administration of slightly larger diet particles throughout larval development. Increasing the diet particle size throughout larval development improves feeding efficiency, survival, and reduction of waste in the form of uneaten food (Hartman 1983; Charlon and Bergot 1984; Walford et al. 1991; Cahu and Zambonino Infante 2001). The relative homogeneity of particle sizes in each stage of formulated larval fish diets can prevent the administration of food that is too large to be consumed, is too small for detection, or does not trigger a feeding response (Walford et al. 1991; Cahu and Zambonino Infante 2001).

The survival rate of lab-reared Red Shiners was similar to that reported for larvae reared by Islam (1972) at the same temperature. Red Shiners experience highest mortality in the first 60 d, which is unsurprising because high larval and juvenile mortality rates are seen in most teleosts (Bailey and Houde 1989; Lorenzen 1996). Islam (1972) reported the highest survival rate of larval Red Shiners when fish were reared at 22°C. Rearing Red Shiners at 26–27°C resulted in faster growth, but it may have reduced overall survival. The mean survival rate to 62 dph was 56%, but if higher larvae survival rates are desired, then lowering temperatures to 22°C may be helpful. A definitive cause of death could not be identified in the lar- val carcasses submitted to the University of Arizona's Aquaculture Pathology Lab; evidence of specific disease entities, including bacteria, fungi, oomycetes, protozoa, and virus, was absent in the evaluated tissue sections.

Gonadal Differentiation of Red Shiners

In this study, we were able to describe the gonadal differentiation of Red Shiners using histology. These findings show that Red Shiners are gonochoristic, and their testes and ovaries differentiate directly from undifferentiated gonads. Red Shiner ovaries differentiate earlier than testes. This a common pattern in teleost gonadal development (Guraya 1994; Nakamura et al. 1998; Uguz 2008; Benfey 2019; Jiang et al. 2019). The delayed differentiation of testes suggests that gonads that were still undifferentiated by 65 dph were presumptive testes.

Previous histology studies documented a transformation of primordial germ cells to oogonia (Jensen and Shelton 1983; van der Ven and Wester 1999–2003), as well as the development of chromatin-nucleolus stage oocytes (Uguz 2008). We did not observe oogonia or chromatin-nucleolus stage oocytes. Instead, we observed that ovarian differentiation was first detected due to the development of large primary growth oocytes. Oocyte vitellogenesis was seen in Red Shiners as young as 105 dph. Fully developed ovaries in adult females exhibited oocytes at various stages of development, which explains their ability to continuously spawn in the lab and Gale's (1986) finding that they are fractional spawners with indeterminate fecundity.

The onset of testicular differentiation is more difficult to determine than ovarian differentiation. The clustering of presumptive spermatogonia is the first histological pattern observed in testes. The spermatogonia proliferate and are loosely organized into tubules before yielding spermatocytes, spermatids, and spermatozoa. This pattern of testicular maturation has also been described in Fathead Minnow (van der Ven and Wester 1999–2003; Uguz 2008). By 135 dph, gonadal differentiation is complete in both sexes, and some individuals are mature and ripe for spawning by 138 dph.

We observed a large gap in the timing of differentiation in ovaries and testes of Red Shiners. Ovarian structures were histologically identifiable by 45 dph, whereas definitive testicular components were not observed in Red Shiners younger than 105 dph. These findings suggest that sex determination is occurring before 45 dph. Based on the concept of the labile period for fish sex determination (Hackmann and Reinboth 1974; Piferrer 2001), this study shows that the application of exogenous sex hormones or exposure to endocrine disruptors could alter Red Shiner sex differentiation before 45 dph through 105 dph. However, biochemically, differentiation is well underway before phenotypic changes can be observed histologically (Haffrey et al. 2009). Sacobie and Benfey (2005) and Jensen and Shelton (1983) observed that anatomical differentiation in gonads preceded cytological differentiation. Using cuts along the sagittal plane in samples under 100 dph might have prevented us from observing early anatomical differences in developing ovaries and testes. If exogenous sex reversal of Red Shiners is intended, as is required in the production of TSC carriers, we recommend hormone exposure be initiated by at least 30 dph. However, some salmonids have been successfully sex reversed with only a single immersion treatment when roughly half of the eggs from a spawn have hatched (Feist et al. 1996), so an even earlier exposure period might also be considered.

In conclusion, we were able to close the life cycle of the Red Shiner using simple, indoor aquaculture systems and commercially available diets. Methods described here

resulted in consistent Red Shiner production and created an F_2 generation in captivity. The short generation time and ease of care make Red Shiner an appealing species for a variety of studies. We also were able to determine when histologically observable gonadal differentiation starts in juvenile Red Shiners and describe gonadal development into adulthood. Understanding gonadal development and creating aquaculture protocols for Red Shiners may contribute to development of methods to assist with eradication of nonnative populations in the form of TSC strategies, promote native population reestablishment, and facilitate the future use of this species in a variety of studies.

ACKNOWLEDGMENTS

We thank William Stewart of the U.S. Bureau of Reclamation for funding this project. We thank Colby Roberts, Gabriella Buonavolonta, Kylee Rath, and Suhailah Hussein of the University of Arizona for assistance building and maintaining the aquaculture systems and assisting with data collection. We thank Heidi Blasius with the U.S. Bureau of Land Management for assisting in the collection of Red Shiners. We thank Zach Nemec, Larissa Lee, Taylor Ulrich, Ashleigh Grogan, Emily Freed, Chris Jenney, Joshua Grant, Steven Ingram, and Erin Tracy of the University of Arizona for research advice and assistance with the laboratory methods. We thank Arun Dhar and Luis Fernando Arunguren with the University of Arizona Aquaculture Pathology Laboratory for assessing fish health and performing histopathology on submitted carcasses. We thank Liz Mamer of Idaho Department of Fish and Game for histology assistance. There is no conflict of interest declared in this article.

REFERENCES

Adams, M. A., and P. B. Johnsen. 1986. A solid matrix bioassay for determining chemical feeding stimulants. Progressive Fish Culturist 48:147–149.

Ali, A. B. 1993. Aspects of the fecundity of the Feral Catfish, *Clarias macrocephalus* (Gunther), population obtained from the rice fields used for rice-fish farming in Malaysia. Hydrobiologia 254:81–89.

Arezo, M. J., S. D. Alessandro, N. Papa, R. de Sá, and N. Berois. 2007. Sex differentiation pattern in the annual fish *Austrolebias charrua* (Cyprinodontiformes: Rivulidae). Tissue and Cell 39:89–98.

Bailey, K. M., and E. D. Houde. 1989. Predation on eggs and larvae of marine fishes and the recruitment problem. Advances in Marine Biology 25:1–83.

Benfey, T. J. 2019. Sex determination, differentiation, and control in Atlantic Cod. Pages 725–734 *in* H.-P. Wang, F. Piferrer, S.-L. Chen, and Z.-G. Shen, editors. Sex control in aquaculture. Wiley, Hoboken, New Jersey.

Brummett, R. E. 1995. Environmental regulation of sexual maturation and reproduction in tilapia. Reviews in Fisheries Science 3:231–248.

Cahu, C., and J. Z. Zambonino Infante. 2001. Substitution of live food by formulated diets in marine fish larvae. Aquaculture 200:161–180.

Carr, W. E. S. 1976. Chemoreception and feeding behavior in the Pigfish, *Orthopristis chrysopterus*: characterization and identification of stimulatory substances in a shrimp extract. Comparative Biochemistry and Physiology A: Comparative Physiology 55:153–157.

Charlon, N., and P. Bergot. 1984. Rearing system for feeding fish larvae on dry diets. Trial with carp (*Cyprinus carpio* L.) larvae. Aquaculture 41:1–9.

Clarkson, R. W., and P. C. Marsh. 2010. Effectiveness of the barrier-and-renovate approach to recovery of warmwater native fishes in the Gila River basin. Pages 209–217 *in* T. S. Melis, J. F. Hamill, L. G. Coggins Jr., P. E. Grams, T. A. Kennedy, D. M. Kubly, and B. E. Ralston, editors. Coming together: coordination of science and restoration activities for the Colorado River ecosystem. Proceedings of the Colorado River basin science and resource management symposium. U.S. Geological Survey, Scientific Investigations Report 2010-5135, Reston, Virginia.

Cotton, S., and C. Wedekind. 2007. Introduction of Trojan sex chromosomes to boost population growth. Journal of Theoretical Biology. 249:153–161.

Deacon, J. E. 1988. The endangered Woundfin and water management in the Virgin River, Utah, Arizona, Nevada. Fisheries 13(1):18–24.

Desprez, D., C. Mélard, and J. C. Philippart. 1995. Production of a high percentage of male offspring with 17α-ethynylestradiol sex-reversed *Oreochromis aureus*: II. Comparative reproductive biology of females and F_2 pseudofemales and large-scale production of male progeny. Aquaculture 130:35–41.

Devlin, R. H., and Y. Nagahama. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208:191–364.

Douglas, M. E., P. C. Marsh, and W. L. Minckley. 1994. Indigenous fishes of western North America and the hypothesis of competitive displacement: *Meda fulgida* (Cyprinidae) as a case study. Copeia 1994:9–19.

Ewa-Oboho, I. O., and U. K. Enyenihi. 2001. Aquaculture implications of growth variation in the African catfish: *Heterobranchus longifilis* (Val.) reared under controlled conditions. Journal of Applied Ichthyology 15:111–115.

Farringer, R. T., A. A. Echelle, and S. F. Lehtinen. 1979. Reproductive cycle of the Red Shiner, *Notropis lutrensis*, in central Texas and south-central Oklahoma. Transactions of the American Fisheries Society 108:271–276.

Feist, G., C. B. Schreck, and A. J. Gharrett. 1996. Controlling the sex of salmonids. Oregon Sea Grant, Ploidy/Sex Manipulation Work Group, ORESU-H-96-001, Corvallis.

Gale, W. F. 1986. Indeterminate fecundity and spawning behavior of captive Red Shiners—fractional, crevice spawners. Transactions of the American Fisheries Society 115:429–437.

Gale, W. F., and G. L. Buynak. 1982. Fecundity and spawning frequency of the Fathead Minnow—a fractional spawner. Transactions of the American Fisheries Society 111:35–40.

Gao, Z., H. Wang, D. Rapp, P. O'Bryant, G. Wallat, W. Wang, H. Yao, L. Tiu, and R. MacDonald. 2009. Gonadal sex differentiation in the Bluegill sunfish *Lepomis macrochirus* and its relation to fish size and age. Aquaculture 294:138–146.

Gido, K., J. Schaefer, K. Work, P. Lienesch, E. Marsh-Matthews, and W. Matthews. 1999. Effects of Red Shiner (*Cyprinella lutrensis*) on Red River Pupfish (*Cyprinodon rubrofluviatilis*). Southwestern Naturalist 44:287–295.

Gimeno, S., A. Gerritsen, T. Bowmer, and H. Koman. 1996. Feminization of male carp. Nature 384:221–223.

Gomelsky, B., S. D. Mims, R. J. Onders, and W. B. Bean. 2002. Hormonal sex reversal and evidence of female homogamety in Black Crappie. North American Journal of Aquaculture 64:66–69.

Guraya, S. S. 1994. Gonadal development and production of gametes in fish. Proceedings of the Indian National Science Academy Part B 60:15–32.

Gutierrez, J. B., and J. L. Teem. 2006. A model describing the effect of sex-reversed YY fish in an established wild population: the use of a Trojan Y chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology 241:333–341.

Hackmann, E., and R. Reinboth. 1974. Delimitation of the critical stage of hormone-influenced sex differentiation in *Hemihaplochromis multicolor* (Hilgendorf) (Cichlidae). General and Comparative Endocrinology 22:42–53.

Haffrey, P., V. Petit, Y. Guiguen, E. Quillet, P. Rault, and A. Fostier. 2009. Successful production of monosex female Brook Trout *Salvelinus fontinalis* using gynogenetic sex reversed males by a combination of methyltestosterone immersion and oral treatments. Aquaculture 290:47–52.

Hartman, J. 1983. Two feeding strategies of young fishes. Archiv für Hydrobiologie 96:496–509.

Hubbs, C. L. 1954. Establishment of a forage fish, the Red Shiner (*Notropis lutrensis*), in the lower Colorado system. California Fish and Game 40:287–294.

Hunn, J. B., R. A. Schoettger, and E. W. Whealdon. 1968. Observations on the handling and maintenance of bioassay fish. Progressive Fish Culturist 30:164–167.

Islam, M. A. 1972. The effect of temperature on the reproduction of the Red Shiner, *Notropis lutrensis* (Baird and Girard). Doctoral dissertation. Texas A&M University, College Station.

Jensen, J. L., and W. L. Shelton. 1983. Gonadal differentiation in relation to sex control of Grass Carp, *Ctenopharyngodon Idella* (Pisces: Cyprinidae). Copeia 3:749–755.

Jiang, M., S. Jia, J. Chen, K. Chen, W. Ma, X. Wu, L. Hongrui, L. Yongming, Z. Zuoyan, and W. Hu. 2019. Timing of gonadal development and dimorphic expression of sex-related genes in gonads during early sex differentiation in the Yellow River carp. Aquaculture 518:734825.

Kobayashi, M., P. W. Sorensen, and N. E. Stacey. 2002. Hormonal and pheromonal control of spawning behavior in the Goldfish. Fish Physiology and Biochemistry 26:71–84.

Koehn, K. R. 1965. Development and ecological significance of nuptial tubercles of the Red Shiner *Notropis lutrensis*. Copeia 1965:462–467.

Kwiatkowski, M., D. Żarski, D. Kucharczyk, K. Kupren, K. Marta Jamróz, S. Targońska, A. Krejszeff, R. K. Hakuć-Błażowska, and A. Mamcarz. 2008. Influence of feeding natural and formulated diets on chosen rheophilic cyprinid larvae. Archives of Polish. Fisheries 16:383–396.

Lorenzen, K. 1996. The relationship between body weight and natural mortality in juvenile and adult fish: a comparison of natural ecosystems and aquaculture. Journal of Fish Biology 49:627–642.

Lovell, T. 1989. Nutrition and feeding of fish. Van Nostrand Reinhold, New York.

Lowerre-Barbieri, S., K. Ganias, F. Saborido-Rey, H. Murua, and J. Hunter. 2011. Reproductive timing in marine fishes: variability, temporal scales, and methods. Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science Management 3:71–91.

Marsh-Matthews, E., W. J. Matthews, K. B. Gido, and R. L. Marsh. 2007. Reproduction by young-of-year Red Shiner (*Cyprinella lutrensis*) and its implications for invasion success. Southwestern Naturalist 47:605–610.

Marsh-Matthews, E., W. J. Matthews, and N. R. Franssen. 2011. Can a highly invasive species re-invade its native community? The paradox of the Red Shiner. Biological Invasions 13:2911–2924.

Matthews, W. J., and L. G. Hill. 1977. The tolerance of the Red Shiner, *Notropis lutrensis* (Cyprinidae) to environmental parameters. Southwestern Naturalist 22:89–98.

Matthews, W. J., D. S. Lee, C. R. Gilbert, C. H. Hocutt, R. E. Jenkins, D. E. McAllister, and J. Stauffers Jr., editors. 1980. Atlas of freshwater fishes of North America. North Carolina State Museum, Raleigh.

McGree, M. M., D. L. Winkelman, N. K. M. Vieira, and A. M. Vajda. 2010. Reproductive failure of the Red Shiner (*Cyprinella lutrensis*) after exposure to an exogenous estrogen. Canadian Journal of Fisheries and Aquatic Sciences. 67:1730–1743.

Mizzi, J. A. 1996. Environmental assessment for Red Shiner eradication project to protect the native fishes of the Virgin River, Utah. U.S. Fish and Wildlife Service, Salt Lake City, Utah.

Nakamura, M., T. Kobayashi, X. T. Chang, and Y. Nagahama 1998. Gonadal sex differentiation in teleost fish. Journal of Experimental Biology. 281:362–372.

Piferrer, F. 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197:229–281.

Rach, J. J., G. E. Howe, and T. M. Schreier. 1997. Safety of formalin treatments on warm- and coolwater fish eggs. Aquaculture 149:183–191.

Rinne, J. N. 1991. Habitat use by Spikedace, *Meda fulgida* (Pisces: Cyprinidae) in southwestern streams with reference to probable habitat competition by Red Shiner, *Notropis lutrensis* (Pisces: Cyprinidae). Southwestern Naturalist 36:7–13.

Ruppert, J. B., R. T. Muth, and T. P. Nesler. 1993. Predation on fish larvae by adult Red Shiner, Yampa and Green rivers, Colorado. Southwestern Naturalist 38:397–399.

Sacobie, C. F. D., and T. J. Benfey. 2005. Sex differentiation and early gonadal development in Brook Trout. North American Journal of Aquaculture 67:181–186.

Saksena, V. P. 1962. The post-hatching stages of the Red Shiner, *Notropis lutrensis*. Copeia 1962:539–544.

Schill, D. J., J. A. Heindel, M. R. Campbell, K. A. Meyer, and E. R. J. M. Mamer. 2016. Production of a YY male Brook Trout broodstock for potential eradication of undesired Brook Trout populations. North American Journal of Aquaculture 78:72–83.

Schill, D. J., K. A. Meyer, and M. J. Hansen. 2017. Simulated effects of YY-male stocking and manual suppression for eradicating nonnative Brook Trout populations. North American Journal of Fisheries Management 37:1054–1066.

Segner, H. 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 149:187–195.

Senior, A. M., M. Krkosek, and S. Nakagawa. 2013. The practicality of Trojan sex chromosomes as a biological control: an agent-based model of two highly invasive *Gambusia* species. Biological Invasions 15:1765–1782.

Teem, J., L. Alphey, S. Descamps, M. Edgington, O. Edwards, N. Gemmell, T. Harvey-Samuel, R. Melnick, K. Oh, A. Piaggio, S. Royden, D. Schill, P. Thomas, T. Smith, and A. Roberts. 2020. Genetic biocontrol for invasive species. Frontiers in Bioengineering and Biotechnology 8:452.

Treves-Brown, K. M. 2000. Methods of drug administration. Pages 1–15 *in* Applied fish pharmacology. Springer, Dordrecht, The Netherlands.

Uguz, C. 2008. Histological evaluation of gonadal differentiation in Fathead Minnows (*Pimephales promelas)*. Tissue and Cell 40:299–306.

Uguz, C., M. Iscan, and I. Togan. 2003. Developmental genetics and physiology of sex differentiation in vertebrates. Environmental Toxicology and Pharmacology 14:9–16.

van der Ven, L., and P. Wester. 1999–2003. Histology and histopathology atlas of the Zebrafish V2.01. Available: zfin.org/hh_atlas/. (February 2021).

Walford, J., T. M. Lim, and T. J. Lam. 1991. Replacing live foods with microencapsulated diets in the rearing of Sea Bass (*Lates calcarifer*) larvae: do they ingest and digest protein-membrane microcapsules? Aquaculture 92:225–235.

Wedekind, C. 2002. Manipulating sex ratios for conservation: short-term risks and long-term benefits. Animal Conservation 5:13–20.

Yamamoto, T. O. 1963. Induction of reversal in sex differentiation of YY zygotes in the medaka, *Oryzias latipes*. Genetics 48:293–306.

APPENDIX E

THE DEVELOPMENT OF GENETIC SEX IDENTIFICATION MARKERS AND EVIDENCE OF A MALE HETEROGAMETIC SEX DETERMINATION SYSTEM IN RED SHINER *CYPRINELLA LUTRENSIS*

THE DEVELOPMENT OF GENETIC SEX IDENTIFICATION MARKERS AND EVIDENCE OF A MALE HETEROGAMETIC SEX DETERMINATION SYSTEM IN RED SHINER *CYPRINELLA LUTRENSIS*

In press with the North American Journal of Aquaculture

Chad N. Teal[1*], D. Katharine Coykendall[2,3], Matthew R. Campbell[3], Thomas A. Delomas[2,3], Daniel L. Eardley[2,3], John A. Erwin[4], Daniel J. Schill[5], Javan M. Bauder[6], Scott A. Bonar[6] and Melanie Culver[6]

[1] Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2, 1064 E. Lowell St., Tucson, AZ 85721, USA. Email:chadteal@arizona.edu

[2] Pacific States Marine Fisheries Commission, Eagle Fish Genetics Lab, Eagle, ID, USA

[3] Idaho Department of Fish and Game, Eagle Fish Genetics Lab. 1800 Trout Rd., Eagle, ID 83616, USA. Emails:katharine.coykendall@idfg.idaho.gov, dan.eardly@idfg.idaho.gov and matthew.campbell@idfg.idaho.gov

[4]Florida International University College of Law, Rafael Diaz-Balart Hall, 11200 SW 8th St, Miami, FL 33199, Email: jerwin@fiu.edu

[5]Fisheries Management Solutions, Inc., 9950 W. Prairie Road, Boise, ID 83714, USA. Email:dschill@fishmgmtsolns.com

[6]U.S. Geological Survey, Arizona Cooperative Fish and Wildlife Research Unit, School of Natural Resources and the Environment, University of Arizona, ENR2, 1064 E. Lowell St., Tucson, AZ 85721, USA. Emails: jbauder@arizona.edu, sbonar@ag.arizona.edu and culver@ag.arizona.edu

ABSTRACT

Red Shiner *Cyprinella lutrensis* is of increasing management interest as an invasive species which negatively impacts many native fishes throughout North America. Trojan sex chromosome (TSC) carrying individuals could theoretically control invasive fish populations by skewing sex ratios towards 100% male. The efficacy of TSC control programs requires an understanding of a population's sex determination system, yet such information is lacking for Red Shiner. We used single-digest restriction-site associated DNA sequencing (RAD-Seq) to discover sex-linked single-nucleotide polymorphisms (SNPs) in addition to a series of breeding experiments to uncover their sex determination system. All candidate sex-linked SNPs that fit our selection criteria exhibited a pattern of male heterogamety. We developed two sex identification (sex-ID) marker assays, XY_248 and XY_170, which showed a phenotype-genotype concordance score of 77.00% and 84.35%, respectively. These sex-ID markers exhibited a relatively high phenotype-genotype concordance in females (XY_248 = 96.30%, XY_170 = 98.61%) which allowed for selective breeding of phenotypically feminized genetic males. We observed a 3:1 male to female sex ratio in spawns from feminized males crossed with wild-type males indicative of a male heterogametic sex determination system (i.e., XY-male/XX-female). The discovery of a male heterogametic sex determination system in combination with our two markers increases the likelihood of developing an effective Trojan sex chromosome eradication strategy for invasive Red Shiner populations.

INTRODUCTION

Red shiner *Cyprinella lutrensis* is a species of increasing management interest due to their human-aided invasions across North America and their negative impacts on fishes of high conservation concern (Douglas 1994; Herrington 2004; Clarkson and Marsh 2008). By contrast,

the reestablishment of some Red Shiner populations within their native range has been challenging (Marsh-Matthews et al. 2011) despite their persistent invasive populations and ability to thrive in harsh environmental conditions (Matthews and Hill 1977). This makes Red Shiner both a target of biological control initiatives in areas where it has become invasive and, potentially, a target for conservation in its native range.

Over the last decade, an increasing number of models have shown that releases of fish carrying Trojan sex chromosomes (TSC) could eradicate invasive populations (Gutierrez and Teem 2006; Senior et al. 2013; Schill et al. 2017; Teem et al. 2020; McCormick et al. 2021) by skewing a population's sex ratio towards male. Trojan sex chromosome carriers are either YY individuals, males or females that are homozygous for the male-coding region of the genome in fishes with a chromosomal XY-male/XX-female sex determination (SD) system, or ZZ females, phenotypically feminized males in fishes with a chromosomal ZW-female/ZZ-male SD system (Gutierrez and Teem 2006; Senior et al. 2013; Schill et al. 2017; McCormick et al. 2021). Spawns involving YY individuals or ZZ females produce all male offspring (Senior et al. 2013). If a large enough portion of the invasive population is composed of TSC carriers, then the population can theoretically crash over varying time frames (Gutierrez and Teem 2006; Schill et al. 2017). Conversely, this same technique can theoretically be used to boost struggling populations (Cotton and Wedekind 2007a) via skewing a population's sex ratio towards female. Population enhancement could occur by increasing the number of females in the population through the release of either phenotypically masculinized XX-males or WW individuals, masculinized WW-males or WW-females that are homozygous for the female coding region of the genome (Cotton and Wedekind 2007a), though this concept has received relatively little interest from the conservation community.

However, before a TSC eradication or conservation strategy can be assessed, it is necessary to explore the mechanisms that direct SD in the species of interest (Stelkens and Wedekind 2010). Fish exhibit a myriad of SD systems (Devlin and Nagahama 2002). These SD systems can be seemingly simple and chromosomal, such as male-specific master SD genes (sdY and DMRT1) as seen in many Salmonids (Marchand et al. 2000; Yano et al. 2013; Bertho et al. 2018). Alternatively, fishes' SD system can be much more complicated, where a combination of polygenic and environmental factors may direct SD, such as the SD of Bluegill *Lepomis macrochirus* (Shen et al. 2016). The mechanisms of these SD systems are not always conserved within families (Schultheis et al. 2009), or even within a species (Gammerdinger and Kocher 2018). Therefore, investigating which, if either, sex is heterogametic in a species or population targeted for TSC efforts is necessary.

Genetic sex identification (sex-ID) markers are an important tool for identifying SD systems in fish. Sex-ID markers can determine if either sex is heterogametic (Lamatsch et al. 2015; Fowler and Buonaccorsi 2016), even in species with homomorphic sex chromosomes (Baroiller and d'Cotta 2016; Palmer et al. 2019). Sex-ID markers can allow researchers to uncover the evolution of sex chromosomes, SD system transitions within families, and genetic factors involved in sex differences (Gamble et al. 2015; Lamatsch et al. 2015). Sex-ID markers can also facilitate the production of monosex cohorts to increase aquaculture yield (Goudie et al. 1994; Beardmore et al. 2001; Piferrer 2001; Liu et al. 2013) or for commercial and recreational fisheries monitoring (Fowler and Buonaccorsi 2016; Nelson 2018; Vu et al. 2019). Yet the SD systems for many fish species with relatively low commercial or recreational value, such as Red Shiner, remain largely unexplored, and efforts at developing sex ID markers for these species are rare. However, the potential efficacy of a TSC strategy for eradicating invasive populations has

broadened the interest in the mechanisms of SD in more species, including taxa outside of fishes (Cotton and Wedekind 2007b).

The development of restriction-site associated DNA sequencing (RAD-seq) provides an important tool for identifying potential sex-ID markers by allowing for genotyping of single nucleotide polymorphisms (SNPs) and loci across multiple individuals in previously uninvestigated species (Davey et al. 2011; Peterson et al. 2012). The RAD-seq technique allows for genome-wide sequencing of loci adjacent to restriction enzyme cut sites (Miller et al. 2007). The short sequences (~100-150 bp) that RAD-seq generates are often consistent among the investigated genomes, allowing for the discovery and comparison of single nucleotide polymorphisms (SNPs) in many individuals at a time (Davey et al. 2011; Peterson et al. 2012). Therefore, RAD-seq is often used for developing sex-ID markers (Gamble and Zarkower 2014; Brown et al. 2016; Fowler and Buonaccorsi 2016; Gamble 2016; Nelson 2018; Vu et al. 2019). SNPs that are always heterozygous for a particular allele in one sex and fixed in the other can be used as a sex-ID markers, providing insights into the SD system of the species (Vu et al. 2019; Feron et al. 2021). However, since RAD-seq produces a reduced representation of the genome, the identification of such SNPs using solely RAD-seq approaches may not always be possible if the species' sex-specific region or regions are small (Teal et al. 2022).

Identifying sex-ID markers can facilitate the production of TSC carriers (Schill et al. 2016). Sex-ID markers aid in the selective breeding of hormonally sex reversed individuals with wild-type individuals, which can be a necessary step in the production of YY male or ZZ female TSC carriers (Senior et al. 2013; Baroiller and d'Cotta et al. 2016; Schill et al. 2016). Without sex-ID markers time consuming progeny testing is often necessary to differentiate sex reversed individuals from wild-type individuals (Sun et al. 2014; Schill et al. 2016). The spawning of

these sex reversed individuals with wild-type individuals can provide evidence for the SD systems in fishes (Hopkins et al. 1979; Gomelsky et al. 2002). For example, when a phenotypically feminized male (neofemale), a genetic male that has a female phenotype via hormone treatments, is crossed with a wild-type male, the sex ratio of the progeny can provide evidence as to whether the male or the female is the heterogametic sex (Piferrer 2001). If ~75% of the progeny from this cross are male, then this is evidence of an XY-male/XX-female SD system (Figure 1; Hunter et al. 1982). However, if all the progeny from this cross are male, then the species may exhibit a ZW-female/ZZ-male SD system (Hopkins et al. 1979).

Genetic differences between male and female Red Shiner have not been explored. The objectives of this study were to 1) identify, develop, and test several candidate sex-ID markers in this species using RAD-seq data, 2) use these sex-ID markers to identify neofemales in hormonally sex reversed cohorts, 3) assess the sex ratio and sex-ID marker segregation in progeny from neofemales and wild-type male crosses to provide evidence for the SD system found in Red Shiner. Developing sex-ID markers for Red Shiner and uncovering their SD system will allow for an initial examination into the feasibility of utilizing TSC methods for either enhancing or eradicating their populations, as well as provide more insights into the reproductive biology and evolution of SD systems in the *Leuciscidae* family.

METHODS

DNA Extraction and RAD-seq Library Construction

We extracted DNA using a Nexttec extraction kit with Proteinase K (Nexttec Biotechnology GmbH, Hilgershausen, Germany) from 11 phenotypic male and 11 phenotypic female Red Shiner that were collected on June 23, 2018, from the Gila Box Riparian National

Conservation Area in Arizona, USA (GPS coordinates 32°53'0.8872" N, 109°30'0.39489" W). Phenotypic sex was determined by examining secondary sexual characteristics (McGree et al. 2010) in conjunction with dissection and inspection of the gonads. Two RAD-seq libraries were constructed at Idaho Department of Fish and Game's Eagle Fish Genetics Lab in Eagle, Idaho using the methods described by Ali et al. (2016), Vu et al. (2019), and Teal et al. (2022). One library (library 1) contained DNA from six male and six female Red Shiner, along with DNA from 20 Green Sunfish *Lepomis cyanellus* used in another project (Teal et al. 2022). The other library (library 2) contained DNA from five male and five female Red Shiner. Extracted DNA was quantified using a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, California, USA) and Qubit 2.0 fluorometer. The DNA was normalized to 10 ng/µl and 10 µl per sample was used for library construction. We used the *PstI* restriction enzyme, which features a six base-pair cut site recognition sequence, to conduct a single-digest RAD-seq. We chose this restriction enzyme because it was estimated to provide roughly 40X coverage per locus using 22 Red Shiner samples. Sequencing was performed at Eagle Fish Genetics Lab on an Illumina NextSeq 500 (Illumina, San Diego, California, USA). Library 1 was sequenced using a NextSeq 500/550 300 cycle High Output Kit and library 2 was sequenced using a NextSeq 500/550 300 cycle Mid Output Kit. In both cases sequencing yielded 150 bp paired-end reads.

Bioinformatics

Sequence Data Processing and De Novo Assembly of Loci

We used the Stacks v2.4 pipeline (Catchen et al. 2013) on raw sequence data to build catalogues of loci and discover SNPs. First, we ran the Stacks program *process_radtags* with flags *q* and *r- bestrad* to remove low-quality reads, distinguish barcodes, and demultiplex the library. We then ran the *clone_filter* program to remove PCR duplicates. We consolidated

demultiplexed reads from the two libraries for further analysis. No reference genome was available for Red Shiner, so we used the Stacks *de novo* pipeline to identify and genotype loci. In the *ustacks* program we designated a minimum depth of read coverage (*m*) of five to be considered legitimate loci and confidently call SNPs (Catchen et al. 2013) and we set the maximum distance in nucleotides allowed between reads within an individual at a locus (*M*) to be 2, 3, 4, 5, 6, 7, and 8. In *cstacks* we set the maximum number of mismatches among individuals (*n*) to be *M, M*+1, and *M*-1 for each *M* value. Sequence data analyzed with larger *M* and *n* values in *ustacks* and *cstacks* will result in fewer loci identified, because these parameters allow for more nucleotide mismatches within and among individuals per locus, whereas smaller *M* and *n* values will result in more identified loci because reads with number of mismatches above the designated *M* and *n* values will be separated as different loci. We used this broad range of parameter tolerances in *ustacks* and *cstacks* (resulting in 21 separate Stacks runs) to maximize the odds of finding sex-linked SNPs (Mussmann et al. 2022). Validation and further testing via TaqMan assays would filter out false positives accrued with our sex-linked SNP discovery methods. Validation in our study was done by testing the sex-ID markers on individuals used to construct the RAD libraries and therefore have a known genotype. We used default settings in the *sstacks*, *tsv2bam,* and *gstacks* programs. In the *populations* program we specified the minimum percentage of individuals in a population required to process a locus for that population (*r*) to be 0.2.

Sequence Data Analysis

To obtain the libraries' mean weighted read depth of coverages and total number of SNPs genotyped, we used the output from Stacks using moderately permissive tolerances, $M = 4$ and $n = 4$. Since we used *M* parameters ranging from 2-8, obtaining summary statistics of our libraries

with *M* of 4 and *n* of 4 provided midrange values of the aforementioned library characteristics while providing leniency for mismatches and genotyping errors.

Sex-Linked SNP Discovery

After the Stacks pipeline, we used a custom Python (v. 3.0) script (https://github.com/delomast/stacks_to_sex_marker/blob/master/vcf_sex_markers.py) to examine the Stacks *populations* program's SNPs VCF files generated for each *M* and *n* value to find candidate biallelic sex-linked SNPs (sex-ID candidates). We considered SNPs sex-ID candidates if they were fixed for one allele in one sex (homogametic sex) and heterozygous with the same alternate allele in the other sex (heterogametic sex). This Python script created a list of XY sex-ID candidates, where the male is heterozygous for the SNP and the female is homozygous, and a list of ZW sex-ID candidates, where the female is the heterozygous for the SNP and the male is homozygous. We used another Python script (https://github.com/dollykc/Teal-et-al-2022-red-shiner-sex-marker/blob/main/MaxGenoXY.py) to compile all these sex-ID candidates that were genotyped in 50% or more of the individuals used for sequencing.

Candidate Sex-ID Assay Development and Testing

We selected sex-ID candidates for TaqMan assay development if the SNP was genotyped in ≥ 18 of 22 individuals and if the SNP was called in ≥ three of seven *M*-values. We chose this selection criteria to attempt to eliminate false-positive sex-linked SNPs. We chose sex-ID candidates that had ≤ three other SNPs within 20 base-pairs upstream or downstream of the sex-linked SNP to ensure the designed primers and probes would bind and amplify. We also chose sex-ID candidates that featured the potential sex-linked SNP away from the ends of RAD-tag

sequence leaving ≥ 18 base pairs on either end of the SNP to allow for the design of forward and reverse primers. We designed primers and fluorescently labeled hydrolysis probes of the four sex-ID candidates that met our selection criteria sex using the ThermoFisher Custom TaqMan Assay Design Tool with default parameters (https://www.thermofisher.com/order/custom-genomic-products/tools/genotyping/).

TaqMan assays of the four sex-ID candidates (Table 1) were conducted by the University of Arizona Genetics Core (UAGC). The thermocycling protocol used for all assay reactions was (1) 95 ºC for 5 minutes, (2) 95 ºC for 15 seconds, (3) 58 ºC for 1 minute, with steps two and three repeating 40 times for annealing and extending. The assays were comprised of 7.5 µL TaqMan Fast Advance Master Mix, 0.375 µL 40x TaqMan Genotyping assay (SNP candidate), 5.125 µL PCR water, and 2.0 µL DNA. The assays were run on an Applied Biosystems 7900 HT Fast Real-Time PCR System (ThermoFisher).

We first validated all four developed sex-ID candidates on six males and six females that were used in the RAD-seq library construction to ensure the assays genotyped as expected based on our bioinformatic discovery. Along with these 12 individuals of presumably known genotypes, the four sex-ID candidates (XY_248, XY_170, XY_251, XY_73; Table 1) were tested with eight phenotypic males and eight phenotypic females of unknown genotypes (Table 2 – round 1). Two of these sex-ID candidates (XY_248 and XY_170) were selected for a second testing round (Table 2- round 2) based on the genotype-phenotype concordance results of the initial assay. These two sex-ID candidates were tested on an additional 32 phenotypic males and 40 phenotypic females with unknown genotypes for the second round of testing. A total of fifty individuals from this second round of testing were progeny randomly sampled from one of five broodstock tanks in our hatchery, with ten individuals being derived from each of the five

broodstock tanks. The other 22 individuals used in this test were unrelated wild-caught Red Shiner from Gila Box Riparian National Conservation Area in Arizona. Marker XY_170 was chosen for a third testing round (Table 2- round 3) on 47 unrelated wild-caught individuals (23 males and 24 females) due its higher concordance with phenotypic sex in the second round of testing.

Neofemale Crosses (XY-Female) and Progeny Analysis

We used genetic sex-ID marker, XY_170, to select 17β-estradiol treated putative neofemales to cross with wild-type males. Neofemales were produced by feeding treated diets of 100 mg 17β- estradiol per kg of diet to developing Red Shiner from 20 – 120 d post-hatch (dph). Red Shiner were produced following aquaculture protocols developed by Teal et al. (2021). Three putative neofemales and two wild-type males that were heterozygous for the XY_170 SNP were stocked in one of six 37.9 L broodstock aquaria. We used neofemales from three different broodstock lineages, with the broodstock of each neofemale lineage being presumably unrelated, wild-caught adults from the Gila Box Riparian National Conservation Area in Arizona. Three tile spawning stacks constructed using methods described by McGree et al. (2010) were placed in each neofemale broodstock aquarium. Two to four spawns containing ≥ 26 eggs from each neofemale lineage (n = total of nine spawns used) were reared until 180 dph when phenotypic sex could be determined via histological examination of differentiated gonads. In total, 58 offspring of putative neofemales, with four to ten fish coming from each spawn, and 13-23 coming from each neofemale lineage, were submitted for histological processing to detect the phenotypic sex and caudal fin clips were taken for genotyping for marker XY_170. All fish from neofemale spawns that had ≤ 8 fish alive by 180 dph were euthanized and submitted for histological processing, whereas a random sample of 5-10 fish were taken from those that had

more than 8 fish surviving to 180 dph. This was done to retain at least five live fish in each tank for further study. Fish were euthanized by immersion for 10 minutes in an aqueous solution of 75 ppm tricaine mesylate (MS 222) and 150 ppm sodium bicarbonate. Research was conducted in compliance with the University of Arizona's Institutional Animal Care and Use Committee protocol 18-413.

Progeny Data Analysis

We used proportional binomial generalized linear models (GLMs) to compare the mean percentage of males from all neofemale lineage spawns with the mean percentage of males from wild-type female spawns. Data from wild-type female spawns were obtained in a previous study using spawns from one wild-caught male and three wild-caught females in four broodstock tanks (Teal et al. 2021). To test for an effect of lineage on percentage of male progeny from neofemale lineages, we fit a proportional binomial GLM with neofemale lineage as a three-level categorical variable and then compared this model to an intercept-only (i.e., null) model using a likelihood-ratio test. We then combined all fish sampled for histology from these neofemale spawns and used a chi-square goodness-of-fit test (χ^2) see if the sex ratio was significantly different from a 3:1 male to female ratio expected from an XY/XY cross. We used marker XY_170 on sampled fish to see if genotypes were concordant with phenotypes. We used $\alpha = 0.05$ for all statistical tests.

RESULTS

Sequencing, Candidate SNP Selection and Candidate SNP Testing

We generated 865,345,120 and 318,967,560 raw reads from libraries 1 and 2, respectively. We retained a higher proportion of reads (77.7%) from library 1 than from library 2

(50.0%) after removing low quality reads, PCR duplicates, and ambiguous enzyme cut sites. We detected 883,651 SNPs using moderately permissive nucleotide mismatch tolerances (Stacks parameters $M = 4$, $n = 4$). When using 18 out of 22 as the minimum number of individuals genotyped for the SNP as a threshold for sex-ID marker selection, we found seven SNPs which had a fixed allele in one sex and was always heterozygous in the other. All these SNPs fit the pattern of male heterogamety (i.e., female has fixed allele and male is heterozygous). No SNPs were genotyped in all 22 individuals used to build the library. Two males were not genotyped in any of the discovered SNPs. The mean weighted read depth of coverages of these two males were 45.53% (95% CI = 39.32%-50.58%) and 30.31% (95% CI = 22.36% - 36.78%), lower than the library's mean weighted read depth of coverage ($\bar{X}$ = 26.98x; standard deviation = 6.23x; Stacks parameters $M = 4$, $n = 4$). When testing the sex-ID marker assays, we saw that these two males were genotyped as heterozygous using assays XY_248 and XY_170.

Four of the seven sex-ID SNP candidates were chosen for TaqMan assay development due to their position in the RAD-tag sequence and the paucity of SNPs flanking the candidate sex-linked SNP. Two of the sex-ID candidates scored 89.29% (25/28) and 82.14% (23/28) in their concordance with phenotypic sex and failed validation of one sample used during the first round of tests, so these two sex-ID candidates were not used for further testing. During this first test, XY_248 and XY_170 were concordant for phenotypic sex 92.86% (26/28) of the time and genotyped all samples used for validation with the expected genotype and were therefore selected for further testing. Candidate sex-ID marker XY_248 genotyped in most males as heterozygous for base pairs C/T and genotyped in most females as homozygous for base pairs C/C at the SNP locus. Candidate sex-ID marker XY_170 genotyped in most males as heterozygous for base pairs A/G and genotyped in most females as homozygous for base pairs

A/A at the SNP locus. However, one wild-caught individual was genotyped as homozygous for the alternate base pair (putatively Y-linked) with both assays (T/T for XY_248 and G/G for XY_170). During the first two tests combined, assays XY_248 and XY_170 scored a 73.86% (65/88) and 77.27% (68/88), respectively, in their concordance with sexual phenotype in Red Shiner samples not used in the RAD-seq library. Both assays showed much higher concordance in phenotypic females than phenotypic males. When removing samples used for validation, with which they scored 100% concordance with their phenotypic sex, XY_248 scored a 95.83% (46/48) in concordance with females and a 47.50% (19/40) in concordance with males and XY_170 scored a 97.92% (47/48) in concordance with females and a 52.50% (21/40) in concordance with males during their first two tests. All males (n = 11) in the second round of testing that were progeny from two brood stock tanks were homozygous (putative XX). In the third test, XY_170's concordance rate was 100% (24 out of 24) for genotyping unrelated, wild-caught, females and 86.96%% (20 out of 23) for genotyping males, for a total concordance rate of 93.62% (44/47). The XY_248 assay was not tested in the third round of testing due to its lower genotype-phenotype concordance than XY_170 in round 2 of testing. During all the assay trials only one individual had an undetermined genotype, which occurred during the third XY_170 assay trial.

These sex-ID candidates were genotyped as homozygous for fixed alleles (either C/C for XY_248 or A/A for XY_170; i.e. putative XX-females) in both males and females, but when genotyped as heterozygous (either C/T for XY_248 or A/G for XY_170; i.e. putative XY-males), the probability the individual was a phenotypic male was high (Table 2). In our second round of testing, we saw that males from two broodstock lineages were homozygous (putative XX) for the SNPs, whereas the SNPs had a much higher concordance with the individuals' phenotype in

wild-caught Red Shiner. When including all rounds of testing, the randomly sampled male concordance for XY_248 and XY_170 increases from 47.5% to 65.5% and 64.1% to 77.4%, respectively, when we remove males from these two broodstock lineages.

Neofemale Crosses (XY-Female) and Progeny Analysis

Originally, we had six broodstock aquariums, but consistently nonviable spawn from two broodstock aquaria containing wild-type males from a particular lineage prevented us from utilizing these crosses' offspring. The mean percentage of Red Shiner that were phenotypic males from neofemale spawns ($\bar{X}$ = 77.08%, 95% CI = 63.98% - 87.37%, n = 9 spawns) was significantly greater (GLM, Z = 2.765, P value = 0.0057) than the mean percentage of fish that were males from wild-type female spawns ($\bar{X}$ = 48.89%, 95% CI = 34.65% - 63.25%, n = 4 spawns). The mean percentages of progeny of neofemales that were male from each lineage ($\bar{X}$ = 60.0%, 76.5% and 85.7%) were not significantly different (likelihood-ratio test, $\chi^2 = 2.4383$, df = 2, P value = 0.2955). The phenotypic sex ratio of the progeny from neofemales was not significantly different from a 3:1 male to female sex ratio ($\chi^2 = 0.1111$, df = 1, P value = 0.7389). Almost all progeny from neofemales had the expected genotype-phenotype concordance using marker XY_170, with 100% of the phenotypic males having either an A/G (wild-type male) or G/G (YY male) genotype, but one phenotypic female was genotyped as A/G instead of the expected A/A genotype.

DISCUSSION

Our two TaqMan assays, XY_248 and XY_170, appear to genotype partially sex-linked SNPs. The concordance between genotypic and phenotypic sex appeared sensitive to the inclusion of two broodstock lineages. This variance in concordance can occur due to sex-linked

markers showing specificity based on family lineages or populations (Ezaz et al., 2004; Chen et al., 2009; Gao et al., 2010). This lineage specificity is a testament to the many polymorphisms that can surround potential sex determining regions and the frequency of sex chromosome recombination and turnovers that can occur in species with homomorphic sex chromosomes (Gamble et al. 2015; Brelsford et al. 2017; Palmer et al. 2019). Incomplete linkage disequilibrium and recombination often stops evolutionary strata from developing in the sex determining chromosomes (Palmer et al. 2019; Qiu et al. 2022) which can prevent allelic fixation in a sex and result in only partially sex-linked markers. In addition, the variance in the concordance of a putatively sex-linked marker with phenotype can be due to deletions, insertions, mutations, and null alleles that occur on or near sex-linked loci (Volff and Schartl 2001; Gao et al. 2010; Fontaine et al. 2017). Our designed TaqMan assays are limited in the detection of null alleles since a null allele in a probe site may result in an erroneous homozygous genotyping for the SNP the probe was designed for. However, if enough sequence data is obtained upstream and downstream of the putatively sex-linked locus, then Sanger sequencing amplicons from this locus may detect the deletions, insertions, mutations, and null alleles that can cause variation in genotype-phenotype concordance. For our purposes, we did not need to further investigate our candidate sex-linked loci since our markers appeared to effectively select neofemales from our sex reversed cohorts. Our concordance results suggest that the developed sex-ID markers are likely not within a sex determining locus. Rather, they are likely a partially sex-linked locus on the presumed Y chromosome close enough to the sex determining locus to be in strong linkage disequilibrium (López-Fernández and Bolnick 2007; Palmer et al. 2019).

The genotype-phenotype concordance of sex-ID markers can vary and is rarely 100% (Vu et al. 2019; Mussmann et al. 2022; Xu et al. 2022). This can be due to the aforementioned

genetic variation within or among populations (Sun et al. 2014), as well as sequencing errors or misidentification of phenotypic sex (Palmer et al. 2019; Mussmann et al. 2022; Xu et al. 2022). Due to the low population sizes and bioinformatic filter settings often used in the development of sex-ID markers, candidate sex-ID markers for contrasting SD systems (e.g., male heterogamety vs female heterogamety) can be discovered and many of the discovered sex-ID markers are often false positives. Validation using many samples of known sexual phenotype is a necessary step to filter out false positive. In the current study, we have the added evidence of obtaining the expected mean sex ratios of progeny and high genotype-phenotype concordance in progeny from neofemales using XY_170 that bolsters our conclusion for the SD system in Red Shiner and validates the accuracy of our sex-ID marker in most individuals.

More assays should be conducted with XY_248 and XY_170 on Red Shiner populations from various geographic origins to assess if these markers are present and partially sex-linked across the range of this species. The wide-spread utility of these sex-ID markers would facilitate assessing sex ratios on TSC targeted populations as well as assist in the future production of YY Red Shiner if this eradication strategy is implemented. This is of special interest in the Western United States where Red Shiner are considered one of the fastest spreading aquatic invaders (Olden and Poff 2005) and where there has been possible hybridization leading to a divergent Red Shiner lineage (Glotzbecker et al. 2016).

The sex ratio of the progeny from neofemales provides strong evidence that the sampled Red Shiner population possess an XY-male/XX-female SD system. The 3:1 male to female sex ratio we observed in progeny from neofemales is similar to what other researchers have observed from progeny of neofemales in species with an XY-male/XX-female SD system such as Rainbow Trout *Oncorhynchus mykiss* (Johnstone et al. 1979), Coho Salmon *Oncorhynchus*

kisutch (Hunter et al. 1982), and Goldfish *Carassius auratus* (Yamamoto and Kajishima 1968). This ratio fits the theoretical Mendelian ratio expected from an XY x XY cross if all genotypes have equal viability.

We presume that males which were homozygous for the alternate allele (T/T for XY_248 or G/G for XY_170; Figure 1) from our neofemale crosses are YY males. Determining the viability and relative fitness of these putative YY male Red Shiner is crucial for assessing their potential efficacy in a TSC eradication strategy (Senior et al. 2013; Schill et al. 2017). Due to homomorphic sex chromosomes, YY individuals are viable in most fishes (Penman and Piferrer 2008; Cnaani and Levavi-Sivan 2009). However, we found consistently nonviable spawns between neofemales from two different family lineages and males from a specific family lineage, which can be an indication that these broodstock were closely related. Another study observed nonviable YY male guppies using neofemale crosses, unless the neofemales came from different lineages than the wild-type males (Volf and Schartl 2001). Nevertheless, results from the other crosses show that potential YY Red Shiner males from the other lineages are viable. Further progeny testing should be conducted to ensure that individuals homozygous for the male allele truly are homozygous for the male-determining gene. If the SD system is solely chromosomal, then these YY males should only be capable of producing male offspring when crossed with wild-type females (Mair et al. 1997). The presence of female progeny from putative YY individuals would suggest a polygenic sex determination system or environmental sex determination and consequently reduce the effectiveness of a TSC eradication strategy. It is possible that autosomal sex modifying genes may result in occasional females in the progeny of YY males (Mair et al. 1997; Sun et al. 2014). Future research should validate putative YY males by conducting crosses with wild-type females and then assessing sex ratios and genotype-

phenotype concordance of the resulting progeny. Progenies with only males from putative YY individuals would increase the confidence of a TSC eradication strategy and would confirm our results, as opposed to a polygenic SD in Red Shiner.

Cotton and Wedekind (2007a) explained the theoretical ability to boost a small population using Trojan sex chromosome carriers, i.e., WW females, in species where the female is the heterogametic sex. We have discovered a male heterogametic SD system in Red Shiner, therefore a TSC strategy used to increase the number of females in struggling populations would only be feasible if the target populations have an inverse SD from what we have observed. Alternatively, the release of phenotypically masculinized females (XX males) may be able to increase the proportion of females in a population. This is the first study that has investigated the SD system of Red Shiner. It is unknown if all populations exhibit a chromosomal XY-male/XX-female SD system, as the one in this study appears to, or if this population and others may exhibit autosomal and/or environmental effects on SD.

In conclusion, we discovered two sex-ID markers that allowed for the selective breeding of neofemales with wild-type males. These markers can provide researchers and aquaculturists a valuable tool for producing YY Red Shiner broodstock if a TSC eradication strategy is attempted to control specific invasive populations. We also provided strong evidence that our sampled Red Shiner population exhibit a male heterogametic SD system, which is the only SD system currently being attempted for TSC field trials (Kennedy et al. 2018; Teem et al. 2020). We recommend further testing with these sex-ID markers within additional populations across their invasive and native range to determine if these alleles are present and partially sex-linked across the geographic distribution. This is especially important in the Southwestern United States where Red Shiner are considered one of the most ecologically devastating and fastest expanding

invaders (Rinne 1991; Ruppert et al. 1993; Douglas et al. 1994; Gido et al. 1999; Olden and Poff 2008).

ACKNOWLEDGEMENTS

William Stewart of the United States Bureau of Reclamation solicited and provided funding for this research (United States Bureau of Reclamation, grant number G18AC0061 and modification G19AC0039). Ellie Wisely, Natalie Mercer and Karla Vargas with the University of Arizona Conservation Genetics Laboratory provided advice on project design, methods, and results interpretation. Colby Roberts with the University of Arizona assisted with Red Shiner tissue collection and larval rearing. We thank Heidi Blasius with U.S. Bureau of Land Management for assisting in the collection of Red Shiner and for providing the image of the male Red Shiner used in Figure 1. We thank Kevin Fitzsimmons and Guilherme Caeiro Dias for reviewing our manuscript. There is no conflict of interest declared in this study.

REFERENCES

Ali, O. A., O'Rourke, S. M., Amish, S. J., Meek, M. H., Luikart, G., Jeffres, C., and M.R. Miller. 2016. RAD capture (Rapture): flexible and efficient sequence-based genotyping. Genetics 202:389-400.

Baroiller, J. F., and H. d'Cotta. 2016. The reversible sex of gonochoristic fish: insights and consequences. Sexual Development, 10:242-266.

Beardmore, J.A., Mair, G.C. and R.I. Lewis. 2001. Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects. Reproductive Biotechnology in Finfish Aquaculture 283-301.

Bertho, S., Herpin, A., Branthonne, A., Jouanno, E., Yano, A., Nicol, B., Muller, T., Pannetier, M., Pailhoux, E., Miwa, M., Yoshizaki, G., Schartl, M., and Y. Guiguen. 2018. The unusual rainbow trout sex determination gene hijacked the canonical vertebrate gonadal differentiation pathway. Proceedings of the National Academy of Sciences 115: 12781–12786.

Brelsford, A., Lavanchy, G., Sermier, R., Rausch, A. and N. Perrin. 2017. Identifying homomorphic sex chromosomes from wild-caught adults with limited genomic resources. Molecular Ecology Resources 17:752-759.

Brown, J. K., Taggart, J. B., Bekaert, M., Wehner, S., Palaiokostas, C., Setiawan, A. N., Palaiokostas, C., Setiawan, A.N., Symonds, J.E., and D.J. Penman. 2016. Mapping the sex determination locus in the hāpuku (*Polyprion oxygeneios*) using ddRAD sequencing. BMC Genomics 17 article number: 448. https://doi.org/10.1186/s12864-016-2773-4

Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A., and W.A. Cresko. 2013. Stacks: an analysis tool set for population genomics. Molecular Ecology 22:3124–3140.

Chen, J. J., Wang, Y. L., Yue, Y. Y., Xia, X. H., Du, Q. Y. and Z.J. Chang. 2009. A novel male-specific DNA sequence in the common carp, *Cyprinus carpio*. Molecular and Cellular Probes, 23: 235–239.

Clarkson, R.W., and P.C. Marsh. 2008. Effectiveness of the Barrier-and-Renovate approach to recovery of warmwater native fishes in the Gila River Basin. Proceedings of the Colorado River Basin Science and Resource Management Symposium. USGS, Scottsdale, Arizona.

Cnaani A, and B. Levavi-Sivan. 2009. Sexual development in fish, practical applications for aquaculture. Sexual Development 3:164–175

Cotton, S., and C. Wedekind. 2007a. Introduction of Trojan sex chromosomes to boost population growth. Journal of Theoretical Biology. 249:153-161.

Cotton, S. and C. Wedekind. 2007b. Control of introduced species using Trojan sex chromosomes. Trends in Ecology & Evolution 22:441-443.

Davey, J. W., Hohenlohe, P. A., Etter, P. D., Boone, J. Q., Catchen, J. M., and M.L. Blaxter. 2011. Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nature Reviews- Genetics 12:499–510.

Devlin, R.H., and Y. Nagahama. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191–364.

Douglas, M**.** E., P**.** C. Marsh, and W. L. Minckley. 1994. Indigenous fishes of western North America and the hypothesis of competitive displacement: *Meda fulgida* (Cyprinidae) as a case study. Copeia 1994:9-19.

Ezaz, M. T., Harvey, S. C., Boonphakdee, C., Teale, A. J., McAndrew, B. J. and D.J. Penman. 2004. Isolation and physical mapping of sex-linked AFLP markers in Nile tilapia (*Oreochromis niloticus L.*). Marine Biotechnology 6:435–445.

Feron, R., Pan, Q., Wen, M., Imarazene, B., Jouanno, E., Anderson, J., Herpin, A., Journot, L., Parrinello, H., Klopp, C. and V.A. Kottler. 2021. RADSex: A computational workflow to

study sex determination using restriction site-associated DNA sequencing data. Molecular Ecology Resources 21:1715-1731.

Fowler, B. L. S., and V.P. Buonaccorsi. 2016. Genomic characterization of sex-identification markers in *Sebastes carnatus* and *Sebastes chrysomelas* rockfishes. Molecular Ecology 25:2165–2175.

Gamble, T., and D. Zarkower. 2014. Identification of sex-specific molecular markers using restriction site-associated DNA sequencing. Molecular Ecology Resources 14:902–913.

Gamble, T., Coryell, J., Ezaz, T., Lynch, J., Scantlebury, D. P., and D. Zarkower. 2015. Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. Molecular Biology and Evolution 32:1296–1309.

Gamble, T. 2016. Using RAD—seq to recognize sex-specific markers and sex chromosome systems. Molecular Ecology 25:2114–2116.

Gammerdinger, W. J., and T.D. Kocher. 2018. Unusual diversity of sex chromosomes in African cichlid fishes. Genes 9:480.

Gao, Z. X., Wang, H. P., Yao, H., Tiu, L., and W.M. Wang. 2010. No sex-specific markers detected in Bluegill Sunfish *Lepomis macrochirus* by AFLP. Journal of Fish Biology 76: 408–414.

Gido, K., J. Schaefer, K. Work, P. Lienesch, E. Marsh-Matthews, and W. Matthews. 1999. Effects of Red Shiner (*Cyprinella lutrensis*) on Red River Pupfish (*Cyprinodon rubrofluviatilis*). The Southwestern Naturalist 44: 287-295.

Glotzbecker, G.J., Alda, F., Broughton, R.E., Neely, D.A., Mayden, R.L. and M.J. Blum. 2016. Geographic independence and phylogenetic diversity of Red Shiner introductions. Conservation Genetics 17:795-809.

Gomelsky, B., Mims, S.D., Onders, R.J., and W.B. Bean. 2002. Hormonal sex reversal and evidence of female homogamety in Black Crappie. North American Journal of Aquaculture 64:66-69.

Goudie, C.A., Simco, B.A., Davis, K.B. and G.J. Carmichael. 1994. Growth of Channel Catfish in mixed sex and monosex pond culture. Aquaculture 128:97-104.

Gutierrez, J.B., and T.L. Teem. 2006. A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology 241:333-41.

Herrington, S.J. 2004. Ecology and impacts of nonindigenous Red Shiner in the Chattahoochee River drainage, Georgia. Dissertation. Auburn University.

Hopkins, K. D., Shelton, W. L., and C.R. Engle. 1979. Estrogen sex-reversal of *Tilapia aurea.* Aquaculture 18:263–268.

Hunter, G.A., Donaldson, E.M., Goetz, F.W., and P.R. Edgell. 1982. Production of all-female and sterile Coho Salmon, and experimental evidence for male heterogamety. Transactions of the American Fisheries Society 111:367-372.

Johnstone, R., Simpson, T.H., Youngson, A.F. and C. Whitehead. 1979. Sex reversal in salmonid culture: Part II. The progeny of sex-reversed Rainbow Trout. Aquaculture 18:13-19.

Kennedy, P.A., Meyer, K.A., Schill, D.J., Campbell, M.R. and N.V. Vu. 2018. Survival and reproductive success of hatchery YY male Brook Trout stocked in Idaho streams. Transactions of the American Fisheries Society 147:419-430.

Lamatsch, D.K., Adolfsson, S., Senior, A.M., Christiansen, G., Pichler, M., Ozaki, Y., Smeds, L., Schartl, M. and S. Nakagawa. 2015. A transcriptome derived female-specific marker from the invasive Western Mosquitofish (*Gambusia affinis*). PLoS One 10 p.e0118214.

Liu, F., Sun, F., Li, J., Xia, J.H., Lin, G., Tu, R.J. and G.H. Yue. 2013. A microsatellite-based linkage map of salt tolerant tilapia (*Oreochromis mossambicus* x *Oreochromis* spp.) and mapping of sex-determining loci. BMC Genomics 14:1-14.

López-Fernández, H. and D.I. Bolnick. 2007. What causes partial F1 hybrid viability? Incomplete penetrance versus genetic variation. PLoS One 2 p.e1294.

Mair, G.C., Abucay, J.S., Abella, T.A., Beardmore, J.A. and D.O.F. Skibinski. 1997. Genetic manipulation of sex ratio for the large-scale production of all-male Tilapia *Oreochromis niloticus*. Canadian Journal of Fisheries and Aquatic Sciences 54:396-404.

Marchand O, Govoroun M, D'Cotta H, McMeel, O., Lareyre, J.J., Bernot, A., Laudet, V., and Y. Guiguen. 2000. *DMRT1* expression during gonadal differentiation and spermatogenesis in the Rainbow Trout, *Oncorhynchus mykiss*. Biochimica Et Biophysica Acta-Gene Structure and Expression 1493:180–187

Marsh-Matthews, E., W.J. Matthews, and N.R. Franssen. 2011. Can a highly invasive species re-invade its native community? The paradox of the Red Shiner. Biological Invasions 13:2911–2924.

Matthews, W. J., and L.G. Hill. 1977. Tolerance of the Red Shiner, *Notropis lutrensis* (Cyprinidae) to environmental parameters. The Southwestern Naturalist 89-98.

McCormick, J.L., Schill, D.J., and K.A. Meyer. 2021. Simulated use of YY male stocking and suppression for eradicating Common Carp populations. North American Journal of Fisheries Management. 41:366-382.

McGree, M. M., Winkelman, D.L., Winkelman, Vieira, N.K.M., and A.M. Vajda. 2010. Reproductive failure of the Red Shiner (*Cyprinella lutrensis*) after exposure to an exogenous estrogen. Canadian Journal of Fisheries and Aquatic Sciences. 67:1730–1743.

Miller, M.R., Dunham, J.P., Amores, A., Cresko, W.A., and E.A. Johnson. 2007. Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers. Genome Research 17:240–248.

Mussmann, S.M., Saltzgiver, M.J., Delomas, T.A., Campbell, M.R., Diver, T.A., and W.D. Wilson. 2022. Rapid molecular determination of sex for western North American Chub (*Gila* spp.). North American Journal of Fisheries Management. 42:73-84.

Nelson, E. J. 2018. Sex determination and population genetics of Smallmouth Bass (*Micropterus dolomieu*) in eastern Lake Ontario. Doctoral dissertation, Queen's University (Canada).

Olden, J. D. and N.L. Poff. 2005. Long–term trends of native and non–native fish faunas in the American Southwest. Animal Biodiversity and Conservation 28:75–89.

Palmer, D.H., Rogers, T.F., Dean, R., and A.E. Wright. 2019. How to identify sex chromosomes and their turnover. Molecular Ecology 28:4709-4724.

Penman, D.J., and F. Piferrer. 2008. Fish gonadogenesis. Part I. Genetic and environmental mechanisms of sex determination. Reviews in Fisheries Science 16:16–34

Peterson, B.K., Weber, J.N., Kay, E.H., Fisher, H.S., and H.E. Hoekstra. 2012. Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. PLoS One 7 e37135.https://doi.org/10.1371/journal.pone.0037135

Piferrer, F. 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197:229–281.

Qiu, S., Yong, L., Wilson, A., Croft, D.P., C. Graham and D. Charlesworth. 2022. Partial sex linkage and linkage disequilibrium on the guppy sex chromosome. Molecular Ecology 00: 1-14. https://doi.org/10.1111/mec.16674

Rinne, J. N. 1991. Habitat use by Spikedace, *Meda fulgida* (Pisces: Cyprinidae) in southwestern streams with reference to probable habitat competition by Red Shiner, *Notropis lutrensis* (Pisces: Cyprinidae). Southwestern Naturalist 36:7-13.

Ruppert, J. B., R. T. Muth, and T. P. Nesler. 1993. Predation on fish larvae by adult Red Shiner, Yampa and Green rivers, Colorado. Southwestern Naturalist 38:397-399.

Schill, D. J., Heindel, J.A., Campbell, M.R., Meyer, K.A., and E.R.J.M. Mamer. 2016. Production of a YY male Brook Trout broodstock for potential eradication of undesired brook trout populations. North American Journal of Aquaculture 78:72–83.

Schill, D.J., Meyer, K.A., and M.J. Hansen. 2017. Simulated effects of YY-male stocking and manual suppression for eradicating nonnative Brook Trout populations. North American Journal of Fisheries Management 37:1054-1066.

Schultheis, C., Böhne, A., Schartl, M., Volff, J.N. and D. Galiana-Arnoux. 2009. Sex determination diversity and sex chromosome evolution in Poeciliid fish. Sexual Development 3:68-77.

Senior, A. M., Krkosek, M., and S. Nakagawa. 2013. The practicality of Trojan sex chromosomes as a biological control: An agent based model of two highly invasive *Gambusia* species. Biological Invasions 15:1765–1782.

Shen, Z.G., Wang, H.P., Yao, H., O'Bryant, P., Rapp, D., and K.Q. Zhu. 2016. Sex determination in Bluegill Sunfish *Lepomis macrochirus*: Effect of temperature on sex ratio of four geographic strains. Biological Bulletin 230:197–208.

Stelkens, R. B., and C. Wedekind. 2010. Environmental sex reversal, Trojan sex genes, and sex ratio adjustment: conditions and population consequences. Molecular Ecology 19:627–646.

Sun, Y.L., Jiang, D.N., Zeng, S., Hu, C.J., Ye, K., Yang, C., Yang, S.J., Li, M.H. and D.S. Wang, 2014. Screening and characterization of sex-linked DNA markers and marker-assisted selection in the Nile tilapia (*Oreochromis niloticus*). Aquaculture 433:19-27.

Teal, C.N., Schill, D.J., Fogelson, S.B. and S.A. Bonar. 2021. Development of aquaculture protocols and gonadal differentiation of Red Shiner. North American Journal of Aquaculture 83:145-154.

Teal, C.N., Coykendall, D.K., Campbell, M.R., Eardley, D.L., Delomas, T.A., Shira, J.T., Schill, D.J., Bonar, S.A., and Culver, M. 2022. Sex-specific markers undetected in Green Sunfish *Lepomis cyanellus* using restriction-site associated DNA sequencing. Journal of Fish Biology 100:1528-1540.

Teem, J., Alphey, L., Descamps, S., Edgington, M., Edwards, O., Gemmell, N., Harvey-Samuel, T., Melnick, R., Oh, K., Piaggio, A., Royden, S., Schill, D., Thomas, P., Smith, T., and A. Roberts. 2020. Genetic biocontrol for invasive species. Frontiers in Bioengineering and Biotechnology 8:1-18.

Volff, J.N., and M. Schartl. 2001. Variability of genetic sex determination in Poeciliid fishes. Genetica 111:101–110.

Vu, N.V., Eardley, D.L., Delomas, T.A., and M.R. Campbell. 2019. Identification of sex-specific SNPS in burbot *Lota lota* using RAD sequencing: conservation and management applications. Fisheries and Aquatic Sciences 22 article number: 18.

Xu, Y., Du, Z., Liu, J., Su, H., Ning, F., Cui, S., Wang, L., Liu, J., Ren, C., Di, S. and X. Bai. 2022. Male heterogametic sex determination in *Rana dybowskii* based on sex-linked molecular markers. Integrative Zoology 17:105-114.

Yamamoto, T.O. and T. Kajishima. 1968. Sex hormone induction of sex reversal in the Goldfish and evidence for male heterogamety. Journal of Experimental Zoology 168:215-221.

Yano, A., Nicol, B., Jouanno, E., Quillet, E., Fostier, A., Guyomard, R., and Y. Guiguen. 2013. The sexually dimorphic on the Y-chromosome gene (sdY) is a conserved male-specific Y-chromosome sequence in many Salmonids. Evolutionary applications 6:486-496.

TABLES

Sex-linked SNP (TaqMan assay)	Template sequence	Forward primer sequence	Reverse primer sequence	VIC probe	FAM probe
XY_248	**GACGCGCATGTTCG TACAC**CCNTCCTTNA GAGAATGGCT[C/T]AT ATGGCGAG**AGGAGG GAGAGAAGACCAAG TT**	GACGCGCAT GTTCGTACA C	AACTTGGT CTTCTCTC CCTCCT	CTCGCC ATATGA GCCAT	CTCGCC ATATAA GCCAT
XY_170	**GCGAGCACAGCTAT TGATAAAAGTC**ATAT TTTA[A/G]CATTGGCT GGGGTGTCGTTCATC TGAAAAATGGCCGNT GTGTNTGACAGCNAG GGTTGGCNGTGC**AGA GACATGGTGCTTTA TTAGGCC**	GCGAGCACA GCTATTGAT AAAAGTC	GGCCTAAT AAAGCACC ATGTCTCT	CCAGCC AATGTT AAAAT	CAGCCA ATGCTA AAAT
XY_251	**TGCAGTATTTGGCA GTTCCTGATT**GTGCT GGTGGNACACGTCC[T/G]TTAGAAAGTCAT TGAATAAATA**TTGTC TTATGGTATGTTCAT TTTTAAAGTTTAAG GG**	TGCAGTATT TGGCAGTTC CTGATT	CCCTTAAA CTTTAAAA ATGAACAT ACCATAAG ACAA	CAATGA CTTTCTA AAGGAC GTG	ATGACT TTCTAAC GGACGT G
XY_73	**TGGAGCACGGACTC ATCCT**NCCGCTGGCT CCTAAAC[C/T]CTGCA CACACAACCANTGTN GGGGGTCACTCAGTN CATNAGAGTTACGN**A ATCAGATTACTTTTC AAGTAACTGGTAAA**	TGGAGCACG GACTCATCC T	TTTACCAG TTACTTGA AAAGTAAT CTGATT	TGTGTG CAGGGT TTAG	TGTGTG CAGAGT TTAG

Table E.1. TaqMan assays developed for genetic sex identification markers in Red Shiner *Cyprinella lutrensis* collected from Gila River, Arizona.

Sex-ID SNP candidate	Testing round	Phenotype	Samples from RAD-seq library-validation (N)	Validation phenotype concordance score (%)	Randomly sampled (N)	Randomly sampled phenotype concordance score (%)	Total undetermined genotype	Total score (%)
XY_248	1	Male	6	100.00%	8	75.00%	0	85.71%
		Female	6	100.00%	8	100.00%	0	100.00%
		Total	12	100.00%	16	87.50%	0	92.86%
XY_248	2	Male	-	-	32	43.75%	0	43.75%
		Female	-	-	40	95.00%	0	95.00%
		Total	-	-	72	72.00%	0	72.00%
XY_248	Grand total	Male	6	100.00%	40	47.50%	0	54.34%
		Female	6	100.00%	48	95.83%	0	96.30%
		Total	12	100.00%	88	73.86%	0	77.00%
XY_170	1	Male	6	100.00%	8	75.00%	0	85.71%
		Female	6	100.00%	8	100.00%	0	100.00%
		Total	12	100.00%	16	87.50%	0	92.86%
XY_170	2	Male	-	-	32	46.88%	0	46.88%
		Female	-	-	40	97.50%	0	97.50%
		Total	-	-	72	75.00%	0	75.00%
XY_170	3	Male	-	-	23	86.96%	1	86.96%
		Female	-	-	24	100.00%	0	100.00%
		Total	-	-	47	91.49%	0	91.49%
XY_170	Grand total	Male	6	100.00%	63	65.08 %	1	68.12 %
		Female	6	100.00%	72	98.6 %	0	98.61 %
		Total	12	100.00%	135	82.96 %	0	84.35 %

Table E.2. Phenotype-genotype concordance scores for two sex identification (sex-ID) markers for Red Shiner *Cyprinella lutrensis*

FIGURES

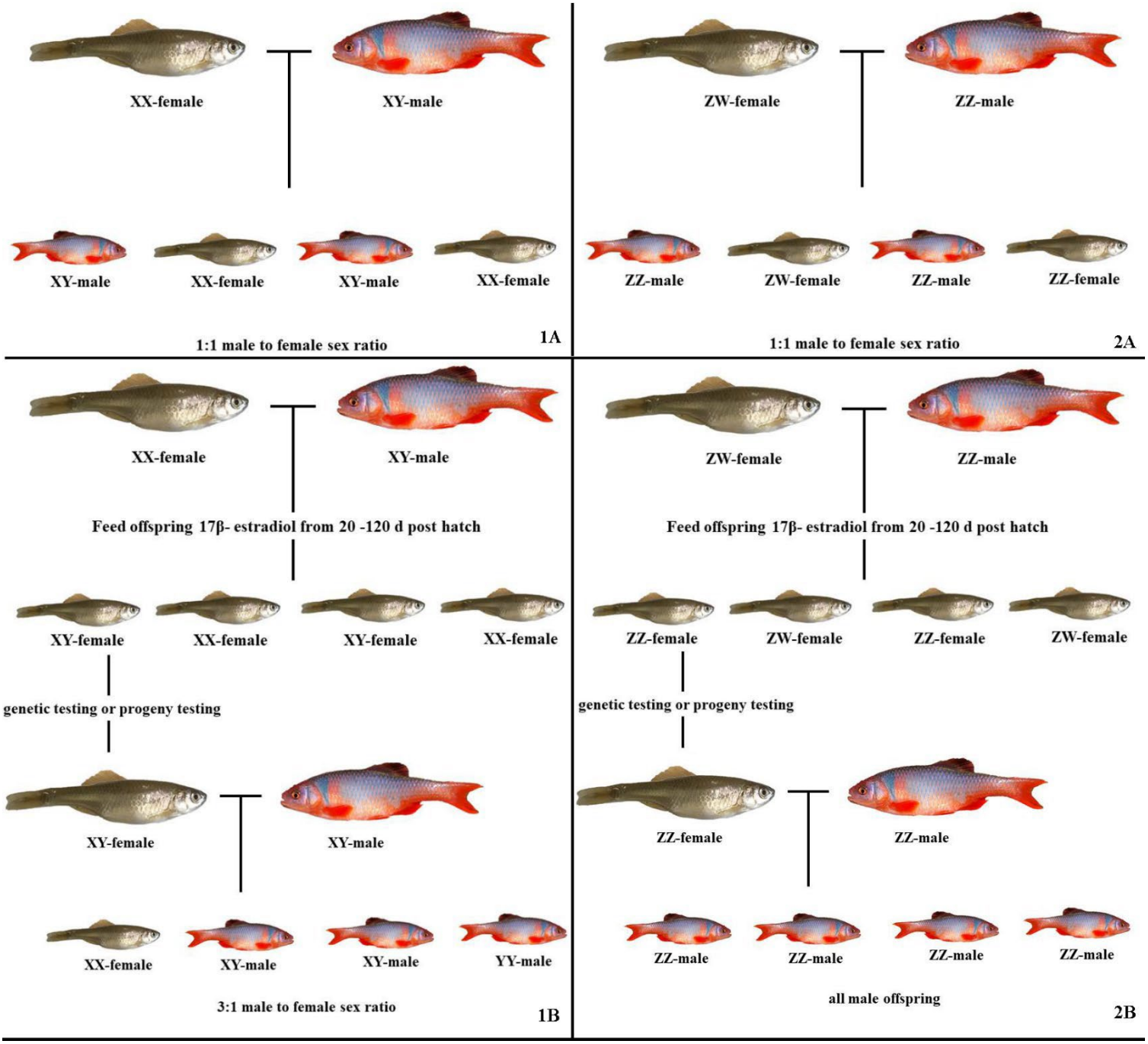

Figure E.1. Whether a species possesses a male heterogametic sex determination system (1A and 1B) or a female heterogametic sex determination system (2A and 2B), the sex ratio of males to females in the offspring will be roughly 1:1 in wild-type crosses (1A and 2A),

barring any non-chromosomal influence on sex determination or sex differentiation. Assuming YY males are viable, a 3:1 male to female sex ratio will be observed in progeny from hormonally feminized males if the males in the species are heterogametic (1B). All progeny from hormonally feminized males will be male if the females in the species are heterogametic (2B). Without genetic sex-identification markers, observing deviations from a 1:1 male to female sex ratio via progeny testing is necessary to determine which individuals are feminized males as opposed to wild-type females (1B and 2B).

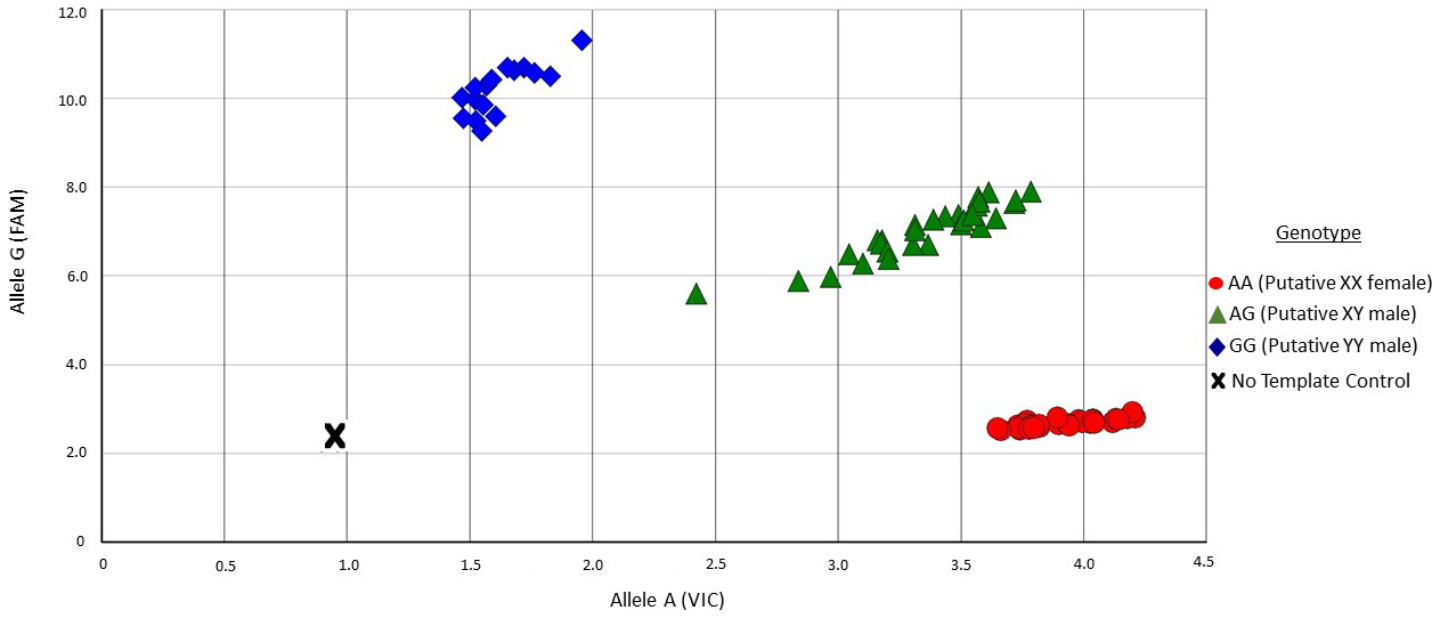

Figure E.2. An allelic discrimination plot from the TaqMan assay using Red Shiner *Cyprinella lutrensis* sex identification marker XY_170 on progeny from a neofemale (XY-female) and wild-type male (XY-male) cross. The individuals homozygous for the A allele (red circles) are putative XX females. The individuals heterozygous for the A allele (green triangles) are putative XY males and the individuals homozygous for the G allele (blue diamonds) are

putative YY males. Only one female was genotyped as heterozygous, whereas all other samples exhibited the expected genotype-phenotype concordance.

APPENDIX F

THE EFFECTS OF ESTRADIOL-17B ON THE SEX REVERSAL, SURVIVAL, AND GROWTH OF RED SHINER *CYPRINELLA LUTRENSIS* AND ITS USE IN THE DEVELOPMENT OF YY INDIVIDUALS

THE EFFECTS OF ESTRADIOL-17B ON THE SEX REVERSAL, SURVIVAL, AND GROWTH OF RED SHINER *CYPRINELLA LUTRENSIS* AND ITS USE IN THE DEVELOPMENT OF YY INDIVIDUALS

Prepared for the North American Journal of Aquaculture

ABSTRACT

Red Shiner is one of the most prolific and ecologically destructive invasive fish species in the Southwestern United States. The production and release of YY individuals can theoretically eradicate invasive fish populations. The production of YY fish is typically accomplished through a series of hormonally induced sex reversals and selective breeding of subsequently feminized males. We tested three dosages of estradiol-17β (E2) treated diets (50 mg E2 per kg of diet, 100 mg E2 per kg of diet, and 150 mg E2 per kg of diet) administered to developing Red Shiner for various durations to determine their effectiveness at feminizing Red Shiner cohorts. We assessed how these E2 treatments affected survival, growth, and gonadal development. Under our rearing conditions, the 50 mg of E2/kg of diet treatment lasting from 2-120 d post-hatch (dph) and the 100 mg of E2/kg of diet treatment lasting from 20-120 dph were highly effective at feminizing wild-type Red Shiner, with no males observed in these treatment groups. Since the 50 mg of E2/kg of diet treatment from E2-2 resulted in 100% female cohorts with a lower total amount of E2 administered than the 100 mg of E2/kg of diet fed from 20-120 dph we suggest using the 50 mg of E2 concentrated diet for 2-120 dph under our rearing conditions for feminizing wild-type male Red Shiner. These treatments had minimal, if any, detrimental effects on the growth and gonadal development of Red Shiner ($P > 0.05$). However, we saw increased mortality and gonadosomatic indexes in the 100 mg of E2/kg of diet lasting from 2-62 dph when compared to this treatment's control group ($P < 0.05$). Some YY males developed from crossing XY females with XY males were less susceptible to feminization with the 100 mg of E2/kg of diet treatment lasting from 20-120 dph than XY males. YY verification crosses ($n = 14$) resulted in all male

offspring (140 offspring) except for a single female that may have resulted from an inbred cross between an XY-male and YY females. More progeny tests with inbred and outbred crosses should be conducted to determine the prevalence of female offspring from YY individuals and how this may impact an eradication strategy featuring releases of YY Red Shiner.

INTRODUCTION

Red Shiner *Cyprinella lutrensis* is an abundant minnow species with a native range encompassing the Great Plains, tributaries of the Mississippi River, and the western Coastal Plain drainages of the Rio Grande River (Matthews 1980; Glotzecker et al. 2016). Due to introductions from aquaculture and their use as bait by anglers, invasive populations of Red Shiner now exist across the United States, extending from California to North Carolina (Glotzbecker et al. 2016). Red Shiner are considered highly invasive when introduced outside of their native range due to their aggressive behavior, high vagility, and ability to thrive in a variety of habitats (Matthews 1987; Marsh-Matthews et al. 2011; Glotzbecker et al. 2015; Glotzbecker et al. 2016). Their high heat tolerance and ability to survive in disturbed habitats makes them particularly detrimental to native fish populations in the Southwestern United States (Poulos et al. 2012). Red Shiner have been implicated in the decline of many native fish populations due to larvae predation (Ruppert et al. 1993), competition (Rinne 1991), hybridization (Johnston 1999) and transmission of parasites (Heckmann et al., 1986). Red Shiner introductions have been associated with declines in populations of Red River Pupfish *Cyprinodon rubrofluviatilis*, Razorback Sucker *Xyrauchen texanus*, Spikedace *Meda fulgida*, Colorado Pikeminnow *Ptychocheilus lucius*, Redside Shiner *Richardsonius balteatus*, Loach Minnow *Tiaroga cobitus* and Woundfin *Plagopterus argentissimus*, among others (Minckley 1973; Holden and Stalnacker 1975; Deacon 1988; Rinne 1991; Ruppert et al. 1993; Gido et al. 1999; Carpenter and Mueller 2008). This species has been deemed the worst invasive species for the survival of indigenous Southwestern U.S. fishes, second only to Western Mosquitofish *Gambusia affinis* (Dill and

Cordone 1997), and conservation of native minnow species in the Midwestern and Southeastern U.S. is threatened due to hybridization with introduced Red Shiner (Wallace and Ramsey 1982; Johnstone 1999; Glotzbecker et al. 2016).

Controlling invasive Red Shiner populations is difficult and traditional removal efforts are rarely effective (U.S. Fish and Wildlife Service 1996). Novel methods, such as releases of individuals that are homozygous for the male-coding region of the genome (YY males or YY females), can theoretically reduce and even eliminate nuisance fish populations by their spawning with wild-type fish and skewing the sex ratio of the population towards all male (Gutierrez and Teem 2006; Senior et al. 2013; Schill et al. 2017; McCormick et al. 2021). This strategy of extirpating nuisance populations via YY males or YY females is referred to as the Trojan Y chromosome strategy or the Trojan sex chromosome (TSC) eradication strategy (Gutierrez and Teem 2006; Cotton and Wedekind 2007; Senior et al. 2013).

The production of YY fish has been conducted for decades and has been accomplished in a diversity of species (Schill et al. 2016; Beardmore et al. 2001). Although typically pursued to enhance aquaculture performance (Beardmore et al. 2001; Piferrer 2001; Arslan et al. 2004; Wang et al. 2008) or to obtain all male cohorts for the ornamental fish industry (Pandian and Sheela 1995; Piferrer and Lim 1997), the development of YY individuals for commercially unimportant taxa has gained more interest due to their possible implications in invasive population control (Cotton and Wedekind 2007). In species where the male is the heterogametic sex, such as in Red Shiner (Teal et al. *in press*), the development of YY fish is often accomplished by selectively breeding feminized males (neofemales) with wild-type males (Beardmore et al. 2001; Cotton and Wedekind 2007; Senior et al. 2013; Schill et al. 2016). Progeny from this cross will feature three genotypes: XX-females, XY males, and YY males. These YY males can also be feminized to develop YY females with functional ovaries. Progeny testing or genetic sex identification markers can be used to select non-feminized YY males and

feminized YY females for spawning (Cotton and Wedekind 2007; Mair et al. 1997) with the use of genetic markers preferential to progeny testing because it requires two less generations to build the broodstocks (Schill et al. 2016). The resulting progeny require no genotyping since 100% of the offspring will be YY individuals. These YY individuals can then be stocked into nuisance populations to help extirpate the population.

The development of YY broodstock requires full feminization of XY males and YY males to produce sexually functional females. The estrogen hormone, estradiol-17β (E2), is a commonly used exogenous estrogen used for the male to female sex reversal of fishes (Pandian and Sheela 1995; Piferrer 2001). Gonochoristic species, such as Red Shiner (Teal et al. 2021), are often able to have their sexual phenotypes permanently altered if an appropriately dosed hormone treatment is started prior to gonadal differentiation and lasts until cytological gonadal differentiation is first observable through histology (Hackmann and Reinboth 1974; Piferrer 2001). The administration of E2 is often done by feeding larval and juvenile fish hormone treated diets (Yamamoto and Matsuda 1963; Al-Ablani 1997; Piferrer 2001; Schill et al. 2016; Teal et al. 2022). Susceptibility to E2 varies depending on the species (Piferrer, 2001; Wang et al., 2008). E2 diets have never been administered to Red Shiner. McGree et al. (2010) observed feminization and subsequent reproductive failure of male Red Shiner when immersed in 70 ng of E2/L. After these adult male Red Shiner were removed from the dissolved E2 their male attributes (e.g. coloration and courtship behavior) and reproductive functions restored. Effective, non-reversible, E2 feminization treatments can range in dosage from 1 mg E2 per kg of diet up to 750 mg E2 per kg of diet and treatment durations can range from six days to six months depending on the species (Kavumpurath and Pandian 1992; Colombo and Grandi 1995; Piferrer 2001). However, E2 treatments can be detrimental to the gonadal development, survival, and growth rates of fish if an exposure threshold is surpassed (Hunter et al. 1986; George and Pandian 1996; Piferrer 2001; Wang et al. 2008; Teal et al. 2022).

The production of YY Red Shiner had never been attempted and the viability and fitness of YY Red Shiner was uninvestigated before this study. Some species, such as Nile Tilapia *Oreochromis niloticus* and guppies *Poecilia reticulata*, exhibit reduced survival or viability of YY males (Piferrer 2001; Mair et al. 1997; Cotton and Wedekind 2007). Davis et al. (1995) observed that putative YY × YY crosses yield < 1% viable eggs in Channel Catfish *Ictalurus punctatus*. However, YY broodstock exhibiting comparable viability to wild-type counterparts have been produced for species such as Brook Trout (Schill et al. 2016; Kennedy et al. 2018), Common Carp *Cyprinus carpio* (Jiang et al. 2018), and Yellow Catfish *Pelteobagrus fulvidraco* (Liu et al. 2013). Determining the viability and fitness of YY individuals for the species of interest is crucial in assessing their potential use for invasive population control (Cotton and Wedekind 2007; Stelkens and Wedekind 2010; Senior et al. 2013; Schill et al 2017). In addition, some species possess autosomal sex modifying genes that may result in occasional females in the progeny of YY males (Mair et al. 1997) and occasional males in the progeny of XX males (Arslan 2018). Influence of sex modifying autosomes resulting in females from YY spawns would reduce the effectiveness of skewing the sex ratio of a population towards all-male using YY individuals. Therefore, the production, viability assessment, fitness assessment, and progeny testing of Red Shiner with YY genotypes should be conducted to determine the feasibility of using the Trojan Y eradication strategy for eradicating invasive populations.

In the current study, we tested three dosages of E2 treated diets fed to larval and juvenile Red Shiner administered for various durations to assess their effects on sexual development, survival, and growth. Our objectives were to 1) use E2 for the development of YY Red Shiner, 2) assess YY genotype viability and relative fitness as compared to wild-type Red Shiner, and 3) perform YY verification crosses to ensure all offspring from YY individuals are male.

METHODS

Production of Neofemales (XY females)

Spawning and Hatching

Red Shiner broodstock were collected via stream seine from the Gila Box Riparian National Conservation Area in Arizona, USA (GPS coordinates 32°53'0.8872" N, 109°30'0.39489" W) and stocked in four 37.9-L glass aquariums. Spawning and hatching protocols followed methods described by Teal et al. (2021). Briefly, one adult male and three adult females were stocked in each broodstock aquarium. Each aquarium was outfitted with an AquaClear 50 Power Filter (Hagen, Mansfield, Massachusetts) and an EHEIM Jager 100-W heater (EHEIM GmbH & Co, Deizisau, Germany) and the water quality parameters were maintained withing the following: water temperature 27 – 29º C, ammonia < 0.25 ppm, nitrite < 1.0 ppm, and pH 8.0 – 8.2. Broodstock aquariums were exposed to an 18-h lights on and 6-h lights off cycle to cue consistent spawning (Gale 1986; Teal et al. 2021). Each broodstock aquarium contained three stacks of spawning tiles fitting the description used in previous Red Shiner studies (Gale 1986; Teal et al. 2021).

Spawns containing 100 eggs or more were used for the subsequent section of the study. Eggs deposited in the spawning stacks were first inspected for clarity and/or eyes, to assess viability and fertilization rates (Franklin and Smith, 1963). Viable spawns were given a 30 min 100-ppm formalin bath in an aerated 18.9-L bucket. After the formalin treatment, the spawning stacks were placed in a 37.9-L larval rearing aquarium filled with dechlorinated water. Upon hatching, 100 larvae from the spawns from the broodstock aquariums were stocked in one of 16 37.9-L larval rearing aquariums.

17-β Estradiol (E2) Treatments

One hundred larvae originating from a spawn from one of the four broodstock aquariums

were given E2 treated diet or a control diet for varying durations of their grow out. Spawns with 200 larvae or more were split so only 100 larvae from a broodstock aquarium was receiving a treatment level and specific duration of treatment. Surplus larvae leftover from these increments of 100 were euthanized by immersion for 10 minutes in 75 ppm of tricaine mesylate (MS 222; Pentair Aquatic Eco-Systems) buffered with 150 ppm sodium bicarbonate (Teal et al. 2021). The treatment levels were 50 mg E2 per kg of diet (low-dose), 100 mg E2 per kg of diet (medium-dose), and 150 mg E2 per kg of diet (high-dose)(Table 1). Red Shiner were fed all five stages of Zeigler AP 100 larval diet (Zeigler Bros., Gardners, Pennsylvania) during the treatments. The diet particle sizes were stage 1: < 50 µm, stage 2: < 100 µm, stage 3: 100-150 µm, stage 4: 150-250 µm, and stage 5: 250-400 µm. E2 treated diets were formulated by dissolving E2 in 100% ethanol, mixing the E2 and ethanol solution with the diets, and then wicking off the ethanol in a fume hood (Wang et al. 2008; Teal et al. 2022). The control diets were treated the same way except without the addition of the hormone.

Diets were fed four times a day via an EHEIM automated fish feeder and twice a day ad libitum. The amount of feed dispersed to each tank was 63.30 mg (n = 20 measurements of diet dispersed, 95% CI = 13.94 – 70.86 mg), 69.60 mg (n = 20, 95% CI = 55.77 – 83.43 mg), 124.80 mg (n = 20, 95% CI = 116.14 – 133.46 mg), 94.20 mg (n = 20, 95% CI = 81.23 – 107.17 mg), 165.00 mg (n = 20, 95% CI = 144.40 – 185.60 mg) diet daily from 2 – 12 dph, 13 – 24 dph, 25 – 36 dph, 37 – 48 dph, and 49 – 150 dph, respectively. The amount to feed ad libitum was based on how much diet the fish could consume within two minutes without diet settling on the bottom of the aquariums (Teal et al. 2021). The estimated feed rates in percent body weight per day were 8% - 37% for fish 25-48 dph and 3%-4% for fish 49 dph and older. Accurate weights in fish younger than 25 dph were not obtained.

Different E2 treatments occurred in two batches on different spawns, roughly a year apart (Fall 2018 and Fall 2019). The first batch of E2 treatments (E2-1) consisted of feeding low-dose

or medium-dose E2 treated diets from 2-62 d post-hatch (dph). The 2-62 dph treatment dosage and duration was based on successful sex reversal methods used for Goldfish *Carassius auratus* (Yamamoto and Kajishima 1968). The second batch of E2 treatments (E2-2) consisted of feeding the low-dose, medium-dose, or high-dose from 2-120 dph. E2-2 also featured a delayed start treatment with the medium-dose being administered from 20-120 dph. E2-2 treatment duration was extended after observing the timing of gonadal differentiation in developing Red Shiner (Teal et al. 2021). Each treatment level and duration had 3 or 4 replicates, each replicate was derived from a spawn from a different broodstock aquarium. The low-dose treatment group and medium-dose treatment group from E2-1 (2-62 dph treatment) was fed a total of 0.309 mg of E2 (95% CI = 0.248-0.347 mg) and 0.618 mg of E2 (95% CI = 0.496-0.694 mg), respectively. The low-dose treatment group, medium-dose treatment group, medium-dose treatment group with a delayed start, and high-dose treatment group from E2-2 (2-120 or 20-120 dph treatment) was fed a total of 0.788 mg of E2 (95% CI= 0.667-0.885 mg), 1.575 mg of E2 (95% CI=1.333-1.771 mg), 1.464 mg of E2 (95% CI=1.285-1.643), and 2.363 mg (95% CI=2.000-2.656 mg), respectively. The treatment tanks used for E2-1 were 37.9 L glass aquariums featuring an airstone, a QANVEE Bio Sponge filter (Taian Qanvee Aquarium Equipment Co., Ltd., Shandong, China), and an EHEIM Jager 100-W heater (EHEIM GmbH & Co, Deizisau, Germany). The E2-1 water quality parameters were maintained withing the following: 24 – 28º C; ammonia < 0.25 ppm; nitrite < 1.0 ppm; and pH 8.0 – 8.4.

The treatment tanks for E2-2 had an identical configuration, except with the removal of the heater and the addition of a filter bag containing 10 g of activated carbon. The heaters were removed to increase the survival rates of the larvae by decreasing the water temperature (Islam 1972) and the activated carbon was added to adsorb E2 leaching out of uneaten food or feces and into the water (Teal et al., 2022). E2-2's water quality parameters were maintained within the following: 16 – 23º C; ammonia < 0.25 ppm; nitrite < 1.0 ppm; and pH 8.0 – 8.4. For both E2-1

and E2-2, a 10% water change was conducted at least once a week and uneaten diet and feces were siphoned out each larval rearing tank daily.

Total length measurements were taken throughout the first year of growout. Fifteen randomly selected larvae from E2-1 were measured upon hatching to obtain an average length at hatch for Red Shiner. All shiner from E2-1 of were measured at the end of the E2 treatment (62 dph), as well as at 125 dph and 262 dph. All shiner from E2-2 were measured at the end of the E2 treatment (121 dph), as well as at 282 dph.

At 125 dph E2-1 was transferred to six 473-L aquariums with a screen partition in the center for the duration of their growout. Each of the 473-L tanks housed one or two of the replicates, each replicate was randomly assigned to a tank and a side. Each tank had two 300-W EHEIM drop in heaters and two AquaClear 110 Power Filters. For the growout of E2-2, shiner were transferred to sixteen 757-L round fiberglass tanks that comprised a recirculating aquaculture system (RAS) located in a greenhouse with limited temperature control and a natural lighting regimen. The RAS was composed of thirty 757-L round fiberglass tanks connected to a filtration system featuring a Lifegard ¾ hp in-line pump, a DF-6 Polygeyser bead filter (Aquaculture Systems Technologies, Louisiana, U.S.A.), an Emperor 750 W UV sterilizer (Pentair Aquatic Eco-Systems), and a Dayton ½ hp in-line pump (Dayton Electric Mfg. Co., Illinois, U.S.A.). Aeration to each tank was provided by a blower (WW80 Whitewater, Pentair Aquatic Eco-Systems).

Assessment of Sex Reversal

At 387 dph, 5-13 shiner from each replicate of E2-1, 26-40 fish from each treatment group, were randomly selected and euthanized by immersion for 10 minutes in 75 ppm of MS 222 buffered with 150 ppm sodium bicarbonate. The fish were measured for total length and weight. The gonads were removed, weighed, and fixed in 10% neutral buffered formalin

(hereafter just referred to as "euthanized"). When shiner from E2-2 were 451-515 dph we randomly selected and euthanized 3-11 shiner from each replicate, 23-31 fish from each treatment group. One E2-2 replicate only had three fish remaining in the tank, so all fish were euthanized for histology from this replicate. We removed 5-11 fish from all other replicates in E2-2. For shiner in E2-2, we weighed the gonads and then performed a gonadal squash (Guerrero and Shelton 1974) on one of the gonads and fixed the other gonad in 10% neutral buffered formalin. The fixed E2-2 gonads were used for histology to validate observations using the gonadal squash method and to provide higher resolution images of sexually ambiguous tissue. All fixed carcasses and gonads from E2-1 and E2-2 were submitted to Fishhead Labs (Florida, U.S.A.) for histology processing and hematoxylin and eosin staining. Samples were cut along the transverse plane. Histological sections and squashed gonads were examined using an AmScope 40x-2000x 3W LED Seidentopf trinocular compound microscope. For identifying phenotypic sex, the gonads were deemed undifferentiated, ovary, testi, or intersex based on comparisons to images and descriptions from relevant literature (Jensen and Shelton 1983; Sacobie and Benfey 2005; Arezo et al. 2007; Uguz 2008; Gao et al 2009; Lowerre-Barbieri et al. 2011; van der Ven and Wester, no date). Gonads that were deemed undifferentiated were left out of sex ratio analysis, although gonads still undifferentiated by 150 dph are presumptive testes (Teal et al. 2021).

Tagging and Genotyping Neofemales

We selected 16 females from an E2-1 low-dose replicate tank, as well as 74 females from the 2-62 dph, 2-120 dph, and 20-120 dph medium-dose treatment groups from E2-1 and E2-2 for tagging and genetic sex identification genotyping. The females were derived from three different broodstock lineages, with 24 females from one broodstock lineage, 24 females from a different broodstock lineage, and 26 females from a third broodstock lineage. We selected 17 males from the E2-1 control group and 25 wild-caught males from the Gila Box Riparian National

Conservation Area in Arizona, USA (GPS coordinates 32°53'0.8872" N, 109°30'0.39489" W), for tagging and genetic sex identification genotyping. The males from the E2-1 control group were derived from two different broodstock lineages, with eight being from one lineage and nine from the other. Methods for the surgical implanting of PIT tags were adapted from Archdeacon et al. (2009). Before tagging, we anesthetized 3-5 shiner at a time by immersion in 50 ppm MS 222 buffered with 150 ppm sodium bicarbonate in a 19-L bucket filled with dechlorinated tap water. After at least two minutes of immobile anesthesia, we removed a fish from the bucket and used a number 11 surgical blade to make a 2-3 mm incision on the ventral side of the body, ~3 mm above the left pelvic fin attachment. We then inserted the 8 mm Biomark RFID (pit) tag (Bio8.B.041V1 PL HPT 8 PL, Biomark, Boise, ID, USA) into the incision at a 15 – 30 º angle. The tag was gently massaged back over the incision so the broadside of the tag laid across the incision. We retrieved a caudal fin clip for genetic sex identification. Tagged and clipped fish were placed in a 19-L bucket filled with 9.5-L of dechlorinated water and 2 ml of API Stress Coat (Mars, Inc. VA, USA). After 30 minutes, tagged fish were transferred to 37.9 L aquariums that contained a prophylactic treatment of 15 ppm nitrofurazone and 1 ppt salt. After 8 hours in the nitrofurazone a 50% water change was conducted. After 24 hours the shiner were evenly divided and transferred to three 473-L aquariums.

Fin clips were submitted to the University of Arizona Genetics Core (UAGC) for DNA extraction and genotyping via single nucleotide polymorphism (SNP) TaqMan assays. Individuals who were heterozygous (A/G) for the male sex-linked SNP with the sex identification marker XY_170 (Teal et al., *in press*), were broodstock candidates for the next phase of spawning.

Production of YY Red Shiner

Twelve tagged and genotyped males (M_{XY}) were selected to spawn with 18 tagged and genotyped neofemales (F_{XY}). These broodstock were stocked in six 37.9 L aquariums that had

identical configurations and similar water quality parameters as the broodstock aquariums used in the previous section “Spawning and Hatching”. These broodstock aquariums were stocked at a ratio of two males to three neofemales. Each tank contained three tile spawning stacks. Spawns containing over 20 eggs were inspected for viability as previously described. Viable eggs were given a 30 minute 100 ppm formalin treatment and stocked in one of sixteen 37.9-L larval rearing tanks. These larval rearing tanks had identical configurations and water quality parameters as E2-1 except with the addition of 10 g of activated carbon. The heaters were added back to the larval rearing tanks for these progeny in an attempt to increase growth rates and maturation time (Teal et al. 2021). Nine spawns were fed untreated Zeigler AP 100 larval diet and 7 spawns were fed medium-dose Zeigler E2 diets from 20-120 dph with the intention of producing YY females. The neofemale broodstock for these spawns were from three different broodstock lineages to prevent inbreeding when crossing YY males with YY females in the next phase of spawning. Neofemales were from a different broodstock lineage than the wild-type males they spawned with and were presumably unrelated.

When progeny from neofemale crosses were 180 dph 4-10 fish were randomly sampled from each tank and euthanized. The total number of untreated fish sampled was 70 and the total number of E2 treated fish sampled was 47. All fish were sampled from the tank if no more than four fish remained upon sampling, otherwise a subsample of at least four fish was taken from the tank. This was done to ensure enough fish remained for further study and to prevent stressful holding conditions by keeping enough fish for them to school (McGree et al. 2010). Sampled fish were measured for total length (mm) and weight (g). A fin clip was taken for genetic testing with marker XY_170 and carcasses were fixed in 10% neutral buffered formalin for histological examination of gonads. The gonads were deemed undifferentiated, ovary, testi, or intersex using previously described methods. Gonads that were deemed undifferentiated were left out of sex ratio analysis.

When progeny from neofemale crosses were between 239-306 dph (32-57 mm TL) phenotypic males from the untreated tanks and all fish from the medium-dose E2 tanks were pit tagged and fin clipped for genotyping. Males and females that were homozygous for the alternate allele with the XY_170 TaqMan assay (i.e., G/G) were deemed YY individuals.

YY Verification Crosses

To validate that the individuals that were homozygous for the alternate allele using sex identification marker XY_170 were YY individuals, and should only be capable of producing male offspring, we crossed putative YY males with wild-type females (M_{YY} x F_{XX}) and YY females with wild-type males (F_{YY} x M_{XY}). We also wanted to assess the viability of YY broodstock so we crossed putative YY males with YY females (M_{YY} x F_{YY}). In each cross, two phenotypic males and three phenotypic females were placed in a 37.9 glass aquarium with identical configurations of previously described spawning tanks. The YY males were from two different broodstock lineages and the YY females from two different broodstock lineages. The wild-type males and females were either sourced from untreated control tanks from previously described methods or wild-caught from the Gila River. No presumably related individuals were crossed with each other, except for one spawn that may have been an M_{XY} father spawning with F_{YY} daughters.

Over a two-month duration spawns from the various crosses were counted and stocked in larval rearing aquariums with identical configurations from the neofemale spawns. Egg counts and viability were assessed for all spawns, but spawns with at least 22 eggs were stocked in the larval rearing tanks after being given a 30-min 100 ppm formalin treatment. Water quality parameters were maintained as followed: temperature 26 – 29º C; ammonia < 0.25 ppm; nitrite < 1.0 ppm; and pH 8.2 – 8.4. A total of four M_{YY} x F_{XX}, seven M_{XY} x F_{YY}, and three M_{YY} x F_{YY} spawns were grown to 150 dph. At 150 dph 10 fish from each larval rearing tank were fin clipped, euthanized, and fixed in 10% neutral buffered formalin for histological examination of gonads. Two M_{YY} x F_{YY} spawns only had one or two fish left in the tank by 150 dph so all fish

from these tanks were euthanized and processed for histology. The gonads were deemed undifferentiated, ovary, testi, or intersex using previously described methods. Gonads that were deemed undifferentiated were left out of sex ratio analysis.

Data Analysis

Data analysis was conducted with Microsoft Excel V 2102 and R Studio V 3.6.1 (R Core Team, 2013). No comparisons in sex ratios, gonadosomatic indexes (GSIs), total length, or weights were made between E2-1 and E2-2 due to rearing differences and differences in the sampling schedule. Only ovaries were able to be assessed for GSI since testes were too small to obtain accurate weights with our scale. To assess the effectiveness of the various E2 treatments we used proportional binomial generalized linear models (GLMs) to compare mean proportion of fish that were females in E2-1 and E2-2 treated groups with the mean proportion of fish that were females in their respective control group. We also used a GLM to compare mean proportion of fish that were males among the various cross types to obtain adjusted means and confidence intervals. We used a chi-square test on all sampled fish from the E2-1 control group and all fish from the E2-2 control group to determine if each control group's sex ratio was significantly divergent from a 1:1, male to female, sex ratio. GSIs were calculated by dividing the fish's gonad weight by the fish's total weight and multiplying by 100 (Gabr et al. 1998).

We used generalized linear mixed models (GLMMs) with Gaussian error distributions and 'tank' as a random effect (random intercept) with the other metrics of interest (GSI, length, and weight) and treatment groups as fixed effects to account for the variation among fish that came from the same tank while avoiding pseudoreplication. We then conducted a Tukey post-hoc analysis with the GLMMs using the Kenward-Roger method for calculating degrees of freedom to compare means of the various metrics of interest among the various treatment groups. We used a GLMM with 'tank' as a random effect to compare the lengths and weights of YY males

and wild-type males that resulted from spawning neofemales with wild-type males. The weight of the YY males and XY males in progeny from neofemales were log transformed to improve normality of data before conducting the GLMMs. We used a GLMM with Gamma error distribution and 'tank' and 'date' nested within 'tank' as random effects to compare egg count per day among spawns between wild-type females and wild-type males (M_{XY} x F_{XX}), wild-type males and neofemales (M_{XY} x F_{XY}), YY males and wild-type females (M_{YY} x F_{XX}), YY males and YY females (M_{YY} x F_{YY}), and finally, wild-type males and YY females (M_{XY} x F_{YY}). We then conducted a Tukey post hoc analysis with the egg count GLMMs using the Kenward-Roger method for calculating degrees of freedom to compare egg count per day among the different breeding crosses. We used egg count per day as an index of fecundity because the number of days the broodstock groups had access to spawning stacks varied. Dividing the eggs by the number of days the tanks contained spawning stacks standardized our comparison. We used a GLM to compare mean proportion of spawns that were viable among the genotype crosses.

We used a beta generalized linear model (BGLM) to compare the mean proportion of fish that survived among the various treatment groups for E2-1. We then fit an additional BGLM to conduct a post hoc analysis comparing the survival rates between the low-dose group and the medium-dose group and used a Holm-Bonferroni (Holm 1979) correction to adjust *P* values for experiment-wise error.

No analysis was conducted on the survival rate of E2-2 due to inaccurate larval counts during larval stocking. One low-dose replicate from E2-1 was left out of analysis due to a large die off we attributed to water contamination that occurred early in the E2 treatment of that tank.

RESULTS

Effects of E2 on Sex Ratios, Gonadosomatic Index, and Ovary Condition

Based on the histology and gonadal squash data, we saw that the percentages of shiner that developed into phenotypic females in the E2 treated groups were significantly larger in both E2-1 (GLM, $Z > 2.80$, P value < 0.005) and E2-2 (GLM, $Z > 3.391$, P value < 0.001) as compared to their control groups. We saw no male individuals in the sampled fish from the low-dose treatment (50 mg E2 per kg of diet) group of E2-2 ($n = 25$ fish) or the medium-dose treatment (100 mg E2 per kg of diet) group of E2-2 ($n = 23$ fish) that started treatment at 20 dph (Table 1). We did not observe intersex fish in any of the groups. The sex ratios of the E2-1 control group and the E2-2 control group were not significantly divergent from a 1:1, male to female, sex ratio (Chi-Square Test, df = 1, P value $_{E2\text{-}1} = 0.88$; P value $_{E2\text{-}2} = 0.35$).

All sampled XY ($n = 22$ fish) and YY ($n = 8$ fish) progeny of neofemales (not fish included in Table 1) that were given the medium-dose from 20 dph to 120 dph developed into phenotypic females by 180 dph. We observed no evidence of intersex gonadal tissue in these E2 treated fish. However, we noted that four out of 17 YY individuals from the E2 treatment, that originally appeared to have female phenotypes upon stocking in spawning tanks for YY verification crosses, developed male coloration after 30 days. These putative YY females were 279 dph or 287 dph upon stocking. Despite the male coloration, we observed oocytes via the gonad squash method in one YY fish that we euthanized for further inspection. We did not euthanize the three other YY fish that appeared to revert to a male phenotype and we were unable to express gametes so it is unclear what was the state of their gonads.

The mean female GSI among the treatment groups of E2-1 differed significantly (GLMMs, $t_{6.27} > 6.088$, P value < 0.01; Figure 1). The medium-dose treatment in E2-1 had a larger female mean GSI ($\bar{x}_{\text{medium-dose}} = 12.72$, $n = 4$) than the control group ($\bar{x}_{\text{control}} = 5.42$, $n = 4$; GLMM- Tukey, $t_{9.54} = 5.966$, P value = 0.0004) and the low-dose group ($\bar{x}_{\text{low-dose}} = 5.42$, $n = 3$;

GLMM-Tukey, $t_{6.27} = 6.089$, P value = 0.0019). The low-dose group and the control group did not have significantly different female mean GSI's (GLMM-Tukey, $t_{6.80} = 0.084$, P value = 0.9962). Even though the female mean GSIs among the E2 treatment groups ($\bar{x}_{E2} = 2.93 – 3.70$) of E2-2 were larger than the control group ($\bar{x}_{control} = 2.65$, $n = 4$), the differences in means GSIs of E2-2 were small and insignificant (GLMM, $t_{18.33} < 1.185$, P value > 0.7600; Figure 1).

We observed orange, compacted, masses indicative of follicular atresia or obstruction of the outflow tract of the ovaries in five fish from the E2 treatment groups (Figure 2D). One fish was from E2-1 low-dose group, two fish were from E2-2 medium-dose group (one fish was from E2-2 medium-dose group with the delayed start), and one fish was from E2-2 high-dose group. No shiner from the control groups had this condition. We also observed inflammation in the ovaries of two fish from the E2-2 low-dose group (Figure 2C).

Survival of E2-1

Mean percentage of fish from E2-1 that survived to the end of the treatment period differed among the treatment groups (Figure 3). The medium-dose group (100 mg E2/kg diet for 2 – 62 dph) in E2-1 had a significantly lower survival rate ($\bar{x}$ = 26.25% survived, SD = 10.20 %, $n = 4$) than both the low-dose group (50 mg E2/kg diet for 2-62 dph, $\bar{x}$ = 77.00% survived, SD = 14.00 %, $n = 3$; BGLM, $Z = -5.332$, P value < 0.0001) and the control group ($\bar{x}$ = 56.00% survived, SD = 8.98 %, $n = 4$; BGLM, $Z = -3.946$, P value < 0.0001). The mean survival from the low-dose group was significantly higher than the control group (BGLM, $Z = 2.881$, P value = 0.004).

E2 Treatment Growth Rates

E2 treatments had little effect on the growth rates of Red Shiner during the treatment period (Figure 4). Differences in mean total lengths (mm) and mean total weights among E2-1 treatment groups and the control group were small and insignificant at the end of the treatment

period ($GLMM_{lengths}$, $t_{8.34} < 1.832$, P value > 0.2180; $GLMM_{weights}$, $t_{8.25} < 1.839$, P value > 0.2163). Differences in mean total lengths and weights among E2-2 treatment groups and the control group were also small and insignificant at the end of the treatment period ($GLMM_{lengths}$, $t_{11.3} < 0.786$, P value > 0.9296; $GLMM_{weights}$, $t_{10.5} < 1.126$, P value > 0.7901).

Differences in Growth Rates of YY Males and XY Males

At 180 dph the wild-type males and the YY males did not have significantly different mean TLs ($\bar{x}_{XY}$ = 24.04675 mm, 95% CI = 20.08554 – 28.78919 mm, n = 8; $\bar{x}_{YY}$ = 23.33606 mm, 95% CI = 19.29797 – 28.21913 mm, n = 8; GLMM-Tukey, $t_{43} < 1.167$, P value > 0.4795). At 180 dph the wild-type males and the YY males did not have significantly different mean weights ($\bar{x}_{XY}$ = 0.093 g, 95% CI = 0.051 - 0.168, n = 8; $\bar{x}_{YY}$ = 0.088 g, 95% CI = 0.046 - 0.168, n = 8; GLMM-Tukey, $t_{42.8} < 1.270$, P value > 0.4207).

Fecundity in Individuals with putative XX, XY, and YY genotypes

Super crosses (M_{YY} x F_{YY}) had significantly lower mean egg counts per day (Table 2) than M_{XY} x F_{XX} crosses (GLMM, t_{125} = 3.569, P value = 0.0046) and superfemale crosses (M_{XY} x F_{YY})(GLMM, t_{125} = 3.005, P value = 0.0261). Although nonviable spawns did occur in M_{XY} x F_{XX} crosses (4 nonviable/19 spawns; Table 2), most nonviable spawns occurred in neofemale crosses (12 nonviable/31 spawns) and super crosses (8 nonviable/16 spawns). All seven spawns collected from superfemale crosses were viable. Differences in mean proportion of spawns that were viable among the various crosses were not statistically significant (GLM, $Z < 1.983$, P value > 0.223).

Sex Ratios of YY Verification Crosses

As expected, we observed 100% phenotypic male offspring from the four M_{YY} x F_{XX} and three M_{YY} x F_{YY} crosses (Table 3). One female was produced in the potentially inbred spawn of

an M_{XY} x F_{YY}. This female was heterozygous (A/G) for the sex-linked marker XY_170 which is typical of phenotypic males (Teal et al., *in press*). Four out of 140 individuals still had undifferentiated gonads at 150 dph and all these individuals came from M_{XY} x F_{YY} crosses.

DISCUSSION

Under our rearing conditions, the 50 mg of E2 per kg of diet treatment lasting from 2-120 dph and the 100 mg of E2 per kg of diet treatment lasting from 20-120 dph were highly effective at feminizing wild-type Red Shiner. The other E2 treatment groups contained heavily female-skewed sex ratios ranging from 88.0% female to 97.7% female (Table 1), typically with only one or two males being sampled within a replicate tank or a treatment group. Every treatment group had at least one replicate tank where all fish sampled were female. Therefore, all these treatment regimens likely produced feminized XY males that could be used in the production of YY Red Shiner. Surprisingly, the E2-2 medium-dose treatment group and the E2-2 high-dose treatment group did not result in 100% female cohorts, whereas the low-dose treatment group and the medium-dose treatment group with the delayed start did. Sexual differentiation in fishes is often a function of length, not age (Colombo et al. 1984; Malison et al. 1986; Blázquez et al. 2001; Gao et al. 2009). Even though E2 did not have a significant influence on length or weight (Figure 4), it is possible that starting the medium-dose and high-dose at the first feeding had an unobserved influence on treated Red Shiner that may have resulted in a delayed sexual differentiation for some individuals. Exogenous estrogens are known to cause a variety of developmental effects in other species, including: teratological phenomenon (McBride and Van Overbeek 1971; Piferrer 2001), growth depression (Wang et al. 2008; Schill et al. 2016), and changes in liver metabolism (Mommsen and Walsh 1988). Either starting the low-dose treatment at first feeding or delaying the E2 medium-dose treatment start until at least 20 dph may help ameliorate any adverse developmental effects E2 could have on developing Red Shiner and appears to facilitate their feminization as compared to our other E2 treatment regimens.

It may be necessary to increase the dosage and/or the duration for feminizing YY Red Shiner. Despite all putative YY Red Shiner given 100 mg E2/kg of diet possessing ovaries when sampled at 180 dph, we observed four individuals develop male coloration after 300 dph. Teal et al. (2021) observed that all their sampled Red Shiner gonads had sexually differentiated by 105 dph, and spawns occurred with fish as young as 138 dph. Based on the concept of labile period (Hackmann and Reinboth 1974; Piferrer 2001), the timing of our treatment should have been appropriate for causing irreversible sex reversal in our YY Red Shiner. Schill et al. (2016) observed that XY males were more amenable to E2 feminization than YY males, with 223 of 224 XY males being fully feminized given a particular E2 regimen and 45 of 48 YY males being intersex given the same E2 regimen. We only inspected the gonads of one of the four YY fish that developed male coloration upon stocking for spawning trials and this fish appeared to have normal oocyte development despite its male coloration. This seeming reversion to a male external phenotype was unexpected and was not observed in any males with an XY genotype. The effects of appropriately timed and dosed hormone treatments typically result in permanent sex reversal (Piferrer 2001). However, androgen treatments in Malabar Grouper *Epinephelus malabaricus*, which are protogynous hermaphrodites, and Rainbow Trout *Oncorhynchus mykiss*, which are differentiated gonochorists, have resulted in temporary masculinization (including spermatogenesis) of treated cohorts with reversion to their genetic sex months after treatments ended (Olito and Brock 1991; Murata et al. 2014). McGree et al. (2010) exposed adult male Red Shiner to 70 ng of E2/L of water and saw temporary feminization and temporary reproductive failure of E2 treated males. Unlike McGree et al.'s Red Shiner, our Red Shiner were treated with E2 throughout the onset of gonadal differentiation which resulted in permanent sex reversals of all XY genotypes and most YY genotypes. Abucay and Mair (1997) reported that XY and YY genotypes differed in their susceptibility to diethylstilbestrol based on which family lineage the treated males were derived from (Mair et al. 1997). All the YY females that reverted to male coloration were from the same broodstock lineage. We do not have strong evidence for family-

specific resistance to E2 feminization because, we only had one YY female from a different broodstock lineage and this female died soon upon stocking for YY verification crosses.

The effects that E2 treatments have on ovary development and condition varies depending on the species. Most species, including Stickleback *Gasterosteus aculeatus*, Goldfish and Common Carp *Cyprinius carpio*, exhibit reduced ovary size and GSI's when exposed to exogenous estrogen (Komen et al. 1989; Piferrer 2001; Huang et al. 2015; Roufidou et al. 2019). Typically, an increased GSI is only observed in estrogen treated males while being decreased in females (Lei et al. 2013; Zha et al. 2007). Conversely, European Eels *Anguilla anguilla* exhibited an increased GSI with E2 treatments, which is attributed to an increase in pituitary immunoreactive gonadotropin (Dufour et al. 1983). In addition, Aryani and Suharman (2014) reported significant increases in GSI and fecundity of E2 treated Green Catfish *Hemibagrus nemurus*. The medium-dose group from E2-1 exhibited a significantly larger mean GSI than this batch's low-dose group and control group. The mean GSI among the E2-2 treatment groups did not differ, but E2-2 gonads were sampled from this batch during January, where they were exposed to a natural photoperiod. It is unclear if the E2-2 batch would retain similar GSI's during the Spring and Summer spawning season.

The ovarian pathology we observed in the E2 treated fishes (Figure 2) was present in all E2 treatment dosages and durations, yet was absent in the E2-1 and E2-2 control groups. Ovarian pathologies were rare, and we did not observe higher frequencies of ovarian pathology with increased E2 dosages or treatment durations. It is well documented that exogenous estrogens, androgens, and endocrine disrupting chemicals can increase gonadal pathologies, including excessive follicular atresia, inflammation, and gonadal duct deformities (Renee'Miles-Richardson 1977; Takahashi 1977; Papoulias et al. 2000; Vidal and Dixon 2018; Chukwuka et al. 2019). The most common gonadal abnormalities reported from sex hormone treatments is the presence of intersex gonads in treated fishes (Piferrer 2001; Wang et al. 2008; Schill et al. 2016).

Intersex individuals are typically an indication of an undersized estrogen dosage and treatment duration (Wang et al. 2008; Piferrer 2001), while sterility can result from overexposure to estrogens (Blázquez et al. 1998; Eckstein and Spira 1965; Piferrer 2001). We did not observe any intersex tissue in our sampled gonads. Despite the rare gonadal pathologies we observed, our sex reversed Red Shiner appeared to have normal sexual function, with no evidence of sterilization in any sex reversed shiner selected for spawning. Since we did not see any intersex gonad tissue nor hormone-induced sterility, the dosage and durations were likely within an appropriate range for feminization of this species.

We recommend the addition of activated carbon to E2 treatment systems to 1) reduce any extra E2 immersion the fish may receive leeching out of uneaten feed or feces (Teal et al. 2022) and 2) reduce amounts of E2 in wastewater (Schill et al. 2016). It appears we surpassed a toxic threshold in the medium-dose group of E2-1 since this treatment group exhibited a significantly higher mortality rate than the low-dose group and the control group (Figure 3). Conversely, the E2-1 low-dose group exhibited a significantly higher survival rate than this batch's control group. According to Piferrer's (2001) comprehensive review on hormone treatments in fishes "there is no convincing evidence that oestrogen (e.g. E2) treatment can increase survival". We recommend conducting additional low-dose E2 treatments for short durations to see if E2 may increase survival in Red Shiner.

It is unclear if E2 empirically had a negative impact on the survival of E2-2 treatment groups due to inaccurate larval counts at the beginning of the experiment. Anecdotally, we observed little differences in mortality among the E2-2 treatment groups in fish older than 20 dph. This may be attributed to the addition of 10 g of activated carbon to the treatment tanks.

Since the 50 mg of E2 per kg of diet treatment from E2-2 resulted in 100% female cohorts with a lower total amount of E2 administered than the 100 mg of E2/kg of diet fed from 20-120 dph (Table 1) we suggest using the 50 mg E2 concentrated diet from 2-120 dph under our

rearing conditions for feminizing wild-type male Red Shiner. An additional treatment should be attempted with a 50 mg E2/kg of diet treatment administered 20 – 120 dph since this E2 concentration could be as effective as the 100 mg E2/kg of diet if administered during that time in Red Shiner development. Administering the smallest amount of E2 possible, without sacrificing feminization results, is recommended since E2 may negatively impact fish's health (George and Pandian 1996; Piferrer 2001; Peterson and Davis 2012; Teal et al. 2022) and regulatory concerns with treatment-derived wastewater containing E2 will likely increase with the use of higher dosages (Schill et al. 2016).

Both GSI and growth are often reliable predictors for absolute fecundity, with increased size and GSI having a significant relationship with increased fecundity (Rheman et al. 2002; Shafi 2012). Schill et al. (2016) did not observe significant differences in fecundity between XY females and wild-type females while producing YY Brook Trout *Salvelinus fontinalis* broodstock. In addition, our findings contradict Senior et al.'s (2012) generalization that hormonally sex reversed fish will have reduced GSI's and therefore reduced fecundity. We did not observe a decreased GSI or a significant difference in body size of E2 treated groups among each other or with their control group. However, the effect, if any, of E2 on our Red Shiner's fecundity is still uncertain . Red Shiner fecundity is highly variable, with 24-h clutch sizes ranging from 5 to 613 eggs using our broodstock spawning conditions (Teal et al. 2021). We observed a significant reduction in mean egg count per day in our super crosses as compared to the wild-type crosses and the superfemale crosses (Table 2), but since these same YY females exhibited the highest mean egg count per day when crossed with wild-type males, it may be due to a lack of compatibility with spawning YY males with YY females and not a result of the hormone treatment. In comparison to wild-type crosses we did not see a significant reduction in mean egg count per day when neofemales were crossed with wild-type males or when YY males were crossed with wild-type females. Mean egg count per day was not significantly reduced in YY spawns with wild-type counterparts, and YY females may produce

even more eggs than wild-type females. This is an important observation when considering the efficacy of a TSC eradication strategy for Red Shiner since the release of YY females may result in quicker extirpation than the release of YY males (Teem et al. 2020).

Even though E2 did not appear to influence mean egg count per day, neofemale crosses and super crosses did exhibit the most spawns that were nonviable. This difference in proportion of spawns that were nonviable was not statistically significant, but nevertheless important to consider in its implication for this species' susceptibility to a TSC eradication strategy. Volff and Schartl (2001) observed nonviable YY genotypes in guppies when spawning neofemales with wild-type males. YY guppies are fully viable and fertile if the Y chromosome from the neofemale and the wild-type males were from different strains (Volff and Schartl 2001). Similarly, Davis et al. (1995) observed predominantly nonviable offspring of Channel Catfish when spawning YY males with YY females. The performance of YY individuals is likely related to the extent of chromosome sex differentiation between X and Y chromosomes in the population (Frolov 1991; Stelken and Wedekind 2010). We observed neofemale crosses and super crosses were consistently nonviable only when crossing individuals from specific broodstock lineages. Although only unrelated individuals were selected for spawning with each other, it is possible that the Y chromosomes of these strains resulted in nonviable offspring due to lethal recessive alleles found on these sex chromosomes (Devlin and Nagahama 2002).

Observing one female offspring from a M_{XY} x F_{YY} cross was unexpected, especially since the two other spawns featuring these YY females resulted in all male offspring. We have no reason to believe this could be a mislabeled sample nor a contamination of the histology slide since the individual is the correct size for the age of submission. It is important to note that this is the only spawn in this study that may have been between closely related individuals; a male that sired the females it subsequently spawned with. This inbreeding occurred due to an initial mistake in recording the PIT tag number for this male. Before assessing the sex ratio from this

spawn, we considered removing this spawn from our study because it was the only potentially inbred spawning replicate. Since there were two males in the spawning tank, it is possible that the other, unrelated, male sired this spawn. It is also possible that one of the putative YY females was misgenotyped and actually possessed an XY genotype. However, both of these scenarios are unlikely since the unrelated male was left in the spawning tank, while the father was replaced with a different male for subsequent spawns with these YY females, and these two subsequent spawns only produced males.

Previous studies provide examples of how inbreeding can result in XY females (Eicher and Washburn 1986) or XX-males (Komen et al. 1992). This can be the result of increased homozygosity and concomitant expression of recessive genes (Winge and Ditlevsen 1947; Komen et al. 1992;). Mice possessing an XY chromosome compliment can develop ovaries or ovotestes because of autosomal gene influences on the *Tdy* sex determining gene that result from repeated backcrossing (Eicher and Washburn 1986). XX-male Common Carp (*Cyprinus carpio*) were produced via backcrosses with gynogenetically produced fish (Komen et al. 1992). This XX-maleness in Common Carp is due to an expression of a recessive mutation in a sex determining gene that was not detected in outbred crosses (Komen et al. 1992).

However, inbreeding is not always necessary to produce XY females. Nile Tilapia *Oreochromis niloticus* possess an XX-female/XY-male sex determination system that features a major sex-determining gene on the Y-chromosome (Sun et al. 2014). However, autosomal genes and environmental factors can cause occasional female progeny to develop from crosses with YY males (Mair et al. 1997; Sun et al. 2014). In addition, Palaiokostas et al. (2015) detected quantitative trait loci (QTL) in Nile Tilapia that caused masculinization of XX genotyped individuals. There are many examples within fishes of SD behaving as a complex trait where QTL, often with environmental influence, can result in nonmendelian sex ratios (Martínez et al. 2014; Sun et al. 2014). In these instances, the threshold in which male or female development is

triggered is based on the combination of autosomal genes and possibly environmental factors, such as temperature (Vandeputte and Piferrer 2018; Palmer et al. 2019).

It is unclear what mechanisms caused a female to develop in our YY verification crosses. We recommend additional YY Red Shiner progeny tests with inbred and outbred crosses to gather more information on the prevalence of female offspring from putative YY individuals. This information will be critical in determining the efficacy of a TSC eradication strategy for Red Shiner.

In conclusion, we observed that two of our E2 sex reversal treatments, 50 mg of E2/kg of diet lasting from 2-120 dph and 100 mg of E2/kg of diet lasting from 20-120 dph, resulted in 100% of fish sampled possessing ovaries. These treatments had minimal, if any, detrimental effects on the growth and gonadal development of Red Shiner. However, we saw increased mortality in the 100 mg of E2/kg of diet lasting from 2-62 dph. Overall, our feminization methods allowed us to conduct crosses with feminized males as well as crosses with feminized YY individuals. These crosses allowed for the production of YY individuals and have provided valuable information in the potential for using a TSC eradication strategy for Red Shiner. Only one offspring (out of 140) was female from the 14 crosses we conducted using YY individuals, which may be the result of father-daughter inbreeding. The implication of potential XY females resulting from inbreeding Red Shiner should be considered when assessing the efficacy of a TSC eradication strategy for this species. We recommend conducting additional progeny tests from inbred and outbred crosses to acquire more information on the prevalence of females resulting from putative YY individuals.

ACKNOWLEDGEMENTS

The U.S. Bureau of Reclamation graciously funded this research (United States Bureau of Reclamation, grant number G18AC0061 and modification G19AC0039). Gabriella Buonavolonta, Kylee Rath, Suhailah Hussein, Cora Thomas, Kim Smoot, Jesus Bustamante,

Amy Heiling, and Kaitlyn Tyler assisted with data collection and construction and maintenance of aquaculture systems. Heidi Blasius with the Bureau of Land Management assisted with broodstock and genetic sample collection. Chris Jenney, Kaitlyn Gahl, Zach Nemec, Larissa Lee, Taylor Ulrich, Emily Freed, Joshua Grant, Steven Ingram, and Erin Tracy with the University of Arizona provided assistance with construction of the aquaculture system and provided research advice. Liz Mamer with Idaho Department of Fish and Game assisted in analyzing histology slides. There is no conflict of interest declared in this study.

REFERENCES

Abucay, J.S., and G.C. Mair. 1997. Methods of identifying males with YY genotype in *Oreochromis niloticus* L. In Proceedings of the Second AADCP International Workshop on Genetics in Aquaculture and Fisheries Management, Phuket, Thailand, Nov. 7–11, 1994.

Al-Ablani, S., 1997. Use of synthetic steroids to produce monosex populations of selected species of sunfish Family: Centrarchidae., PhD Thesis, Auburn University, 143 pp.

Archdeacon, T.P., Remshardt, W.J. and T.L. Knecht, 2009. Comparison of two methods for implanting passive integrated transponders in Rio Grande Silvery Minnow. North American Journal of Fisheries Management 29: 346-351

Arslan, T., and R.P. Phelps. 2004. Directing gonadal differentiation in Bluegill, *Lepomis marochirus* (Rafinesque), and Black Crappie, *Pomoxis nigromaculatus* (Lesueur), by periodic estradiol-17β immersions. Aquaculture Research 35: 397–402.

Arslan, T., 2018. Sex-Determining Mechanisms and Control of Sex Differentiation in Largemouth Bass and Crappies. In: Wang, H.P., Piferrer, F., Chen, S.L., Shen, Z.G. (Eds.), Sex Control in Aquaculture. Hoboken, New Jersey, pp. 385–403.

Arezo, M.J., S.D. Alessandro, N. Papa, R. de Sá, and N. Berois. 2007. Sex differentiation pattern in the annual fish *Austrolebias charrua* (Cyprinodontiformes: Rivulidae). Tissue and Cell

39:89–98.

Aryani, N. and I. Suharman. 2014. Effects of 17ß-estradiol on the reproduction of green catfish (*Hemibagrus nemurus*, Bagridae). Journal of Fisheries and Aquaculture 5: 163-166.

Beardmore, J.A., Mair, G.C., and R.I. Lewis. 2001. Monosex male production in finfish as exemplified by Tilapia: applications, problems, and prospects. Reproductive biotechnology in finfish aquaculture, pp.283-301.

Blázquez, M., Zanuy, S., Carrillo, M., and F. Piferrer. 1998. Structural and functional effects of early exposure to estradiol-17β and 17α-ethynylestradiol on the gonads of the gonochoristic teleost *Dicentrarchus labrax*. Fish Physiology and Biochemistry 18: 37-47.

Carpenter, J. and G.A. Mueller. 2008. Small nonnative fishes as predators of larval Razorback Suckers. The Southwestern Naturalist 53: 236-242.

Chukwuka, A., Ogbeide, O., and G. Uhunamure. 2019. Gonad pathology and intersex severity in pelagic (*Tilapia zilli*) and benthic (*Neochanna diversus* and *Clarias gariepinus*) species from a pesticide-impacted agrarian catchment, south-south Nigeria. Chemosphere 225: 535–547.

Colombo, G., Grandi, G., and R. Rossi. 1984. Gonad differentiation and body growth in *Anguilla anguilla* L. Journal of Fish Biology 24:215-228.

Colombo, G., and G. Grandi. 1995. Sex differentiation in the European eel: histological analysis of the effects of sex steroids on the gonad. Journal of Fish Biology. 47: 394–413.

Cotton, S., and C. Wedekind. 2007. Control of introduced species using Trojan sex chromosomes. Trends in Ecology and Evolution 22: 441-443.

Davis, K.B., Goudie, C.A., and B.A. Simco. 1995. The plasticity of sex determining genotypes in Channel Catfish. In Goetz F and Thomas P. The Fifth International Symposium on the

Reproductive Physiology of Fish. The University of Texas at Austin, Texas, USA, pp. 93-95.

Deacon, J.E. 1988. The Endangered Woundfin and Water Management in the Virgin River, Utah, Arizona, Nevada. Fisheries. 13: 18-24.

Devlin, R.H. and Y. Nagahama. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191-364.

Dill, W.A., and A.J. Cordone. 1997. History and status of introduced fishes in California, 1871-1996. Fish Bulletin 178. California Department of Fish and Game, Sacramento.

Dufour, S., Delerue-Le Belle, N., and Y.A. Fontaine. 1983. Effects of steroid hormones on pituitary immunoreactive gonadotropin in European Freshwater Eel, *Anguilla Anguilla L*. General and Comparative Endocrinology 52: 190-197.

Eckstein, B. and M. Spira. 1965. Effect of sex hormones on gonadal differentiation in a cichlid, *Tilapia aurea*. The Biological Bulletin 129: 482-489.

Eicher, E.M. and L.L. Washburn. 1986. Genetic control of primary sex determination in mice. Annual Review of Genetics 20: 327-360.

Franklin, D.R., and L.L. Smith Jr. 1963. Early life history of the Northern Pike, *Esox lucius L*., with special reference to the factors influencing the numerical strength of year classes. Transactions of the American Fisheries Society 92: 91-110.

Frolov, S. V. 1991. Mechanisms of sex chromosome differentiation in Salmonidae. Russian Journal of Marine Biology. 17: 3–17.

Gabr, H.R., Hanlon, R.T., Hanafy, M.H., and S.G. El-Etreby. 1998. Maturation, fecundity and seasonality of reproduction of two commercially valuable cuttlefish, *Sepia pharaonis* and *S. dollfusi*, in the Suez Canal. Fisheries research, 36: 99-115.

Gale, W.F. 1986. Indeterminate fecundity and spawning behavior of captive Red Shiners–fractional, crevice spawners. Transactions of the American Fisheries Society 115: 429-437.

Gao, Z., Wang, H.P., Rapp, D., O'Bryant, P., Wallat, G., Wang, W., Yao, H., Tiu, L. and R. MacDonald. 2009. Gonadal sex differentiation in the Bluegill Sunfish *Lepomis macrochirus* and its relation to fish size and age. Aquaculture 294: 138-146.

George, T., and T. J. Pandian.1996. Hormonal induction of sex reversal and progeny testing in the Zebra Cichlid *Cichlasoma nigrofasciatum*. Journal of Experimental Zoology 275: 374-382.

Gido, K., Schaefer, J., Work, K., Lienesch, P., Marsh-Matthews, E., and W. Matthews. 1999. Effects of Red Shiner (*Cyprinella lutrensis*) on Red River Pupfish (*Cyprinodon rubrofluviatilis*). The Southwestern Naturalist 44: 287-295.

Glotzbecker, G.J., Ward, J.L., Walters, D.M., and M.J. Blum. 2015. Turbidity alters pre-mating social interactions between native and invasive stream fishes. Freshwater Biology 60: 1784-1793.

Glotzbecker, G.J., Alda, F., Broughton, R.E., Neely, D.A., Mayden, R.L., and M.J. Blum. 2016. Geographic independence and phylogenetic diversity of Red Shiner introductions. Conservation Genetics 17: 795–809.

Guerrero, R.D., and W.L. Shelton. 1974. An aceto-carmine squash method for sexing juvenile fishes. The Progressive Fish-Culturist 36: 56

Gutierrez, J.B., and T.L. Teem. 2006. A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology. 241:333-41.

Heckmann, R.A., Deacon, J.E., and P.D. Greger. 1986. Parasites of the Woundfin Minnow, *Plagopterus argentissimus*, and other endemic fishes from the Virgin River, Utah. The Great Basin Naturalist 662-676.

Hackmann, E., and R. Reinboth. 1974. Delimitation of the critical stage of hormone-influenced sex differentiation in *Hemihaplochromis* multicolor (Hilgendorf) (Cichlidae). General and Comparative Endocrinology 22: 42–53.

Holden, P.B. and C.B. Stalnaker. 1975. Distribution and abundance of mainstream fishes of the middle and upper Colorado River basins, 1967-1973. Transactions of the American Fisheries Society 104: 217-231.

Huang, B., Sun, W., Li, X., Liu, J., Li, Q., Wang, R., and X. Pan. 2015. Effects and bioaccumulation of 17β-estradiol and 17α-ethynylestradiol following long-term exposure in Crucian Carp. Ecotoxicology and Environmental Safety 112: 169-176.

Hunter, G.A., Solar, I.I., Baker, I.J., and E.M. Donaldson. 1986. Feminization of Coho Salmon *Oncorhynchus kisutch*. and Chinook Salmon *Oncorhynchus tshawytscha* by immersion of alevins in a solution of estradiol-17b. Aquaculture 53: 295–302.

Islam, M. A. 1972. The effect of temperature on the reproduction of the Red Shiner, *Notropis lutrensis* (Baird and Girard). Doctoral dissertation. Texas A&M University, College Station, Texas.

Jensen, J.L., and W.L. Shelton. 1983. Gonadal differentiation in relation to sex control of Grass Carp, *Ctenopharyngodon Idella* (Pisces: Cyprinidae). Copeia 3:749-755.

Jiang, M., Wu, X., Chen, K., Luo, H., Yu, W., Jia, S., Li, Y., Wang, Y., Yang, P., Zhu, Z. and W. Hu. 2018. Production of YY supermale and XY physiological female Common Carp for potential eradication of this invasive species. Journal of the World Aquaculture Society 49: 315-327.

Johnston, C.E., 1999. The relationship of spawning mode to conservation of North American minnows (Cyprinidae). Environmental Biology of Fishes 55: 21-30.

Kavumpurath, S., and T.J. Pandian. 1992. Production of YY male in the guppy, *Poecilia reticulata*, by endocrine sex reversal and progeny testing. Asian Fisheries Science 5: 265–276.

Kennedy, P.A., Meyer, K.A., Schill, D.J., Campbell, M.R. and N.V. Vu. 2018. Survival and reproductive success of hatchery YY male Brook Trout stocked in Idaho streams. Transactions of the American Fisheries Society 147: 419-430.

Komen, J., De Boer, P., and C.J.J. Richter. 1992. Male sex reversal in gynogenetic XX females of Common Carp (*Cyprinus carpio L.*) by a recessive mutation in a sex-determining gene. Journal of Heredity 83: 431-434.

Lei, B., Kang, J., Yu, Y., Zha, J., Li, W., and Z. Wang. 2013. β-estradiol 17-valerate affects embryonic development and sexual differentiation in Japanese Medaka (*Oryzias latipes*). Aquatic toxicology 134: 128-134.

Liu, H., Guan, B., Xu, J., Hou, C., Tian, H. and H. Chen. 2013. Genetic manipulation of sex ratio for the large-scale breeding of YY super-male and XY all-male Yellow Catfish (*Pelteobagrus fulvidraco* (Richardson)). Marine biotechnology 15: 321-328.

Lowerre-Barbieri, S., K. Ganias, F. Saborido-Rey, H. Murua, and J. Hunter. 2011. Reproductive timing in marine fishes: variability, temporal scales, and methods. Marine and Coastal Fisheries Dynamics Management and Ecosystem Science Management 71–91.

Mair, G.C., Abucay, J.S., Abella, T.A., Beardmore, J.A., and D.O.F. Skibinski. 1997. Genetic manipulation of sex ratio for the large-scale production of all-male tilapia *Oreochromis niloticus*. Canadian Journal of Fisheries and Aquatic Sciences 54: 396-404.

Malison, J.A., Kayes, T.B., Best, C.D., Amundson, C.H., and B.C. Wentworth. 1986. Sexual differentiation and use of hormones to control sex in Yellow Perch (*Perca flavescens*). Canadian Journal of Fisheries and Aquatic Sciences 43: 26-35.

Marsh-Matthews, E., Matthews, W.J., and N.R. Franssen. 2011. Can a highly invasive species re-invade its native community? The paradox of the Red Shiner. Biological Invasions 13:2911–2924.

Martínez, P., Viñas, A.M., Sánchez, L., Díaz, N., Ribas, L., and F. Piferrer. 2014. Genetic architecture of sex determination in fish: applications to sex ratio control in aquaculture. Frontiers in Genetics. 5: 340.

Matthews, W.J. 1980. *Notropis lutrensis*. In: Lee, D.S., Gilbert, C.R., Hocutt, C.H., Jenkins, R.E., McAllister, D.E., and J. Stauffer Jr. (eds) Atlas of freshwater fishes of North America. North Carolina State Museum, Raleigh, pg. 285.

Matthews, W.J., 1987. Physicochemical tolerance and selectivity of stream fishes as related to their geographic ranges and local distributions. Community and Evolutionary Ecology of

North American Stream Fishes pg. 111-120.

McBride, J.R., and A.V. Overbeeke. 1971. Effects of androgens, estrogens, and cortisol on the skin, stomach, liver, pancreas, and kidney in gonadectomized adult Sockeye Salmon (*Oncorhynchus nerka*). Journal of the Fisheries Board of Canada 28: 485-490.

McCormick, J.L., Schill, D.J., and K.A. Meyer. 2021. Simulated use of YY male stocking and suppression for eradicating Common Carp populations. North American Journal of Fisheries Management 41: 366-382.

McGree, M. M., Winkelman, D.L., Vieira, N.K.M., and A.M. Vajda. 2010. Reproductive failure of the Red Shiner (*Cyprinella lutrensis*) after exposure to an exogenous estrogen. Canadian Journal of Fisheries and Aquatic Sciences 67:1730–1743.

Minckley, W.L. 1973. Fishes of Arizona. Arizona Game and Fish Department, Tempe, Arizona.

Mommsen, T.P. and P.J. Walsh. 1988. Vitellogenesis and oocyte assembly. In Fish Physiology. Academic Press. 11: 347-406.

Murata, R., Kobayashi, Y., Karimata, H., Kishimoto, K., Kimura, M. and M. Nakamura. 2014. Transient sex change in the immature Malabar grouper, *Epinephelus malabaricus*, androgen treatment. Biology of Reproduction 91: 25-1.

Olito, C., and I. Brock. 1991. Technical notes: sex reversal of Rainbow Trout: creating an all-female population. The Progressive Fish-Culturist 53: 41-44.

Palaiokostas, C., Bekaert, M., Khan, M.G., Taggart, J.B., Gharbi, K., McAndrew, B.J., and D.J. Penman. 2015. A novel sex-determining QTL in Nile tilapia (*Oreochromis niloticus*). BMC genomics 16:1-10.

Palmer, D. H., Rogers, T. F., Dean, R., and A.E. Wright. 2019. How to identify sex chromosomes and their turnover. Molecular Ecology, 28: 4709-4724.

Pandian, T.J. and S.G. Sheela. 1995. Hormonal induction of sex reversal in fish. Aquaculture, 138: 1-22.

Papoulias, D. M., Noltie, D. B., and D.E. Tillitt. 2000. An in vivo model fish system to test chemical effects on sexual differentiation and development: exposure to ethinyl estradiol. Aquatic Toxicology 48: 37–50.

Peterson, B.C., and K.B. Davis. 2012. Effects of gender and sex hormones on disease susceptibility of Channel Catfish to *Edwardsiella ictaluri*. Journal of the World Aquaculture Society 43: 733-738.

Piferrer, F., and L.C. Lim. 1997. Application of sex reversal technology in ornamental fish culture. Aquarium Sciences and Conservation. 1: 113–118.

Poulos, H.M., B. Chernoff, P.L. Fuller, and D. Butman. 2012. Mapping the potential distribution of the invasive Red Shiner, *Cyprinella lutrensis* (Teleostei: Cyprinidae) across waterways of the conterminous United States. Aquatic Invasions 7: 377-385.

Renee'Miles-Richardson, S., 1997. Effects of estrogenic compounds on the Fathead Minnow (*Pimephales promelas*). Doctoral dissertation. Michigan State University.

Rheman, S., Islam, M.L., Shah, M.M.R., Mondal, S., and M.J. Alam. 2002. Observation on the fecundity and gonadosomatic index (GSI) of Grey Mullet *Liza parsia* (Ham.). Food and Agriculture Organization of the United Nations.

Rinne, J. N. 1991. Habitat use by Spikedace, *Meda fulgida* (Pisces: Cyprinidae) in southwestern streams with reference to probable habitat competition by Red Shiner, *Notropis lutrensis* (Pisces: Cyprinidae). Southwestern Naturalist 36:7-13.

Roufidou, C. and B. Borg. 2019. Ovarian fluid in the Three-spined Stickleback *Gasterosteus aculeatus*: effects of egg overripening and sex steroid treatment. Journal of Fish Biology 94: 446-457.

Ruppert, J. B., Muth, R.T., and T. P. Nesler. 1993. Predation on fish larvae by adult Red Shiner, Yampa and Green rivers, Colorado. Southwestern Naturalist 38:397-399.

Sacobie, C.F.D, and T.J. Benfey. 2005. Sex differentiation and early gonadal development in Brook Trout. North American Journal of Aquaculture 67: 181-186.

Schill, D. J., Heindel, J.A., Campbell, M.R., Meyer, K.A., and E.R.J.M. Mamer. 2016. Production of a YY male brook trout broodstock for potential eradication of undesired brook trout populations. North American Journal of Aquaculture 78:72–83.

Schill, D.J., Meyer, K.A., and M.J. Hansen. 2017. Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. North American Journal of Fisheries Management 37:1054-1066.

Senior, A.M., Lim, J.N., and S. Nakagawa. 2012. The fitness consequences of environmental sex reversal in fish: a quantitative review. Biological Reviews 87: 900-911.

Senior, A. M., Krkosek, M., and S. Nakagawa. 2013. The practicality of Trojan sex chromosomes as a biological control: An agent based model of two highly invasive Gambusia species. Biological Invasions 15:1765–1782.

Shafi, S., 2012. Study on fecundity and GSI of *Carassius carassius* (Linneaus, 1758-introduced) from Dal Lake Kashmir. Journal of Biology, Agriculture and Healthcare, 2: 68-75.

Stelkens, R.B., and C. Wedekind. 2010. Environmental sex reversal, Trojan sex genes, and sex ratio adjustment: conditions and population consequences. Molecular Ecology 19: 627-646.

Sun, Y.L., Jiang, D.N., Zeng, S., Hu, C.J., Ye, K., Yang, C., Yang, S.J., Li, M.H., and D.S. Wang. 2014. Screening and characterization of sex-linked DNA markers and marker-assisted selection in the Nile Tilapia (*Oreochromis niloticus*). Aquaculture 433: 19-27.

Takahashi, H., 1977. Effects of large doses of methyltestosterone on the development of reproductive organs of juvenile guppy, *Poecilia reticulata*. Hokkaido University Fisheries Research Bulletin 28: 6-19.

Teal, C.N., Schill, D.J., Fogelson, S.B., and S.A. Bonar. 2021. Development of aquaculture protocols and gonadal differentiation of Red Shiner. North American Journal of Aquaculture 83: 145-154.

Teal, C.N., Schill, D.J., Fogelson, S.B., Roberts, C.M., Fitzsimmons, K., Bauder, J.M., W.T. Stewart and S.A. Bonar. 2022. The effects of estradiol-17β on the sex reversal, survival, and growth of Green Sunfish *Lepomis cyanellus*. Aquaculture, p.738853.

Teal, C.N., Coykendall, D.K., Matthew, M.R., Delomas, T.A., Eardley, D.L., Erwin, J.A., Schill, D.J., Bauder, J.M., Bonar, S.A., and M. Culver. in press. The development of genetic sex identification markers and evidence of a male heterogametic sex determination system in Red Shiner *Cyprinella lutrensis*. North American Journal of Aquaculture. Accepted for publication October 25, 2022.

Teem, J.L., Alphey, L., Descamps, S., Edgington, M.P., Edwards, O., Gemmell, N., Harvey-Samuel, T., Melnick, R.L., Oh, K.P., Piaggio, A.J., and J.R. Saah. 2020. Genetic biocontrol for invasive species. Frontiers in Bioengineering and Biotechnology 8:452.

Uguz, C. 2008. Histological evaluation of gonadal differentiation in Fathead Minnows (*Pimephales promelas)*. Tissue and Cell 40:299–306.

U.S. Fish and Wildlife Service. 1996. Red Shiner eradication project to protect the native fishes of the Virgin River, Utah. U.S. Fish and Wildlife Service, Salt Lake City, Utah.

van der Ven, L. and P. Wester. Histology and Histopathology Atlas of the Zebrafish V2.01. available: zfin.org/hh_atlas/

Vandeputte, M. and F. Piferrer, 2018. Genetic and environmental components of sex determination in the European Sea Bass. In: Wang, H.P., Piferrer, F., Chen, S.L., Shen, Z.G. (Eds.), Sex Control in Aquaculture. Hoboken, New Jersey, pp. 307–325.

Vidal, J.D., and Dixon, D., 2018. Ovary. In: Suttie, A.W., Leininger, J.R., Bradley, A.E. (Eds.), Boorman's Pathology of the Rat. London, U.K., pp. 523-536

Volff, J.N., and M. Schartl. 2001. Variability of genetic sex determination in Poeciliid fishes. Genetica 111:101–110.

Wallace Jr, R.K. and J.S. Ramsey. 1982. A New Cyprinid Hybrid, *Notropis lutrensis* × *N. callitaenia*, from the Apalachicola Drainage in Alabama. Copeia 214-217.

Wang, H.P., Gao, Z., Beres, B., Ottobre, J., Wallat, G., Tiu, L, Rapp, D., O'Bryant, P., and H. Yao. 2008. Effects of estradiol-17 β on survival, growth performance, sex reversal and gonadal structure of Bluegill Sunfish *Lepomis macrochirus*. Aquaculture 285, 216–223.

Winge, Q., and E. Ditlevsen. 1947. Color inheritance and sex determination in Lebistes. Heredity 1:65-83.

Yamamoto, T., and N. Matsuda. 1963. Effects of estradiol, stilbestrol and some alkyl-varboyil androstanes upon sex differentiation in the Medaka, *Oryzias latipes*. General and Comparative Endocrinology 3: 101–110.

Yamamoto, T.O., and T. Kajishima. 1968. Sex hormone induction of sex reversal in the Goldfish and evidence for male heterogamity. Journal of Experimental Zoology 168: 215-221.

Zha, J., Wang, Z., Wang, N., and C. Ingersoll. 2007. Histological alternation and vitellogenin induction in adult Rare Minnow (*Gobiocypris rarus*) after exposure to ethynylestradiol and nonylphenol. Chemosphere 66: 488-495.

TABLES

Batch	Treatment Level (E2 mg/kg of diet) – Dose Level	N	Number of fish	Treatment Duration (DPH)	Total amount of E2 administered (mg)	Mean Percentage of Females	95% CI for Mean Percentage of Females
E2-1	Control	4	45	2-62	0	51.11%	36.75% – 65.35%
E2-2	Control	3	29	2-120	0	41.38%	24.75% – 59.47%
E2-1	50 - Low	3	33	2-62	0.309	90.90%	74.20% – 97.78%
E2-2	50 - Low	3	25	2-120	0.788	100.00%	NA
E2-1	100 - Medium	4	25	2-62	0.618	88.00%	67.33% – 97.03%
E2-2	100 – Medium	3	29	2-120	1.575	97.54%	87.24% – 99.88%
E2-2	100 – Medium	3	23	20-120	1.464	100.00%	NA
E2-2	150 – High	3	31	2-120	2.362	97.70%	88.20% – 99.88%

Table F.1. Percentage of sampled fish containing ovaries at various treatment durations and E2 feed concentrations.

Genotype of Crosses	Number of Spawns Assessed for Viability	Number of Nonviable Spawns	Adjusted Proportion of Viable Spawns	95% CI for Spawn Viability	Mean Egg Count per Day	95% CI for Egg Count per Day
M_{XY} x F_{XX}	19	4	78.95%	57.65 – 92.94%	40.85	31.50 – 52.98
M_{XY} x F_{XY}	31	12	29.69%	9.22 – 59.92%	25.53	17.64 – 37.34
M_{YY} x F_{YY}	16	8	21.05%	5.27 – 52.74%	14.73	8.85 – 24.78
M_{YY} x F_{XX}	11	1	72.72%	24.88 – 98.26%	33.78	18.36 – 62.18
M_{XY} x F_{YY}	7	0	100%	NA	65.37	28.50 – 151.41

Table F.2. Spawn viability and egg count per day for each genotype cross.

Genotype of Crosses	Number of Spawns Assessed for Sex Ratio	Total Number of Fish Assessed for Sex Ratio	Mean Percentage of Males	95% CI for Mean Percentage of Males
M_{XY} x F_{XX}	7	74	52.70%	41.38 – 63.84%
M_{XY} x F_{XY}	9	58	77.08%	63.98 – 87.37%
M_{YY} x F_{YY}	3	30	100%	NA
M_{YY} x F_{XX}	4	40	100%	NA
M_{XY} x F_{YY}	7	66	97.83%	90.78 – 99.87%

Table F.3. Percentage of sampled fish containing testes among various cross types.

FIGURES

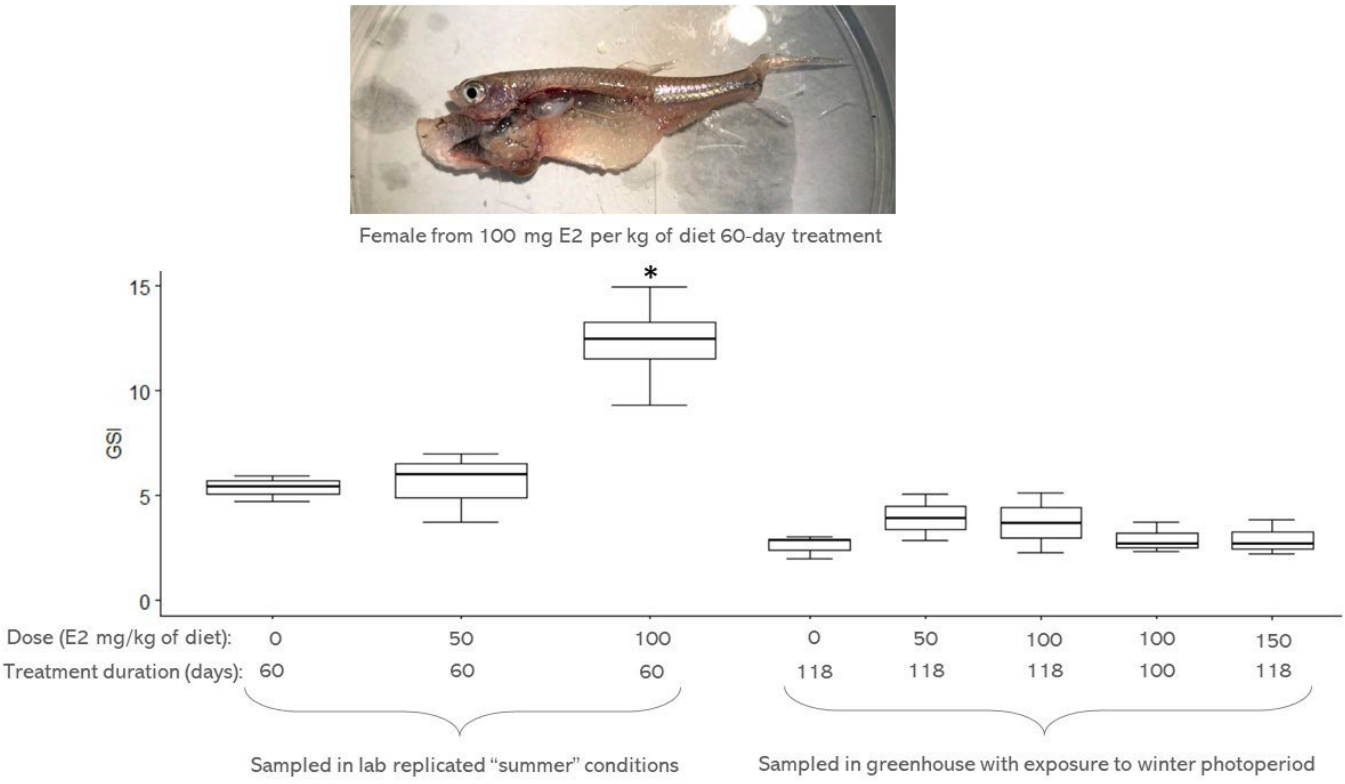

Figure F.1. A comparison of gonadosomatic indexes (GSIs) among E2 treatment groups. No comparisons were made between the 60-day treatments and the other treatments. A significant difference within respective treatment durations is denoted by an asterisk. Many females from the 100 mg E2 per kg of diet group treated for 60 days were enlarged with eggs as depicted in this photograph.

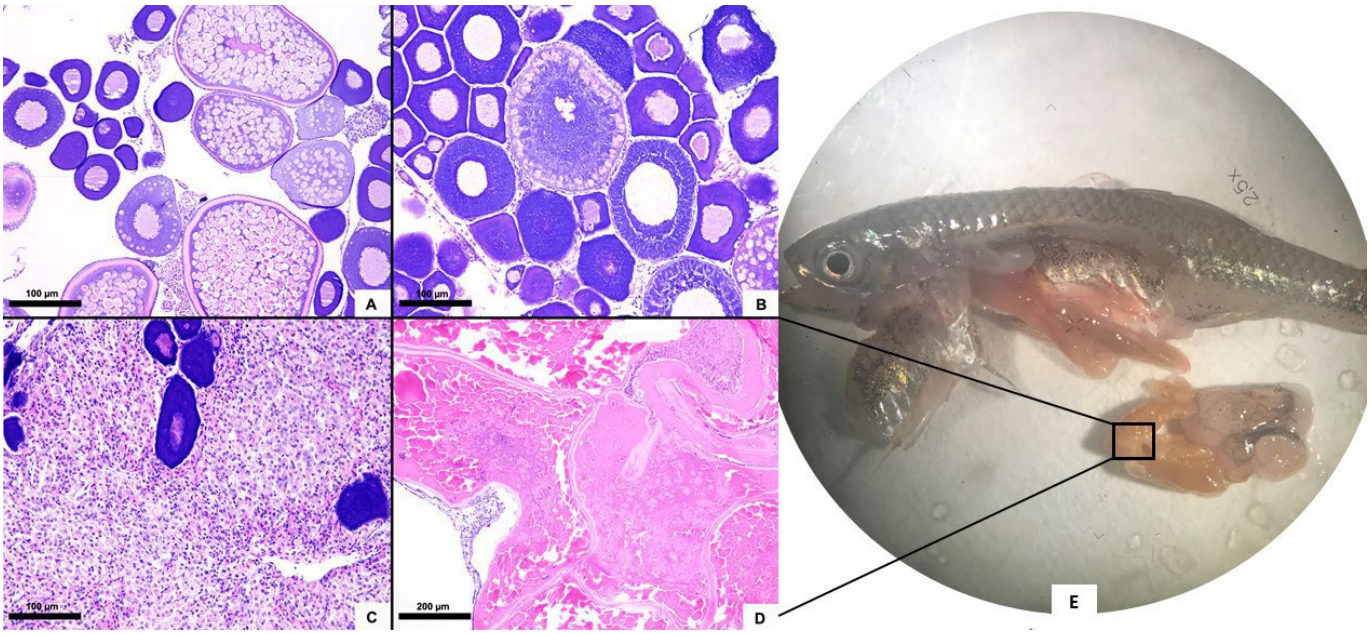

Figure F.2. Photomicrographs of ovaries: control group treatment lasting 60 days (A), 100 mg E2 per kg of diet treatment lasting 100 days (B), 50 mg E2 per kg of diet treatment lasting 118 days (C), and 100 mg E2 per kg of diet treatment lasting 118 days. Figures A and B show normal oocyte development. Figure C is an example of ovarian inflammation. Figure D is an example of follicular atresia with a macrophotograph of how it presents as a hard-compacted orange mass upon dissection (E). Inflammation and follicular atresia were not observed in either the 60 day or 118 day control group.

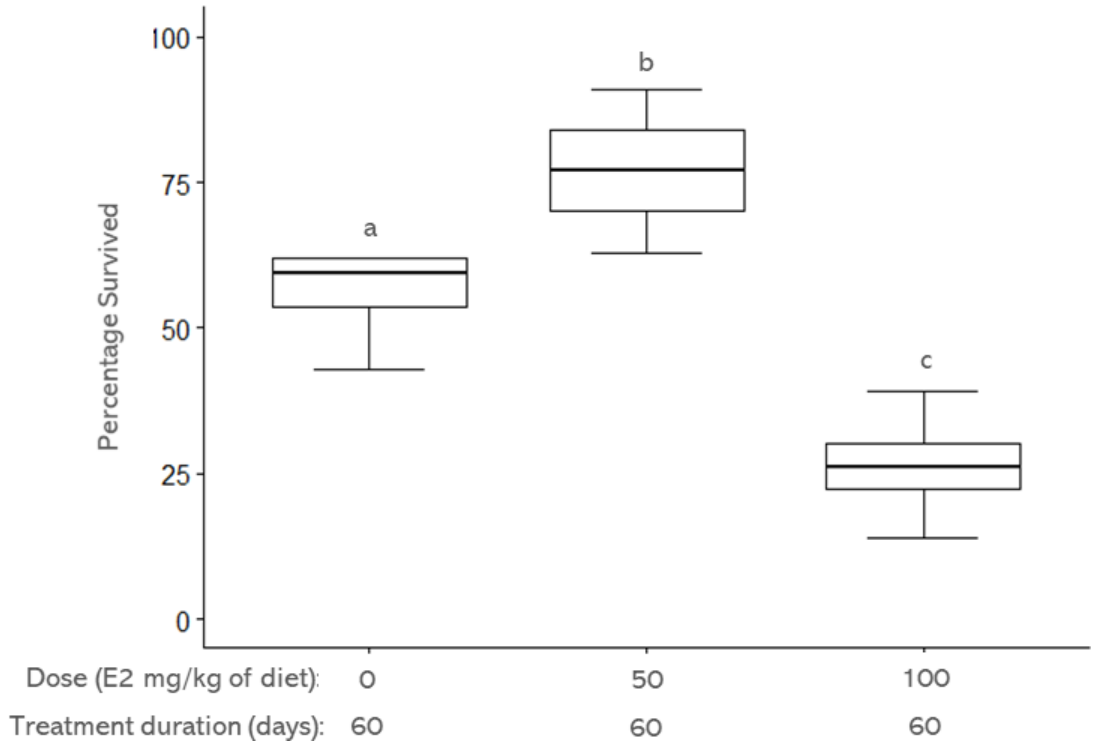

Figure F.3. A comparison of survival among the 60-day E2 treatment groups. Different letters denote significant differences.

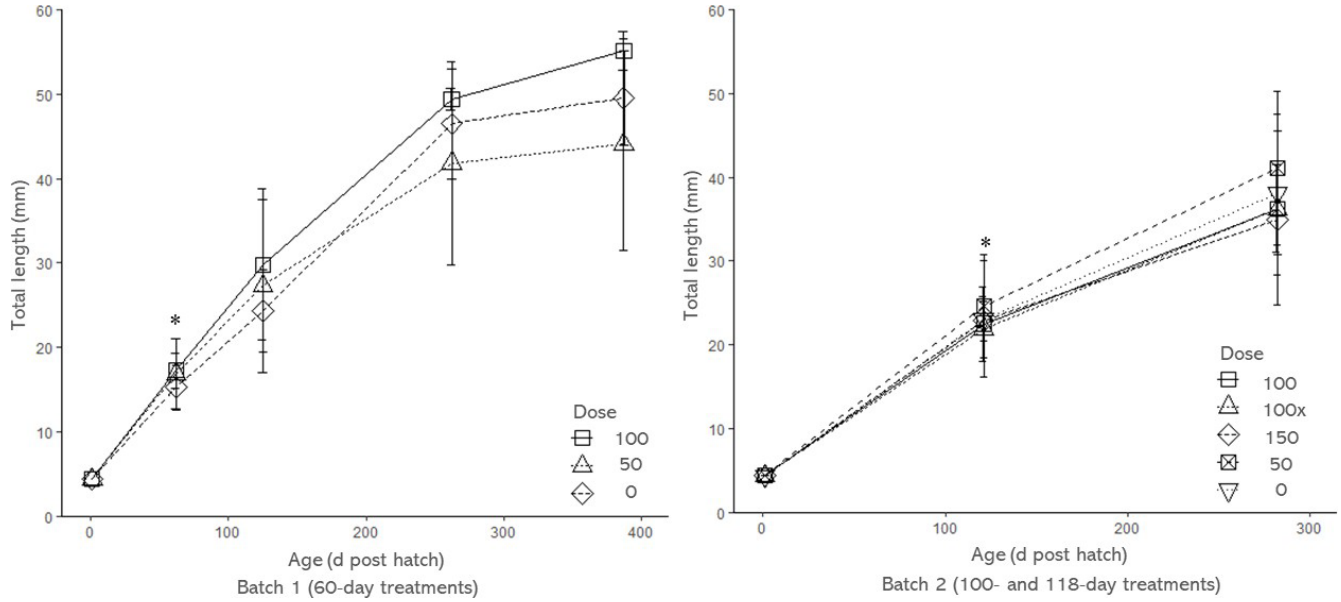

Figure F.4. Batch 1 (60-day treatment; left) and batch 2 (100- and 118-day treatments; right) mean total lengths (mm) throughout the first year of grow out. The "100x" dose in batch 2 was the treatment that lasted from 20-120 dph. Asterisks denotes end of treatment.