Title

The Ecology, Evolution, and Management of Recent Non-Native Hybridization of the Endangered California Tiger Salamander (Ambystoma californiense)

Author

Cooper, Robert David

Publication Date

2021

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The Ecology, Evolution, and Management of Recent Non-Native Hybridization of the Endangered California Tiger Salamander (*Ambystoma californiense*)

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Ecology and Evolutionary Biology

by

Robert David Cooper

2021

ABSTRACT OF THE DISSERTATION

The Ecology, Evolution, and Management of Recent Non-Native Hybridization of the Endangered California Tiger Salamander (*Ambystoma californiense*)

by

Robert David Cooper

Doctor of Philosophy in Ecology and Evolutionary Biology

University of California, Los Angeles, 2021

Professor H. Bradley Shaffer, Co-Chair

Professor Greg Grether, Co-Chair

The introduction of non-native species is a growing threat to biodiversity worldwide. Hybridization between invasive and endangered species severely complicates the management and conservation of threatened taxa. In these situations, it is necessary to understand the forces that drive hybrid success on the landscape in order to employ the most efficient and effective strategies to preserve native diversity. In this thesis, I present three studies that target specific aspects of hybridization between the endangered California tiger salamander (*Ambystoma californiense*) and the introduced barred tiger salamander (*Ambystoma mavortium*). In the first chapter, I use a Critical Thermal Maximum (CTMax) assay to show that hybrid salamanders can function at greater temperatures than either parental species. Complementary analysis of gene expression uncovered extensive transgressive segregation in F1 hybrids, which may explain this

enhanced thermal ability. Increased temperature tolerance in hybrid salamanders may contribute to their success in California. The final two chapters evaluate a potential method for reducing the success of hybrid salamanders in the wild. Previous work has suggested that breeding pond duration (hydroperiod) may confer fitness differences between hybrid and native salamanders. In the second chapter I constructed an array of large, semi-natural experimental ponds to test the effect of hydroperiod on larval survival and mass at metamorphosis. I demonstrate that both hybrid and native larvae benefit from increased pond duration, though hybrids benefit substantially more from each additional day of pond duration. While there were no hydroperiod treatments where native larvae outperformed hybrids, shortened hydroperiods significantly reduced hybrid advantage. In the third chapter, I use data from Chapter 2 to modify a recently developed demographic model for CTS to estimate the effects that hydroperiod manipulation might have on population persistence and hybrid success. Through demographic simulations, I demonstrate that the short hydroperiod treatments do not support a stable CTS population, and do not increase population resistance to hybrid colonization. It appears that healthy native populations near their carrying capacity, supported by long hydroperiod ponds, represent the best tool for deterring hybrid expansion. Conversely, reducing the hydroperiod of primarily non-native ponds may reduce the success of hybrids, decreasing the proliferation of non-native genotypes. Managing non-native hybrids is a difficult, but important conservation priority given the increased performance of hybrids in both temperature tolerance and larval success. Integrative strategies that include targeted hydroperiod management may represent the best strategy for maintaining native CTS diversity in California.

The dissertation of Robert David Cooper is approved.

Jamie Lloyd-Smith

Robert Wayne

Richard Ambrose

Greg Grether, Committee Co-Chair

H. Bradley Shaffer, Committee Co-Chair

University of California, Los Angeles

2021

Dedication Page

I would like to dedicate this dissertation to my parents, Ted and Tanya Cooper who both encouraged me to pursue my dreams, even when they involved working on little critters in cow ponds. I would also like to dedicate this work to my partner, Erin Toffelmier, without whom none of this would be possible. I would not have made it through without her tireless hours helping me in the field, reading over manuscripts, and supporting me through the tumult of graduate school. Finally, I would like to dedicate my dissertation to Barry Sinervo, as my first academic mentor he instilled in me the passion and perseverance necessary to succeed in this field. His brilliance and enthusiasm will certainly be missed, but never forgotten.

Table of Contents

Section	Page Number
Abstract	ii
Committee Page	iv
Dedication Page	v
Table of Contents	vi
List of Figures	vii
Acknowledgements	viii
Biographical Sketch	ix
Chapter 1	1
Chapter 2: Text	19
Chapter 2: Figures	54
Chapter 3: Text	63
Chapter 3: Figures	93
Appendix A	104
Literature Cited	109

List of Figures

Figure	Page Number
2.1	54
2.2	55
2.3	56
2.4	57
2.5	58
2.6	59
2.7	60
2.8	61
2.9	62
3.1	95
3.2	96
3.3	97
3.4	98
3.5	99
3.6	100
3.7	101
3.8	103
3.S1	107
3.S2	108

List of Tables

Table	Page Number
3.1	93
3.2	94

Acknowledgements

I would like to acknowledge the Wiley Journal of Molecular Ecology for allowing me to reprint the published version of my first chapter. I would also like to acknowledge the Shaffer Lab and Grether Lab and several Undergraduate assistants at UCLA, UCSC and CSUMB for support through many aspects of this dissertation. I would like to thank my committee members for their insightful comments and suggestions. I also thank E. Toffelmier, C. Searcy, A. Messerman, J. Smith, and R. Voss for their help and contributions to these projects. I am grateful for the support of agency partners, including C. Darst, M. Oganowski, R. Henry, B. Delgado, E. Morgan, and M. Westphal who ensured the successful completion of this research. I am also grateful for the support of colleagues including T. Anderson, S. Wagoner, J. Miller, and G. Dayton, who were vital in conducting the fieldwork for this study. I would like to acknowledge the UCLA La Kretz Center for California Conservation, the Stunt Ranch UC Natural Reserve, the UCLA Ecology and Evolutionary Biology Department, the Natural Communities Coalition and the UC Conservation Genomics Consortium for partial funding of this dissertation.

Biographical Sketch

Education

University of California at Los Angeles Los Angeles, California
MS in Ecology and Evolutionary Biology**,** 2015
University of California at Santa Cruz Santa Cruz, California
BS in Evolution and Ecology Biology**,** 2012

Work Experience

Natural Communities Coalition and UCLA Orange County, California
Technical Advisor, 2019 – 2020
California State University, Monterey Bay Marina, California
UROC Undergraduate Research Mentor, 2019
University of California at Santa Cruz Santa Cruz, California
Lab Manager / Research Assistant with Dr. Barry Sinervo, 2011-2014
University of California at Santa Cruz Santa Cruz, California
Supplemental Teaching Assistant with Dr. Holly Gritch de Cordova, 2011
University of California at Santa Cruz Santa Cruz, California
Environmental Studies Internship with Dr. Gregory Gilbert, 2010

Publications:

- **Cooper, R.D.**, Shaffer, H.B. (2021). Allele Specific Expression and Gene Regulation Help Explain Transgressive Thermal Tolerance in Non-native Hybrids of the Endangered California Tiger Salamander (*Ambystoma californiense*). Mol. Ecol. https://doi.org/10.1111/mec.15779
- Megía-Palma, R., Paranjpe, D., Blaimont, P., **Cooper, R.**, & Sinervo, B. (2019). To cool or not to cool? Intestinal coccidians disrupt the behavioral hypothermia of lizards in response to tick infestation. Ticks and Tick-Borne Diseases, 101275. doi: 10.1016/j.ttbdis.2019.101275
- Aburto-Oropeza , O., Johnson, A.F., Agha, M., Allen, E.B., Allen, M.F., González, J.A., Arenas Moreno, D.M., Beas-Luna, R., Butterfield, S., Caetano, G., Caselle, J.E., Gaytán, G.C.,. Castorani, M.C.N , Cat, L.A., Cavanaugh, K., Chambers, J.Q., **Cooper, R.D.,** et al. (2018). Harnessing cross-border resources to confront climate change. Environ. Sci. Policy 87, 128–132.
- Megía-Palma, Rodrigo, Paranjpe, D., Reguera, S., Martínez, J., **Cooper, R. D.**, Blaimont, P., … Sinervo, B. (2018). Multiple color patches and parasites in Sceloporus occidentalis: differential relationships by sex and infection. Current Zoology, 64(6), 703–711. doi: 10.1093/cz/zoy007
- Megía-Palma R et al. 2015. Phylogeny of the reptilian Eimeria: are Choleoeimeria and Acroeimeria valid generic names? Zoologica Scripta **44**:684–692.
- Agha M, Augustine B, Lovich JE, Delaney D, Sinervo B, Murphy MO, Ennen JR, Briggs JR, **Cooper R**, Price SJ. 2015. Using motion-sensor camera technology to infer seasonal activity and thermal niche of the desert tortoise (Gopherus agassizii). Journal of thermal biology **49**:119–126.
- Paranjpe DA, Medina D, **Cooper R**, Sinervo B. 2014. Blood parasites and the side-blotched lizards: do the parasites drive negative frequency dependent cycles of three throat color

morphs? Pages E160–E160 Integrative And Comparative Biology. Oxford Univ. Press Inc. Journals Dept., 2001 Evans Rd, Cary, NC. 27513 USA.

- Paranjpe DA, Bastiaans E, Patten A, **Cooper RD**, Sinervo B. 2013. Evidence of maternal effects on temperature preference in side-blotched lizards: implications for evolutionary response to climate change. Ecology and Evolution **3**:1977–1991.

Committees:

- 2020; UCLA Graduate Student Research Award Committee
- 2015; UCLA Incoming Graduate Student Retreat Committee

Honors and Awards:

- 2019; UCLA A.M. Schechtman Distinguished Teaching Award
- 2019; WCB and NCC Spadefoot Toad Habitat Restoration Grant
- 2017; UCLA La Kretz and UCNRS Stunt Reserve Graduate grant
- 2017; UCLA EEB Graduate Summer Fellowship
- 2016; UCLA La Kretz Center Graduate grant
- 2015; NSF Graduate Research Fellowship Program: Honorable Mention
- 2015; UCLA EEB Graduate Research Award
- 2015; UCLA La Kretz Center Graduate grant
- 2014; NSF Graduate Research Fellowship Program: Honorable Mention
- 2013; NSF Graduate Research Fellowship Program: Honorable Mention

Oral Presentations:

- Cooper RD., HB Shaffer 2020 September 24. Managing Pond Hydroperiod for Threatened Amphibians. California Society for Ecological Restoration. SERCAL.
- Cooper RD., HB Shaffer 2019 March 14 The Effect of Hydroperiod and Larval Density on Hybrid Tiger Salamander Success. BLM Coordinated Resource Planning Committee.
- Cooper RD., HB Shaffer 2019 March 11 Exploring Mechanisms Driving Non-native Hybridization in California Tiger Salamanders to Help Inform Management Strategies. CSUMB SNS Seminar
- Cooper RD., HB Shaffer 2018, July 13. The Effect of Hydroperiod and Larval Density on Hybrid Tiger Salamander Success. American Society of Ichthyologists and Herpetologists.
- Cooper RD., HB Shaffer 2017, September 20. Can Managing Pond Hydroperiod Reverse the Spread of Non-Native Genes? California Tiger Salamander Meetings.
- Cooper RD., HB Shaffer. 2017, February 25. Using Pond Hydroperiod to Attenuate the Spread of Invasive Tiger Salamander Alleles. Southwest HerpFest.
- Cooper RD., HB Shaffer. 2017, January 12. Using Pond Hydroperiod to Attenuate the Spread of Invasive Tiger Salamander Alleles. Amphibian Population Task Force Meetings.
- Cooper RD., HB Shaffer 2016, July 8. Connecting Heat Stress and Gene Expression to Explain Pervasive Hybridization of California Tiger Salamanders. American Society of Ichthyologists and Herpetologists.

Received: 20 September 2019 | Revised: 30 November 2020 | Accepted: 11 December 2020

DOI: 10.1111/mec.15779

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Allele-specific expression and gene regulation help explain transgressive thermal tolerance in non-native hybrids of the endangered California tiger salamander (*Ambystoma californiense*)

Robert D. Cooper[1,2] | **H. Bradley Shaffer**[1,2]

[1]Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA, USA

[2]La Kretz Center for California Conservation Science, Institute of the Environment and Sustainability, University of California, Los Angeles, CA, USA

Correspondence
Robert D. Cooper, Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA 90095, USA.
Email: robert.cooper@ucla.edu

Funding information
UCOP Research Catalyst Award, Grant/Award Number: CA-16-376437; NSF, Grant/Award Number: DEB# 0516475

Abstract

Hybridization between native and non-native species is an ongoing global conservation threat. Hybrids that exhibit traits and tolerances that surpass parental values are of particular concern, given their potential to outperform native species. Effective management of hybrid populations requires an understanding of both physiological performance and the underlying mechanisms that drive transgressive hybrid traits. Here, we explore several aspects of the hybridization between the endangered California tiger salamander (*Ambystoma californiense*; CTS) and the introduced barred tiger salamander (*Ambystoma mavortium*; BTS). We assayed critical thermal maximum (CTMax) to compare the ability of CTS, BTS and F1 hybrids to tolerate acute thermal stress, and found that hybrids exhibit a wide range of CTMax values, with 33% (4/12) able to tolerate temperatures greater than either parent. We then quantified the genomic response, measured at the RNA transcript level, of each salamander, to explore the mechanisms underlying thermal tolerance strategies. We found that CTS and BTS have strikingly different values and tissue-specific patterns of overall gene expression, with hybrids expressing intermediate values. F1 hybrids display abundant and variable degrees of allele-specific expression (ASE), likely arising from extensive compensatory evolution in gene regulatory mechanisms between CTS and BTS. We found evidence that the proportion of genes with allelic imbalance in individual hybrids correlates with their CTMax, suggesting a link between ASE and expanded thermal tolerance that may contribute to the success of hybrid salamanders in California. Future climate change may further complicate management of CTS if hybrid salamanders are better equipped to deal with rising temperatures.

KEYWORDS
amphibians, comparative physiology, conservation genetics, hybridization, transcriptomics

1 | INTRODUCTION

The introduction and establishment of invasive species represents one of the most significant threats to vertebrate biodiversity worldwide (Bellard et al., 2016). Non-native species pose immediate threats from competition and/or predation of native flora and fauna (Gurevitch & Padilla, 2004). However, these non-native species pose a more devastating and less well-understood threat when they hybridize with native taxa. Globalization and increased human movement have brought allopatric species into contact that have not evolved intrinsic isolating mechanisms that reduce or eliminate interspecific breeding (Mallet, 2005). Hybridization between divergent species is sometimes halted at the F1 stage due to genomic or behavioural incompatibilities. However, when hybridization produces viable offspring, the result is often genomic introgression. This invasion of non-native genes into local populations typically results in the decline of the native parental species as they are replaced demographically by hybrids (Rhymer & Simberloff, 1996). This may culminate in genomic extinction, where no pure native genotypes persist (Allendorf et al., 2001; Ellstrand & Rieseberg, 2016). A recent review of hybridization across multiple taxa concluded that about half (69 of 143 studies) reported hybridization to be an extinction threat (Todesco et al., 2016).

This 'genetic swamping' can be intensified by anthropogenically induced environmental perturbations, including climate change (Gómez et al., 2015). Successful invaders often have intrinsic abilities that allow them to rapidly adapt to novel environments. Alien species may also bring genes that are specifically adapted to local landscapes where they are introduced, resulting in non-native admixture fortifying hybrid animals against future environmental change (Hamilton & Miller, 2016; Kovach et al., 2016; Pfennig et al., 2016; Rieseberg et al., 1999). This threat can be particularly severe when hybrids possess traits or tolerances that surpass those of their parental species.

1.1 | Transgressive hybrid phenotypes

Transgressive hybrid phenotypes are characterized by trait values in hybrid offspring that are outside the bounds of either parental species. Heterosis and transgressive segregation are two well-documented phenomena that can produce transgressive phenotypes. Heterosis, or hybrid vigour, typically occurs in first-generation (F1) hybrid crosses. Heterosis has been exploited to produce strains of agricultural crops that exhibit superior traits, including increased yield, vigour and disease resistance (Fu et al., 2014; Liu et al., 2020). Despite the importance of heterosis in commercial agriculture, its molecular basis is still widely debated (Comings & MacMurray, 2000; Schnable & Springer, 2013). Exaggerated traits in F1 hybrids are often attributed to individual loci that convey a heterozygote advantage and are not expected to achieve fixation in a population (Hedrick, 2012). However, recent genomic studies have demonstrated that epistasis and epigenetics may play a significant role in producing transgressive hybrids through differential gene regulation (Fujimoto et al., 2012; Landau et al., 2019; Miller et al., 2015), small interfering RNAs (Groszmann et al., 2011; Shen et al., 2017), and DNA methylation (Lauss et al., 2018; Shen et al., 2017). These mechanisms may allow transgressive phenotypes to persist past the F1 stage. In contrast, transgressive segregation occurs at, or after the production of, second-generation hybrid crosses, when meiosis can produce recombinant genotypes. This phenomenon occurs more frequently than previously assumed; 97% of plant and 78% of animal hybrid studies report at least some transgressive phenotypes (Rieseberg et al., 1999). These transgressive traits may be the result of additive or non-additive interactions of loci from the two parental lineages and can become fixed in admixed populations (Dittrich-Reed & Fitzpatrick, 2013).

Though often considered separately, heterosis and transgressive segregation are linked processes that can produce transgressive hybrid phenotypes. Both are predicted to produce more exaggerated phenotypes with increasingly divergent parental lineages (Guindon et al., 2019; Stelkens & Seehausen, 2009). As recent evidence continues to highlight the importance of epistasis and epigenetics in driving transgressive phenotypes at the F1 stage (Jiang et al., 2017), it is likely that these mechanisms contribute to fixed transgressive phenotypes in admixed populations. Furthermore, in many agricultural studies, the extent of observed heterosis helps predict the successful establishment of transgressive phenotypes in subsequent generations (Chahota et al., 2007; Guindon et al., 2019; Khan et al., 2016; Kumar et al., 2017; Sarawat et al., 1993). In the case of wild non-native hybrids, it is similarly useful to analyse phenotypic traits in F1 hybrids to understand the potential advantages and adaptations that may arise through transgressive segregation. These traits may experience positive selection that establishes F1 individuals, a critical step in the early evolution of admixed populations. Furthermore, if the mechanisms that produce transgressive phenotypes in the F1 stage are indeed heritable, then they may facilitate further hybrid crosses with a greater potential of establishing permanent transgressive hybrid populations. In this study, we utilize F1 hybrid phenotypes to understand the forces promoting hybridization between an endangered salamander and an introduced congener in central California.

1.2 | Hybridization in the California tiger salamander system

The California tiger salamander ('CTS', *Ambystoma californiense*) is endemic to the grassland ecosystems of central California (Lannoo, 2005). The species is protected under both state law and federal law (California Department of Fish Game, 2010; U.S. Fish & Wildlife Service, 2004). One of the primary threats to CTS is the rapid introgression of non-native alleles from the introduced barred tiger salamander ('BTS'; *Ambystoma mavortium*). BTS were intentionally introduced into the Salinas Valley (Monterey County, California, USA) in the 1950s and 1960s for the fishing bait industry (Riley et al.,

2003). These introduced BTS may be considered invasive species since they are established, directly compete and hybridize with native CTS, and alter the biodiversity of threatened vernal pool ecosystems (Johnson et al., 2011; Ryan et al., 2009; Searcy et al., 2016). However, their greatest threat is to the genomic integrity of native CTS through introgression.

Hybridization between CTS and BTS occurs readily, hybrids are fertile, and introgression past the F1 stage is common (Johnson et al., 2010). These non-native alleles reach high frequencies in native populations more rapidly than is expected based on models of neutral diffusion (Fitzpatrick & Shaffer, 2007a), and ongoing landscape genomic analyses confirm that the hybrids are expanding and are present in all three of the CTS distinct population segments (McCartney-Melstad and Shaffer, unpublished data). Several studies indicate the presence of transgressive phenotypes in these hybrid salamanders, which may promote their success in the wild. Hybrids were found to have increased larval survival compared with CTS and BTS, affording them a direct fitness advantage (Fitzpatrick & Shaffer, 2007b), and Searcy et al. (2016) concluded that hybrids do not serve the same ecological function as native salamanders, resulting in less productive breeding ponds. This pattern is likely driven by the enhanced growth rate of hybrid larvae, which enables them to prey on larger aquatic organisms compared with native CTS (Ryan et al., 2009; Searcy et al., 2016). This rapid growth rate also results in larger hybrid salamanders at metamorphosis, which has been shown to significantly improve lifetime fitness in CTS (Johnson et al., 2013; Searcy et al., 2014). F1 hybrids also have superior locomotor capabilities compared with either CTS or BTS (Johnson et al., 2010). Taken together, these studies suggest that CTS × BTS hybrids interact with environmental variation in novel ways, often with increased hybrid fitness, likely facilitated by transgressive phenotypes.

1.3 | CTS and thermal stress

In this study, we focus on temperature stress given its critical role in amphibian survival and its potential to promote salamanders with enhanced thermal tolerance in the wild. The sparse rains and high temperatures that characterize semi-arid grasslands are a challenging environment for pond-breeding amphibians, and members of the tiger salamander complex have evolved adaptations to these conditions, which enable them to occupy ecosystems that preclude virtually all other salamanders in North America (Petranka, 1998; Stebbins, 2003). For CTS in particular, limited and highly seasonal annual precipitation leads to shallow breeding ponds that experience rapid, large temperature fluctuations, subjecting newly emerged metamorphs and aquatic larvae to extreme heat (Holland et al., 1990; Pounds, 2001). After metamorphosis, emerging CTS take temporary refuge in nearby rodent burrows while they wait for the necessary autumn rainfall to continue their upland migration (Loredo et al., 1996; Searcy et al., 2013). These initial opportunistic refugia must be adequate for new metamorphs to survive up to 6 months of extreme Central Valley heat since inadequate burrow selection may expose salamanders to lethal temperatures. For the rest of their lives, CTS must also select more permanent burrows to survive extreme summer temperatures (Trenham et al., 2000). It is therefore reasonable to hypothesize that hybrid tiger salamanders with transgressive thermal tolerance may enjoy increased fitness in this challenging landscape. To investigate the role of acute thermal stress on CTS, BTS and hybrid salamanders, we used a critical thermal maximum (CTMax) assay, where CTMax is defined as the upper thermal limit of a species' temperature tolerance, above which the organism is unable to function.

1.4 | Identifying transgressive phenotypes

Here, we briefly outline a research programme that can be used to identify transgressive hybrids at the phenotypic and regulatory levels. First, we investigate the effects of acute thermal stress on CTS, BTS, and hybrid crosses to identify potential differences in thermal tolerance. Second, we quantify the tissue-specific gene expression response at the individual level in all three groups. Differential gene expression (DGE) enables us to compare the expression profiles of CTS, BTS, and hybrids in response to identical thermal stress under controlled conditions. Finally, we examine the regulation of gene expression to better understand transgressive or variable phenotypes in F1 hybrids. Gene regulation is a complex process potentially involving promoters located on the same DNA molecule near the gene of interest (*cis*) and regulatory elements that are not physically linked to the focal gene (*trans*). Analysis of gene expression in the parental species and of parent-specific gene copies in the F1 hybrids allows us to identify genes that are governed by *cis*-, *trans*- or any combination of the two regulatory factors. We employ these methods to assign regulatory modes to each gene of interest in hybrid tiger salamanders, then make inferences about the evolutionary history and mechanisms that are associated with transgressive hybrid phenotypes. From these three levels of analysis, we seek to answer the following questions: (a) At the whole organism physiological level, are there differences in thermal tolerance between CTS, BTS, and hybrids? (b) Are there differences in gene expression between CTS, BTS, and F1 hybrids exposed to acute thermal stress? (c) Can we identify transgressive or variable phenotypes in the F1 hybrids? And (d) if we find transgressive or variable phenotypes in F1 hybrids, what genomic mechanisms are associated with their extreme traits?

2 | MATERIALS AND METHODS

2.1 | Study population and study design

We used tiger salamanders from a captive research colony housed at the University of California, Los Angeles. These individuals were either wild-caught or captive bred and were housed individually in a climate-controlled room for at least 4 years, ensuring that individuals were sexually mature and fully acclimated to an identical thermal regime.

Wild-caught CTS were collected from either Great Valley Grasslands State Park (Merced County, CA) or Jepson Prairie Preserve (Solano County, CA), which are both within the central Distinct Population Segment of CTS in the Central Valley of California and exhibit similar climatic conditions (U.S. Fish & Wildlife Service, 2003). Previous work has shown that CTS from these localities are genetically similar (Shaffer et al., 2004). Pure BTS individuals were collected from Five Star Fish Farms (Lake County, CA), an introduced population founded by salamanders from the initial introduction of BTS in California (Johnson et al., 2011; Riley et al., 2003). Hybrid salamanders were created for use in previous studies by crossing these CTS and BTS individuals in the laboratory (Johnson et al., 2013). Salamanders were fed pinky mice and/or crickets twice per week and housed following approved UCLA animal care protocols (ARC #2013-011-11).

Salamanders of each genotype were randomly assigned to experimental (heated) and control groups. This resulted in 10 CTS (5 control, 5 heat), 12 BTS (6 control, 6 heat), 25 F1-hybrids (13 control, 12 heat), 3 BTS backcross (1 control, 2 heat) and 1 CTS backcross (1 heat) for the CTMax experiment. The F1 hybrids are the offspring of three independent crosses between unrelated CTS and BTS parents. Of these three sib groups, the female parent was CTS in two F1 sib groups (n = 7 and 8) and BTS in one group (n = 10). Given the time (4 years) that animals were maintained in the laboratory, errors in individual identification were possible. We confirmed sib group status with genomic data using the program Colony (Jones & Wang, 2010; see Appendix S1). We analysed potential differences between sib groups using one-way ANOVAs and pooled family groups that were not significantly and consistently different for downstream analyses. We also ran all analyses using linear mixed models that included sib group and female parental genotype as random effects (see Appendix S1). Despite the limited sample size, backcross individuals were included as a separate group in the physiological experiment since they may demonstrate post-F1 transgressive segregation that is not possible in the F1 offspring. However, because we had so few individuals, they were not included in downstream expression analyses.

2.2 | Physiology and CTMax

Critical thermal maximum (CTMax) has been used in many physiological experiments on salamanders to describe a species' tolerance of near-lethal temperatures (Burke & Pough, 1976; Spotila, 1972). CTMax has been shown to correlate well with other measures of thermal tolerance and is a useful tool in predicting population persistence in response to changing climate (Araújo et al., 2013; Huey et al., 2012). We assessed CTMax using the loss of righting response (LRR), a measure that has been used extensively in ectotherms (Delmas et al., 2007; Gvozdík, 2011; Sanabria et al., 2012) including CTS (Johnson, Johnson, et al., 2010). LRR is a whole-animal performance measure with direct ecological significance, given that the inability to right itself represents a loss of function that prevents the organism from escaping stressful conditions or fleeing from a predator (Lutterschmidt & Hutchison, 1997).

To assay CTMax, individuals were placed on a moist paper towel substrate in an opaque plastic container under a 100-w ceramic infrared heat-emitting bulb (Zoo Med brand) (Layne & Claussen, 1982; Young & Gifford, 2013). Salamanders were constantly misted during the heating trials to avoid desiccation and were monitored for signs of abnormal behaviour. Temperatures were constantly monitored with an infrared temperature gun (Amprobe IR-720) positioned 7 cm from the dorsum. After dorsal temperatures reached 30°C, individuals were assayed for loss of righting response (LRR). LRR was achieved when an individual was unable to right itself after 30 s on its back on a moist surface (Lutterschmidt & Hutchison, 1997; Young & Gifford, 2013). At this time, temperature was recorded with an IR temperature gun 1 cm from the dorsal and ventral surface of the body. Control individuals were placed in the same plastic containers in the same room as the CTMax trials and flipped on their back to parallel the LRR assay, but were kept at constant room temperature of 21°C.

Previous work has suggested that measurements taken using non-contact infrared thermometers accurately estimate core body temperature in amphibians, without the need of invasive cloacal probing (Rowley & Alford, 2007; Tracy et al., 2010), though some inconsistencies have been detected in reptiles (Carretero, 2012). We therefore calibrated our temperature measurements using museum specimens to accurately estimate internal core temperature from dorsal and ventral surface measurements. We used eight ethanol-preserved specimens of CTS, BTS and hybrids within the size range of our experimental animals as thermal models. Temperatures were recorded using IR temperature guns positioned 1 cm from the dorsal and ventral sides of each salamander. A thin thermocouple probe was also inserted into the centre of the body cavity to record actual internal temperature. We heated each specimen for 1 h and recorded temperatures every 3 min over this period. We then created linear models that estimate internal body temperature as a function of dorsal and ventral skin temperatures. We identified the best model using the Akaike information criterion (AIC), where models with a $\Delta AIC \geq 3$ were considered superior. All analysis were conducted in R (v3.3.2; R Core Team, 2013).

We analysed CTMax to address our primary question: Do hybrid salamanders possess transgressive thermal tolerance abilities compared with both parental lineages? We first compared the CTMax of CTS and BTS. Since no difference was detected, we pooled CTS and BTS into a parental group and compared the CTMax of the parents with that of the hybrids. We used Welch's t tests to compare mean CTMax values and F tests of the equality of variance to compare the degree of variation in CTMax for each group using custom scripts in R (v3.3.2; R Core Team, 2013).

2.3 | RNA lab protocol

Immediately after reaching CTMax, or after 60 min for controls, individuals were anaesthetized in a 5% benzocaine/water solution

for 5 min and decapitated to ensure instant euthanasia, and brain and muscle tissues were harvested. Entire brains and muscle tissue from the rear left leg were taken and immediately placed in RNAlater (Ambion) solution and stored at −20°C until RNA extraction.

Tissues were homogenized using a bead shaker and extracted using a modified Purelink RNA Mini Kit—TRIzol spin column protocol. Samples were individually marked with dual index iTru barcodes to allow for multiplexing (Glenn et al., 2019). Libraries were prepared using Kapa mRNA Stranded Library Prep kits and standard protocols. Libraries were pooled and split across six lanes of 100-bp single-end reads on an Illumina HiSeq 4000 at the QB3 Genomics Sequencing Laboratory in Berkeley, CA.

2.4 | Trimming and mapping

Raw sequences were trimmed for adapter sequence and low-quality bases using Trimmomatic (Bolger et al., 2014). Overall quality was assessed across all samples using FASTQC (Andrews, 2010). Reads were mapped to the recently published Mexican Axolotl (*Ambystoma mexicanum*) genome (Nowoshilow et al., 2018), a close phylogenetic relative of CTS and BTS (Shaffer & McKnight, 1996, Shaffer and McCartney-Melstad, unpublished). We mapped reads to the *A. mexicanum* genome using STAR (Dobin et al., 2013), which is designed to map transcripts to a genome by efficiently accounting for gap junctions created by intronic sequences. Mapped reads were counted for differential expression analysis using HTSeq-count with exons as the target feature (Anders et al., 2015). Reads that mapped to multiple exons were discarded as ambiguous.

2.5 | Differential expression

Differential expression (DE) was calculated for muscle and brain tissue separately using the R package DESeq2 (Love et al., 2014). A recent comparison demonstrated fewer false discoveries and more consistent identification of DE genes with DESeq2 compared with similar software (Seyednasrollah et al., 2015). We used the exon count output from HTSeq since DESeq2 has an internal method for normalizing expression data based on gene-wide dispersion (Love et al., 2014). We removed transcripts with mean counts less than 10 from the analysis to reduce the number of independent tests performed. We also removed backcross individuals from subsequent expression analyses given the limited sample size of these groups. DESeq2 was then run with a model that included one factor (~groups) with six levels (CTS.heat, CTS.control, BTS.heat, BTS.control, F1.heat and F1.control). This allowed for independent contrasts between levels, allowing us to determine genes differentially expressed in response to temperature within each genotype class (CTS, BTS, F1), as well as the overall response to the temperature treatment for each tissue across the three genotypes.

2.6 | Allele-specific expression

We analysed allele-specific expression (ASE) to identify differences in BTS/CTS allelic expression in F1 hybrids. We first called variant sites following the Genome Analysis Tool Kit (GATK) best practices pipeline (Auwera et al., 2013). We removed duplicate reads using picardtools, then used the mapped reads to call individual genotypes and compared across samples to find consistent single nucleotide polymorphisms (SNPs). ASE analyses are sensitive to read mapping bias that may arise from preferential mapping of the reference allele, a problem that is particularly severe if the reference genome is more closely related to one of the genotypes in the study (Degner et al., 2009; Salavati et al., 2019; Stevenson et al., 2013). Since the axolotl reference genome is more closely related to BTS than to CTS (CTS is the outgroup to the entire tiger salamander complex; see O'Neill et al., 2013; Shaffer & McKnight, 1996), we used the software WASP to remove this potential mapping bias for downstream ASE analyses (van de Geijn et al., 2015). WASP identifies reads that map to regions with one or more SNPs present, simulates artificial reads with every possible alternate allele for that locus, remaps the simulated read to the reference genome and discards reads that do not map to the exact same location. This leaves a conservative set of mapped reads that were used in all downstream ASE analyses.

We filtered variant sites using VCFtools to find alleles that were fixed in the pure genotype samples (fixed different in CTS and BTS), filtering out all other variants. We then kept only SNPs that were also heterozygous in all F1 individuals. This process generated a conservative list of loci that are present in F1s and diagnostic between CTS and BTS. We used this filtered SNP database in the GATK tool ASEReadCounter to count the number of copies of reference and alternate alleles at each locus (Auwera et al., 2013), and assigned parental origin to each allele using the diagnostic BED file using custom scripts in R (v3.3.2; R Core Team, 2013).

We used the software package GENEIASE to identify SNPs and genes that exhibited significant allele-specific expression (Edsgärd et al., 2016). This software uses a beta-binomial distribution to compare the counts of reference and alternate SNPs at heterozygous loci against the null hypothesis that alternate allele counts (altCounts) = reference allele counts (refCounts). The software computes a test statistic (s), which is the log(odds ratio) of the counts of diagnostic alleles. This test statistic is then summarized over all SNPs within a given feature (i.e. annotated gene) using Stouffer's method to determine feature-wide allelic imbalance. This gene-wide allelic imbalance is then compared with a null distribution generated by taking *k*-samples from the beta-binomial distribution and determining the likelihood of the observed imbalance. This yields a probability that the observed allelic ratios for a given gene are equal and that observed differences in allelic contribution are originated by chance, resulting in a list of genes that exhibit significant bias regardless of experimental treatment ('static ASE'). GeneiASE was also used to identify condition-dependent ASE by comparing F1 allelic bias in the heat treatment with the median allelic counts of the control group. Both of these analyses yielded *p*-values for each gene in each individual. We adjusted *p*-values using the Benjamini–Hochberg method (FDR ≤ 0.05; Benjamini

& Hochberg, 2000) and applied a significance threshold of 0.001 to reduce the likelihood of false positives. This yielded a conservative list of genes with significant ASE in each individual.

Following Edsgärd et al. (2016), we used Fisher's method to identify which genes consistently demonstrated significant ASE across all control individuals for use in downstream gene regulation analysis. These resulting *p*-values were adjusted using the Benjamini–Hochberg false discovery correction (FDR ≤ 0.05; Benjamini & Hochberg, 2000). Although geneiASE provides a robust framework for determining genes with significant ASE, it does not report the direction of the bias. We summed the number of CTS and BTS counts for each gene and each sample identified by geneiASE, and took the $\log_2$ ratio of (BTS/CTS) to determine direction and magnitude of ASE. We used analysis of variance (ANOVA) tests to compare the number of genes with significant ASE between treatments (heat vs. control) and tissue type (brain vs. muscle). We also compared the relative magnitude of ASE for each gene between these groups using the paired *t* tests. We used linear regression to identify relationships between individuals' ASE and CTMax. All analyses were performed using custom scripts in R (v3.3.2; R Core Team, 2013).

2.7 | Regulatory mechanisms

We identified the regulatory mechanisms underlying allelic imbalance in F1 hybrids by comparing the relative expression of parental genotypes with their relative expression in hybrid individuals. We corrected for differences in library sequence depth by calculating counts per million (CPM) for each gene and for each sample. To identify genes with significant *trans*-regulatory factors, we followed the protocol of McManus et al. (2010) by comparing ASE ratios in the F1 hybrids with the ratio of BTS/CTS expression in the parental genotypes using Fisher's exact test followed by the Benjamini–Hochberg correction for multiple tests (Benjamini & Hochberg, 2000). We then attributed the following regulatory mechanisms to each gene: 'conserved', '*cis* only', '*trans* only', 'compensatory' or a combination of '*cis* and *trans*' regulation (see Figure 1, Table S1), following the general strategy of McManus et al. (2010). We determined genes that were differentially expressed between CTS and BTS using the output of DESeq2. We also determined genes in F1 offspring with unequal expression of parental alleles using the results of Fishers' method performed on the output of the static geneiASE analysis. This approach provides the opportunity to test for individual-level variation in ASE, which fits well with our sampling design (McManus et al. (2010) used library pools of multiple individuals). We further divided the category of '*cis* and *trans*' into four groups to determine whether *cis*- and *trans*- functioned in opposite or similar directions, and whether *cis*- or *trans*- had a greater effect on expression (see Figure 1, Table S2) following the protocol of Goncalves et al. (2012). All analyses were performed using custom scripts in R (v3.3.2; R Core Team, 2013).

3 | RESULTS

3.1 | Physiology and CTMax

Our use of preserved specimens for internal body temperature calibration demonstrated that linear models containing both dorsal and ventral temperatures were highly significant ($p < 2.2e{-}16$,

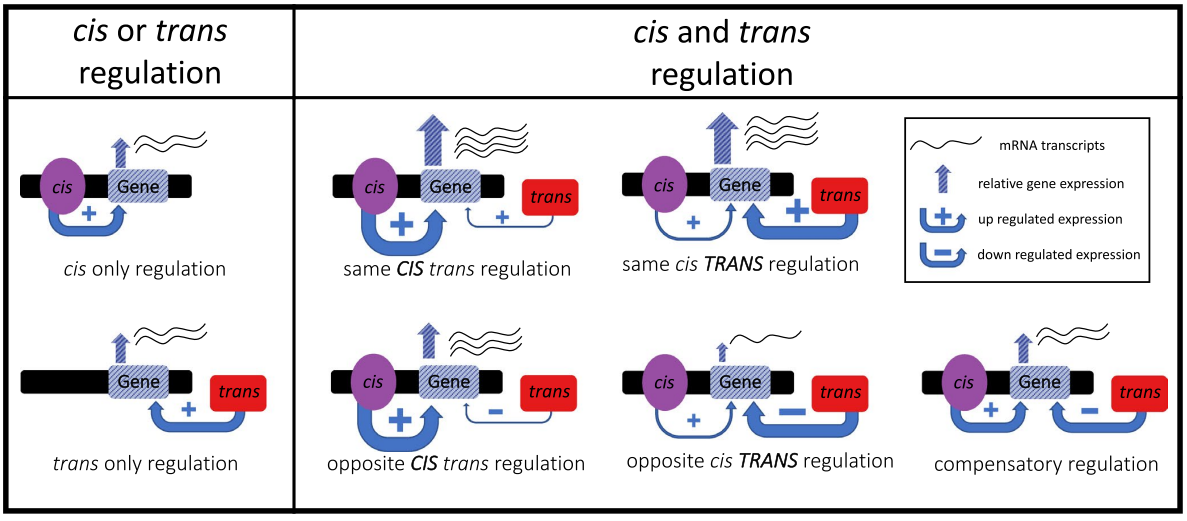

FIGURE 1 Schematic illustrating the different regulatory modes that were assigned to each gene. Blue hatched box represents a focal gene. Purple ovals are *cis*-regulatory factors, which are proximal to the gene of interest. Red squares represent *trans*-regulatory factors that occur elsewhere in the genome, typically unlinked to the focal gene. The '*cis* and *trans* regulation' group has both *cis*- and *trans*-factors acting together in either the same or opposite directions (depicted by the '+'and '–') and with different relative magnitudes (relative size of the arrows, bold text). The overall effect on expression is represented by the size of the blue hatched arrow and number of mRNA transcripts above the focal gene. For complete description of regulatory mode assignment methods, see Tables S1 and S2 [Colour figure can be viewed at wileyonlinelibrary.com]

df = 139, adj R^2 = 0.91) and that the removal of either dorsal or ventral temperature resulted in models with lower explanatory value (ΔAIC = 52.28, 23.21, respectively). We therefore estimated internal body temperatures for each individual using the equation: Internal.Temp = −5.16 + 0.37 * Dorsal.Temp + 0.75 * Ventral.Temp.

In the thermal stress experiment, we detected no difference in CTMax (Welch t test: t = 0.22, *df* = 9.00, *p* = 0.832; Figure 2) between CTS (33.77°C ± 0.39; mean ± SE, *n* = 5) and BTS (33.64°C ± 0.43, *n* = 6). Hybrids (34.24°C ± 0.53, *n* = 15) had a greater mean CTMax than the pooled parental group (33.70°C ± 0.28, *n* = 11; Welch *t* test: *t* = −2.56, *df* = 20.66, *p* = 0.018; Figure 2). F1 hybrids (35.15°C ± 0.61, *n* = 12) also had a greater mean CTMax then the pooled parental group (Welch *t* test: *t* = −2.16, *df* = 15.4, *p* = 0.046) when only F1 hybrids were analysed, and backcross individuals were removed. We detected no difference in CTMax among F1 sib groups or parental genotype (see Appendix S1).

There was also no significant difference in the variance of CTMax between CTS (SD = 0.87, *n* = 5) and BTS (SD = 1.1, *n* = 6; *F* test: *F* = 0.67, *df* = 4, *p* = 0.721). Hybrids (SD = 2.1, *n* = 15) did have greater variance in CTMax compared with the combined parental group (*F* test: *F* = 4.88, *p* = 0.008). When backcross hybrids were removed, F1 hybrids still had greater variance than the parental group (SD = 0.93, *n* = 11; *F* test: *F* = 5.11, *df* = 11, *p* = 0.016). Six of the 15 hybrids (4 F1's, 1 CTS backcross and 1 BTS backcross) displayed a greater thermal tolerance than the highest recorded value in either CTS (*N* = 5) or BTS (*N* = 6). Four F1 hybrids exhibited transgressive CTMax with respect to BTS, with an average of 2.02°C greater tolerance than the highest recorded value in BTS (35.61°C). Five F1 hybrids displayed transgressive CTMax with respect to CTS, with an average of 2.33°C greater thermal tolerance than the highest recorded value in CTS (34.80°C). Despite the increased variance, the lower end of the CTMax range did not differ between groups. Rather, the increased variance was expressed purely as greater CTMax in hybrids.

3.2 | Differential expression

Differential expression analysis using DESeq2 for muscle tissue revealed many genes that responded to acute heat stress. Pooling all genotypes for an overall temperature treatment effect revealed 177 DE genes; 106 were upregulated, and 71 downregulated. When broken down by species, CTS had 359 DE genes (245 up, 114 down),

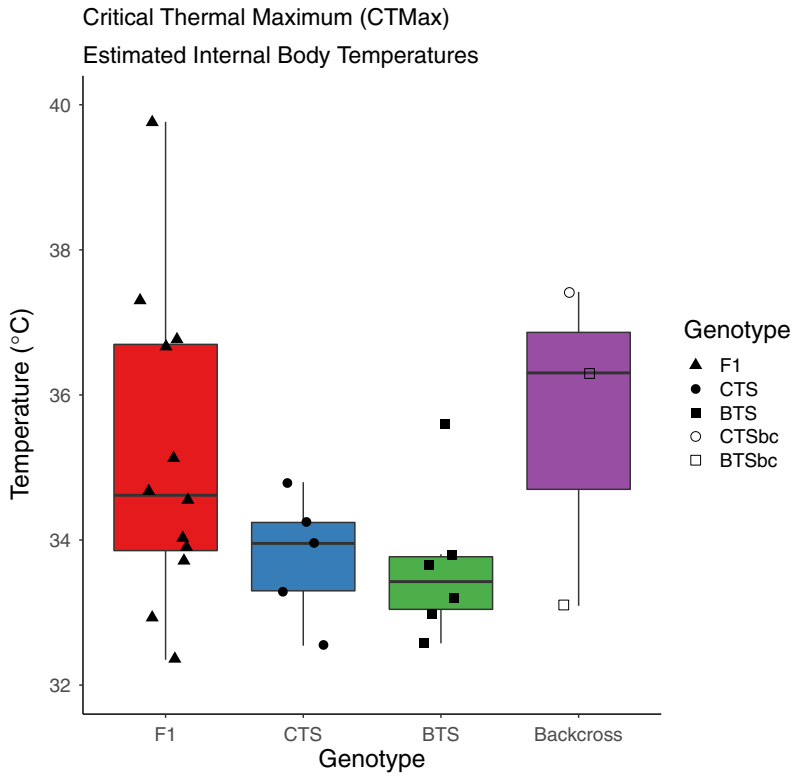

FIGURE 2 Observed values of critical thermal maximum (CTMax) by salamander genotype. Barplots display the mean (black horizontal line) and interquartile range (coloured rectangle) for each group. Internal body temperatures were calculated using a linear model including dorsal and ventral skin temperatures. CTS are pure California tiger salamanders, and BTS are pure barred tiger salamanders. F1 hybrids are first-generation crosses between CTS and BTS parents. There are 3 additional backcross individuals that are included to represent later generation crosses, though these individuals are not included in downstream analyses due to the limited sample size. These backcross individuals are the result of crosses between F1 hybrids and either a BTS ('BTSbc', *N* = 2) or CTS ('CTSbc', *N* = 1) [Colour figure can be viewed at wileyonlinelibrary.com]

BTS had 16 DE genes (8 up, 8 down), and F1 hybrids had 79 DE genes (50 up, 29 down; see Figure 3).

Differential expression in the brain tissue revealed fewer significant genes. Pooling across genotypes yielded 32 DE genes (24 up, 8 down). In CTS, there were 4 DE genes (3 up, 1 down); in BTS, 14 genes were DE (7 up, 7 down); and in hybrids, a total of 13 genes were DE (8 up, 5 down; see Figure 3).

3.3 | Allele-specific expression

GeneiASE revealed many genes with biased parental expression patterns in F1 hybrids. The median ASE across individuals was 21.0% and 11.6% of expressed genes in muscle and brain tissue, respectively. We did not detect an effect of sib group or parent genotype on the percentage of genes that exhibit significant ASE (see Appendix S1). We did identify a significant effect of sib group and parental genotype on the magnitude of ASE, though including these factors as random effects does not qualitatively change results (see Appendix S1). Expression in genes with ASE was slightly biased towards BTS parental copies in both brain ($\log_2$(BTS/CTS) =0.036; Figure 4) and muscle ($\log_2$(BTS/CTS) =0.070; Figure 4), representing a 2.5% and 4.9% increase in the overall expression of BTS gene copies. There were a greater number of genes with significant ASE in muscle than in brain (ANOVA: df = 43, F = 26.4, p = 6.4e^{-6}), and the magnitude of ASE was also greater in the muscle than in the brain (paired t test by gene: df = 1305, t = 3.27, p = 1.1e^{-3}).

We did not detect any significant difference in the percentage of genes with ASE between hybrids in heat and control treatments in either the muscle (ANOVA: df = 21, F = 3.44, p = 0.08), or the brain (ANOVA: df = 20, F = 3.40, p = 0.08). The magnitude of BTS-biased ASE was greater in the control group ($\log_2$ (BTS/CTS) = 0.08) than in the heat treatment ($\log_2$ (BTS/CTS) = 0.06) in muscle tissue (paired t test by gene: df = 3215, p = 5.21e^{-5}). The opposite pattern held for brain where BTS-biased expression was greater in the heat-stressed group ($\log_2$ (BTS/CTS) = 0.04) than in the control group ($\log_2$ (BTS/CTS) = 0.03), although this difference was not significant (paired t test by gene: df = 2659, t = −0.98, p = 0.33).

There was no significant relationship between per cent ASE and CTMax in muscle (linear regression: df = 10, F = 0.17, p = 0.69), or brain (linear regression: df = 8, F = 4.46e^{-7}, p = 0.99). Similarly, there was no significant difference in the magnitude of ASE bias with respect to CTMax in muscle (linear regression: df = 10, F = 0.17, p = 0.69), or brain (linear regression: df = 9, F = 0.57, p = 0.47) tissue.

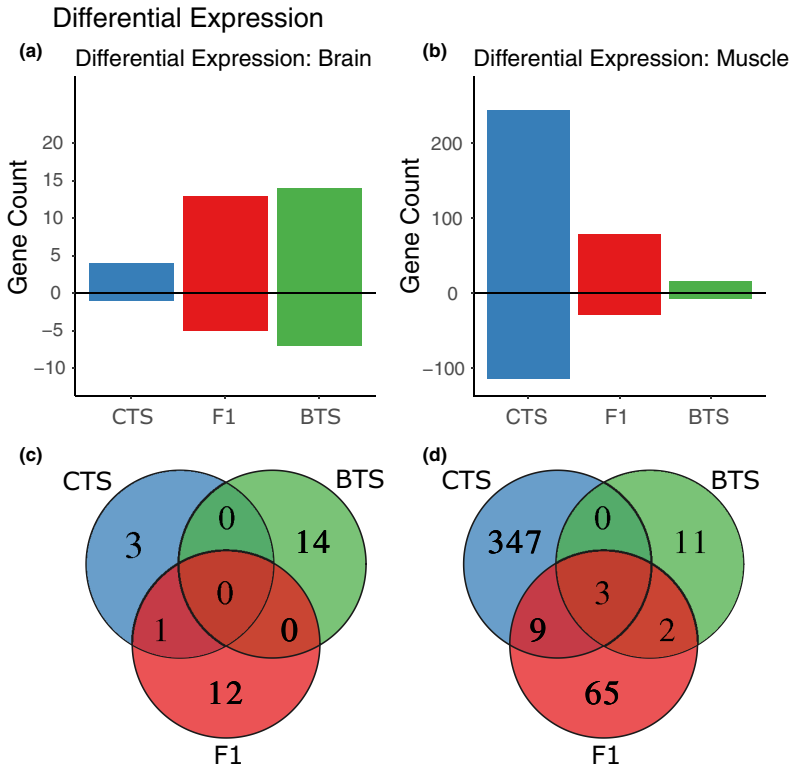

FIGURE 3 Number of genes differentially expressed in response to acute thermal stress. Panels a and b depict the number of genes upregulated (positive) and downregulated (negative) in brain (3a) and muscle tissue (3b) for each genotype. Figure c (brain) and d (muscle) depicts the number of genes that were differentially expressed (DE) in response to temperature stress. Values included in overlapping circles indicate genes that were DE in all overlapping groups [Colour figure can be viewed at wileyonlinelibrary.com]

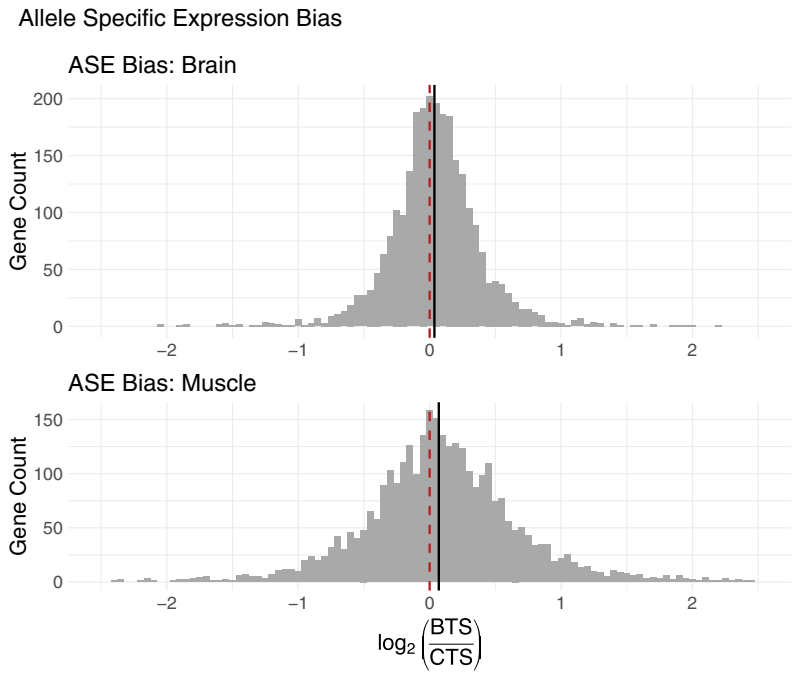

FIGURE 4 Histograms of the direction and magnitude of allele-specific expression (ASE) across all loci that are diagnostic between BTS and CTS for brain (top) and muscle (bottom) tissue. Median $\log_2$ ratios of BTS/CTS allelic expression were taken for each gene in all F1 individuals. Red dashed line at zero ($x = 0$) represents equal expression of BTS and CTS alleles. Black line depicts the observed median of all ASE bias across genes in the brain ($\log_2$(BTS/CTS) = 0.036) and the muscle ($\log_2$(BTS/CTS) = 0.070), representing a 2.5% and 4.9% increase in the overall expression of BTS gene copies. [Colour figure can be viewed at wileyonlinelibrary.com]

The median percentage of genes that exhibited an increase in ASE with response to the heat treatment (condition-dependent) were 0.9% and 0.3% for muscle and brain tissue, respectively. There was significantly more condition-dependent ASE in muscle than in brain (ANOVA: $df = 45$, $F = 48.88$, $p = 1.05e^{-8}$). We found a strong positive correlation between per cent condition-dependent ASE and CTMax in muscle (linear regression: $df = 10$, $F = 10.05$, $p = 0.01$; Figure 5b), which explains a reasonably large fraction of the variance in CTMax ($R^2 = 0.45$, $N = 12$). However, there was no significant relationship between condition-dependent ASE and CTMax in the brain (linear regression: $df = 9$, $F = 0.57$, $p = 0.47$).

3.4 | Regulatory mechanisms

Gene expression in muscle tissue had greater *cis*-only (26.7%) than *trans*-only (2.2%) regulatory differences. Muscle tissue also exhibited a great deal of combined *cis*- and *trans*- divergence (71.1%), which was dominated by compensatory mutations (82.0%). Of the genes with both *cis*- and *trans*-regulatory divergence, most functioned in opposing (16.0%) rather than complimentary directions (2.0%). We found a similar pattern in brain tissue, where there were more *cis*-only (14.3%) than *trans*-only (5.2%) differences in regulation. Additionally, there was a larger number of genes that exhibited a combination of *cis*- and *trans*-regulatory differences (80.5%), again dominated by compensatory regulation (91.2%). In brain tissue, genes regulated by both *cis*- and *trans*-factors predominantly functioned in opposing directions (7.5%) compared with those acting in the same direction (1.3%) (see Table 1 and Figure 6).

4 | DISCUSSION

4.1 | Physiological response (CTMax)

We did not detect a difference in CTMax between CTS (33.77°C ± 0.39) and BTS (33.64°C ± 0.43). This equivalency may result from the physiological constraints that high temperatures impose (Huey & Bennett, 1987; Huey et al., 2012; Lutterschmidt, 1997; Markle, 2015; Youssef et al., 2008). A recent meta-analysis concluded that critical thermal limits are often constrained compared with other physiological tolerances at a global scale (Sunday et al., 2011). However, thermodynamic models have demonstrated that conserved phenotypes, such as thermal tolerance, may mask significant variation in their underlying genetic mechanisms (López-Maury et al., 2008). These mechanistic differences that evolve in isolation may result in unique, but roughly equivalent, strategies for tolerating stressful temperatures. If such independent lineages come back into contact and hybridize, their unique mechanisms can combine to

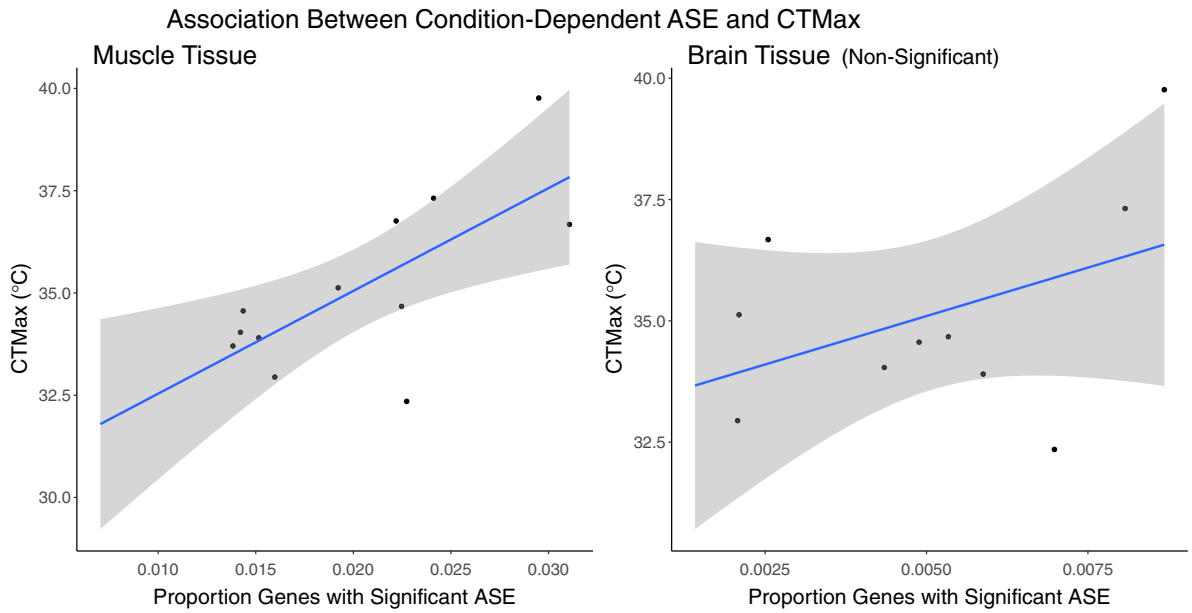

FIGURE 5 Correlation between each F1 individual's condition-dependent allele-specific expression (ASE) and thermal tolerance. Condition-dependent ASE consists of genes that experience a shift in their allelic imbalance when exposed to acute thermal stress. The left figure shows the positive trend between an F1 individual's degree of temperature-dependent ASE bias in muscle tissue and its ability to tolerate acute thermal stress. The right figure shows a similar trend (although non-significant) between F1 individuals' degree of temperature-dependent ASE and thermal tolerance in the brain tissue [Colour figure can be viewed at wileyonlinelibrary.com]

produce phenotypes that exceed either parental species (ex: Alter et al., 2017; Bidani et al., 2007; Perry et al., 2001).

F1 hybrid tiger salamanders had a greater mean thermal tolerance (35.15°C ± 0.61) than the combined parental group (33.69°C ± 0.29). Furthermore, we observed four (out of 12 total) F1-hybrid individuals that maintained full functionality at temperatures greater than the maximum observed in a roughly equal sample of CTS and BTS combined, suggesting that 33% of individuals exhibited transgressive thermal tolerance capacity. This 2.02°C mean increase in thermal tolerance of the four transgressive F1 hybrids is biologically meaningful given the hot arid conditions that these salamanders endure in breeding ponds and during summer aestivation. This increased tolerance may play an important role in the differential survival of hybrid salamanders that has been well documented in the wild and that will almost certainly intensify given current projections of climate change in CTS habitat (Searcy & Shaffer, 2016). Monterey County, which contains the majority of the CTS×BTS hybrid swarm, is expected to experience an overall increase in annual average maximum air temperature of 6.3°C from 2020 to 2100 under the RCP 8.5 scenario (CanESM2 model; Pierce et al., 2018). Given this projection, hybrid salamanders that possess enhanced thermal tolerance may achieve even greater fitness advantages, threatening the persistence of native CTS.

Our results complement previous studies that also document superior CTS x BTS hybrid phenotypes. Johnson et al. (2010) found that F1 hybrids had increased locomotor performance compared with either CTS or BTS and that endurance was heavily influenced by temperature. Those authors were unable to detect a significant interaction between genotype and temperature, suggesting that their effects on locomotor performance were roughly equivalent. However, in the light of our present results, variation in performance among F1 hybrid individuals may have been present, but not identified, in their work. Combining the results of these two studies, it appears that F1 hybrids may enjoy increased dispersal abilities since they can tolerate greater temperatures and have increased mobility at these elevated temperatures.

4.2 | Differential gene expression

Differential expression (DE) analysis revealed substantial differences in gene expression between CTS and BTS in response to acute thermal stress. In muscle tissue, we observed a greater expressional response to heat stress in CTS (359 genes) than in BTS (16 genes). The reverse is true in the brain tissue, although the overall response level was an order of magnitude lower (CTS had only 4 DE genes, and BTS had 14). Additionally, we found an inverted pattern across tissue types, where CTS exhibited a greater expression response in muscle, while BTS showed a greater response in brain. Previous studies have also used patterns of gene expression to identify alternative mechanisms and pathways that species use to survive thermal stress. In non-model species, the majority of these experiments have focused on marine systems (see, for example, Marine snail: Gleason & Burton, 2015; Fishes: Logan & Buckley, 2015; Abalone: Shiel et al.,

TABLE 1 Regulatory mechanisms

	Brain		Muscle	
	Counts	**Percent**	**Counts**	**Percent**
All Genes Considered	7310	100.0%	4130	100.0%
Conserved	3811	52.1%	676	16.4%
Ambiguous	1519	20.8%	882	21.4%
Differential Regulation	1980	27.1%	2572	62.3%
Genes with Differential Regulation	1980	100.0%	2572	100.0%
cis.only	284	14.3%	687	26.7%
trans.only	102	5.2%	57	2.2%
cis.and.trans	1594	80.5%	1828	71.1%
opposite.**CIS**.trans	90	5.6%	181	9.9%
opposite.cis.**TRANS**	30	1.9%	112	6.1%
same.**CIS**.trans	14	0.9%	18	1.0%
same.cis.**TRANS**	7	0.4%	18	1.0%
compensatory	1453	91.2%	1499	82.0%

Counts and percentage of genes that demonstrate significant regulatory divergence between BTS and CTS. Genes with significant differential regulation are broken down into three groups: genes with only *-cis*, only *-trans* and genes with a combination of *-cis* and *-trans* regulation. This group of *-cis* and *-trans* is further divided by the direction and magnitude of the change in expression. Regulatory divergence may operate in the 'opposite' or 'same' direction, and the mechanism with the greatest effect on expression is displayed in bold. See Figure 1 for further explanation of these categories. A more detailed explanation of these assignments is given in the supplemental material (Table S1 and S2).

2015; Trout: Tan et al., 2016; Salmon: Tomalty et al., 2015), although terrestrial species have also recently received some attention (lizards: Campbell-Staton et al., 2020; chicken: Srikanth et al., 2019). These studies have identified genes that are differentially expressed in response to heat stress and genes that are uniquely expressed in species or populations with improved heat tolerance. Together, they demonstrate that species often differ in the mechanisms underlying thermal tolerance. Our work contributes an amphibian system to this body of work and emphasizes that differences in tissue-specific gene expression can and do evolve even among closely related congeners.

4.3 | Transgression and variation in hybrids

Hybrid salamanders exhibited a greater range of thermal tolerances than the combined parental group. The variation in thermal tolerance of F1 hybrids underscores the complexity of mechanisms driving this apparent heterosis. Variation in both CTMax and patterns of ASE among F1 hybrids may be the result of variation in the parental lines. Although we were unable to detect a significant effect of sib group or parental genotype on CTMax or per cent ASE, we did observe an effect on the magnitude of ASE in both the muscle and brain. However, the inclusion of these factors as random effects in did not change any of the qualitative results reported here. It is possible that this family effect does represent an important component of genetic variation, but our limited sample size (three sibling groups) means that we do not have the statistical power to examine it in any meaningful detail. Both variation in segregating loci within species and maternal effects (e.g. Chan et al., 2020) are important targets for future studies using targeted and replicated hybrid crosses.

The F1 hybrids in our study exhibited relatively high levels of static ASE, with 21.0% of genes in the muscle and 11.6% in the brain demonstrating biased expression. These values are higher than ASE levels found in studies on intraspecific crosses (Edsgärd et al., 2016; Kang et al., 2016), but are comparable to other studies on interspecific hybridization (Keane et al., 2011; Zhang & Borevitz, 2009), and hybridization between inbred lines (Zhuo et al., 2017). This highlights the relatively high level of regulatory evolution that has occurred between CTS and BTS since the species diverged 3–5 Ma (Shaffer et al., 2004). In addition to static ASE, we also examined ASE that changed in response to thermal stress. We found that 1.4% of genes in muscle and 0.3% in brain altered their expression of parental gene copies in response to thermal stress, suggesting that there may be a mechanistic link between the CTMax phenotype and altered allelic imbalance in response to temperature stress.

We further investigated the relationship between ASE and thermal tolerance by modelling CTMax as a function of each individual's degree of ASE. We found similar positive correlations between temperature-dependent ASE and CTMax in muscle and brain tissues (Figure 5), though the relationship is only significant for muscle. This may support the intriguing idea that thermal tolerance is affected by an individual's degree of bias in allelic expression. We also observed more overall condition-dependent ASE in the muscle compared with the brain, which may underscore the importance of gene expression in this tissue for tolerating acute thermal stress in these warm-temperature-adapted amphibians. Interactions between allele-specific expression and phenotypic traits have been documented in other species as well, though the causal relationship remains poorly understood (Aguilar-Rangel et al., 2017; Cotroneo et al., 2006; He et al., 2006; Keane et al., 2011). Given our current study design, we cannot demonstrate a causal relationship between allelic imbalance and CTMax. However, our results provide an interesting correlation between two complex phenotypes, which warrant further investigation. In particular, future studies could leverage eQTL (expression quantitative trait loci) experiments to identify specific genes that may afford greater thermal tolerance in hybrids. This would help establish the mechanistic basis, should it exist, between ASE and the CTMax phenotype. Regardless, these patterns of ASE demonstrate unbalanced regulation of parental gene copies in F1 hybrids. Given this result, we examined the *cis*- and *trans*-mechanisms

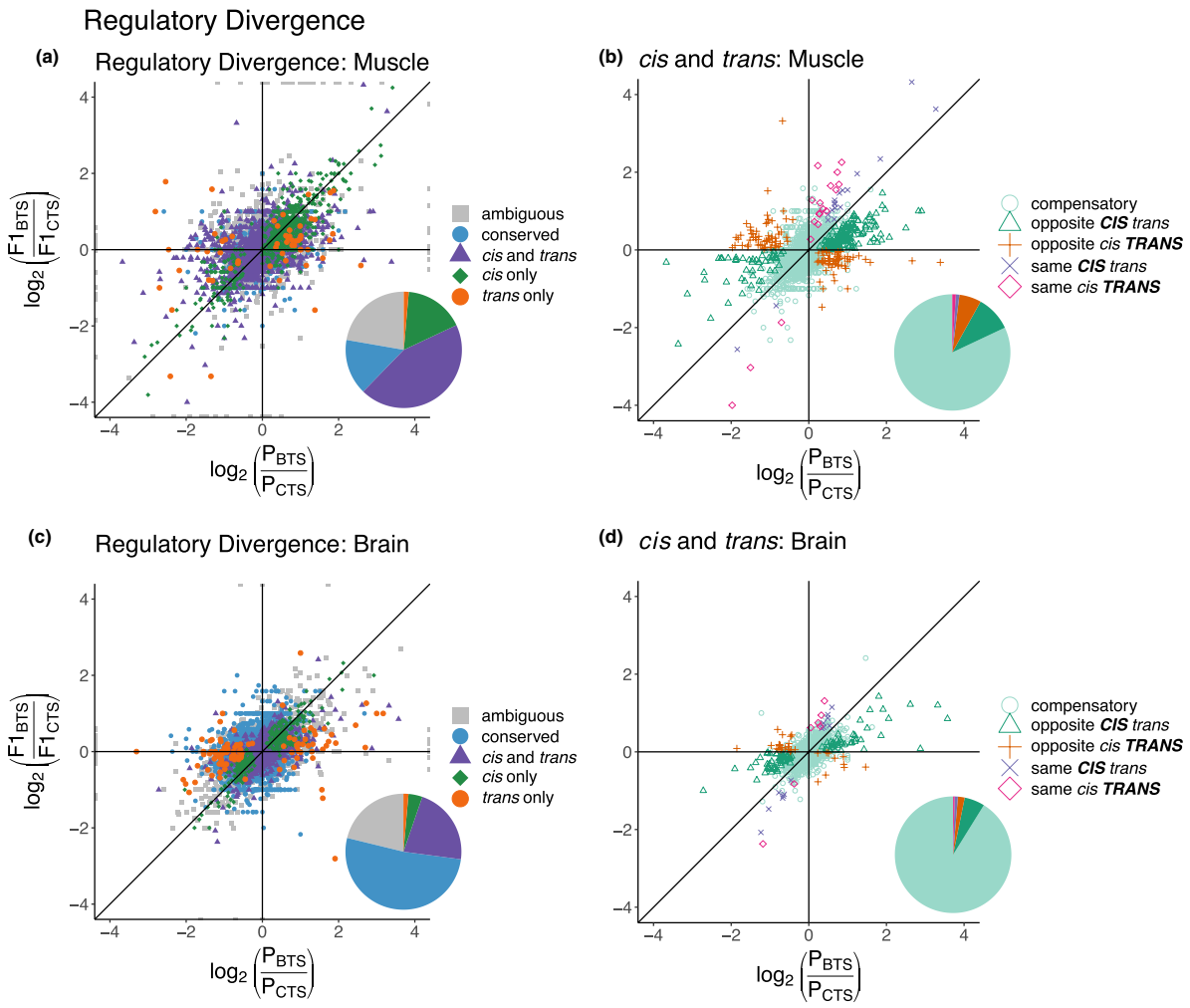

FIGURE 6 Scatter plots showing the relative expression of CTS and BTS genes in pure parental species and of parent-specific gene copies in F1 hybrids. Vertical axis is the $\log_2$ ratio of BTS/CTS-derived gene copies that are simultaneously expressed in F1 hybrids, averaged across all individuals. Horizontal axis is the $\log_2$ ratio of BTS/CTS expression of a specific gene averaged across pure parental individuals. Comparison of these two expression ratios enables us to assign modes of regulation to each gene. Figure 6a and 6c identifies genes that are governed by *cis*-, *trans*- or a combination of the two ('*cis* and *trans*'). Figure 6b and 6d expands on the category '*cis* and *trans*', identifying genes that are more influenced by *cis*- or *trans*-regulation (indicated in bold), and genes where *cis*-regulation and *trans*-regulation act in the 'same' or 'opposite' directions. See Figure 1 and Tables S1 and S2 for further description of the regulatory mode assignment. [Colour figure can be viewed at wileyonlinelibrary.com]

that regulate gene expression to better understand the patterns of ASE in hybrid salamanders.

4.4 | Genomic mechanisms

Analysis of allele-specific expression (ASE) revealed extensive evolutionary divergence in the regulation of gene expression between these two closely related salamander species. Comparing the level of expression of each gene in CTS and BTS with the two parental copies of that gene in F1 hybrids allows us to infer changes in the regulatory mechanisms that govern the expression of that gene between the two parental lineages. Gene expression in both muscle and brain tissue demonstrated more changes in *cis*- than *trans*-regulation. This enrichment of *cis*-regulatory factors is in line with previous model system studies in *Arabidopsis* (Cubillos et al., 2014; Gan et al., 2011), yeast (Kita et al., 2017), and mouse (Goncalves et al., 2012). Studies on variation in human gene regulation confirmed that most allele-specific variation is dominated by *cis*-regulatory elements located near the gene of interest (Pickrell et al., 2010; Tehranchi et al., 2019). This modification of gene expression through *cis*-mutations is thought to play a significant role in the evolution of complex phenotypes (Wray, 2007). In particular, *cis*-regulatory evolution may enable species to 'fine-tune'

their physiological response to dynamic processes, such as thermal stress, with more precision than by relying on coding sequence mutations. A study on *Arabidopsis* identified an excess of *cis*-regulatory mutations that have conferred adaptations to stressful conditions, including cold stress and dehydration (He et al., 2016). Similarly, a recent review highlighted the role of *cis*-regulatory evolution in plants that are adapted to a range of stressful environmental conditions (Jain et al., 2018). Together, this literature demonstrates the importance of *cis*-regulatory evolution in adaptation to physiologically stressful conditions. The present study also identifies extensive *cis*-regulatory differences between CTS and BTS, which likely underscores a similar divergence in expression response to acute thermal stress during allopatric evolution.

Transgressive hybrid gene expression and associated phenotypes may result from extensive compensatory regulation. Compensatory regulation was detected at high levels in both brain (91.2%) and muscle (82.0%) tissues. These are comprised of genes that exhibit significant ASE in hybrids, yet similar overall expression between CTS and BTS. Additionally, regulation where *cis*- and *trans*- were both detected but functioned in opposite directions (another form of incomplete compensation) was detected in both brain (7.5%) and muscle (16.0%). Both of these regulatory mechanisms can lead to expression patterns in hybrids that exceed the range of either parent. This phenomenon has been documented in many model species including *Drosophila* (Michalak & Noor, 2003; Ranz et al., 2004), *Arabidopsis* (Comai et al., 2003; Wang et al., 2004; Yang et al., 2017) and maize (Auger et al., 2005), all of which show transgressive gene expression patterns in hybrids. This process is thought to occur when mutations in *trans*-acting elements have not only a net benefit to an organism (and therefore are selected for) but also a detrimental, pleiotropic effect via other coregulated genes. These negative effects are then reduced through subsequent mutations (likely *cis*-) that shift the expression of these other genes back to their original optimum (reviewed in: Signor & Nuzhdin, 2018). This mechanism maintains the benefit from the original *trans*-mutation, while reducing its negative pleiotropic effects. This may explain why genes exhibit equivalent levels of expression in CTS and BTS, yet have different values in the F1 hybrids. If the expression-level traits that we document here are heritable, then selection on increased CTMax may lead to hybrid salamanders with enhanced thermal tolerances in the wild. Although no studies specifically examine salamander systems, two studies on humans found that 59% of surveyed genes have heritable expression patterns (Wheeler et al., 2016) and that 15% of the variation in gene expression is heritable across multiple tissue types (Price et al., 2011). Furthermore, a recent study in sea turtles found that the variability in heat-shock protein expression was heritable, enabling selection to act on this trait and potentially increase thermal tolerance in the population (Tedeschi et al., 2016). Although these studies are not directly comparable to tiger salamanders, they do establish a mechanism of heritability in gene expression that may function in diverse taxa. Future work examining the CTMax of wild hybrid salamanders that have undergone multiple generations of selection is needed to establish whether this pattern of increased thermal tolerance, or at least variation in CTMax, exists after several dozen generations of selection in nature.

4.5 | Climate change and hybrid persistence

These results suggest that hybrids may be more capable than native CTS of adapting to future climate change. This may have implications for management, depending on the viability of native CTS in the face of warming temperatures. If native CTS populations decline due to temperature stress, then hybrids may become the only viable option for tiger salamander persistence in the hotter regions of the species' range. Despite the disruptive effect of hybrids, extirpation of salamanders from these vernal pool ecosystems has an even greater negative impact (Searcy et al., 2016). It is therefore not advisable to eradicate hybrid salamanders if there are no native CTS left to occupy those habitats. Given this, it may be reasonable to protect hybrids, while simultaneously attempting to restore breeding pool environments to more natural, vernal pool conditions that select for predominantly native traits (e.g. Fitzpatrick & Shaffer, 2007b; Wayne & Shaffer, 2016). This is particularly relevant given recent predictions of large shifts in temperature-dependent habitat suitability by 2070 throughout the CTS range (Searcy & Shaffer, 2016). This study predicts that the only suitable habitat for CTS will be in the central California coastal region where the current hybrid zone is well established and expanding (Searcy & Shaffer, 2016). If increased thermal tolerance is indeed evolving in the hybrid zone and continues to do so as climate warms, then hybrid genotypes may comprise a kind of genetic rescue for CTS as the only viable solution to keep their basic ecological role intact in their native range. If so, it may be time to consider protection for thermally tolerant hybrids as the best option to retain ecologically similar, but not identical, CTS on their remaining natural landscapes.

5 | CONCLUSION

Our study demonstrates how an apparently conserved phenotype shared between two closely related species may conceal mechanistic differences that have accumulated since the species diverged from a common ancestor. New genomic tools, combined with the increased availability of reference genomes for even the most recalcitrant non-model systems such as salamanders, are enabling analyses into complex regulatory mechanisms in species that can be of both eco-evolutionary interest and conservation concern. We have identified thermal tolerance as a potential factor influencing hybrid tiger salamander success. We found substantial variation in hybrid CTMax, with many individuals tolerating hotter temperatures than either parental species, coupled with differential gene expression between CTS, BTS and hybrids suggesting mechanistic differences in response to thermal stress. This apparent transgressive thermal tolerance may be key to the increased fitness that hybrid salamanders enjoy in nature (Fitzpatrick et al., 2009).

ACKNOWLEDGEMENTS

We thank Drs. C. Searcy, R. Voss and J. Smith for assistance with methods and analyses. We thank the Shaffer and Grether Labs for insightful comments. We thank Dr. E. Toffelmier for assistance in the laboratory and with the manuscript, and the La Kretz Center for California Conservation and the Ecology and Evolutionary Biology Department at UCLA, the Natural Communities Coalition, and the University of California Conservation Genomics Consortium (CA-16-376437) and NSF (DEB# 0516475) for partial funding.

CONFLICT OF INTEREST

We have no conflict of interest to report.

AUTHOR CONTRIBUTIONS

R.D. Cooper designed and performed experiment, analysed data and wrote the manuscript. H.B Shaffer contributed to the project design and analysis, and manuscript editing.

DATA AVAILABILITY STATEMENT

All trimmed RNA sequences along with generated count and meta data have been deposited in the NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series Accession no. GSE137607 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE137607) (Cooper and Shaffer, 2019).

ORCID

Robert D. Cooper https://orcid.org/0000-0003-0194-329X
H. Bradley Shaffer https://orcid.org/0000-0002-5795-9242

REFERENCES

Aguilar-Rangel, M. R., Montes, R. A. C., González-Segovia, E., Ross-Ibarra, J., Simpson, J. K., & Sawers, R. J. H. (2017). Allele specific expression analysis identifies regulatory variation associated with stress-related genes in the Mexican highland maize landrace Palomero Toluqueño. *PeerJ*, *5*, e3737. https://doi.org/10.7717/peerj.3737

Allendorf, F. W., Leary, R. F., Spruell, P., & Wenburg, J. K. (2001). The problems with hybrids: Setting conservation guidelines. *Trends in Ecology & Evolution*, *16*(11), 613–622. https://doi.org/10.1016/S0169-5347(01)02290-X

Alter, K., Andrewartha, S. J., Morash, A. J., Clark, T. D., Hellicar, A. D., León, R. I., & Elliott, N. G. (2017). Hybrid abalone are more robust to multi-stressor environments than pure parental species. *Aquaculture*, *478*, 25–34. https://doi.org/10.1016/j.aquaculture.2017.04.035

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2), 166–169. https://doi.org/10.1093/bioinformatics/btu638

Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*.

Araújo, M. B., Ferri-Yáñez, F., Bozinovic, F., Marquet, P. A., Valladares, F., & Chown, S. L. (2013). Heat freezes niche evolution. *Ecology Letters*, *16*(9), 1206–1219. https://doi.org/10.1111/ele.12155

Auger, D. L., Gray, A. D., Ream, T. S., Kato, A., Coe, E. H., & Birchler, J. A. (2005). Nonadditive gene expression in diploid and triploid hybrids of maize. *Genetics*, *169*(1), 389–397.

Auwera, G. A., Carneiro, M. O., Hartl, C., Poplin, R., del Angel, G., Levy-Moonshine, A., Jordan, T., Shakir, K., Roazen, D., Thibault, J., Banks, E., Garimella, K. V., Altshuler, D., Gabriel, S., & DePristo, M. A. (2013). From FastQ data to high-confidence variant calls: The genome analysis toolkit best practices pipeline. *Current Protocols in Bioinformatics*, *43*(1110), 1–33.

Bellard, C., Cassey, P., & Blackburn, T. M. (2016). Alien species as a driver of recent extinctions. *Biology Letters*, *12*(2), 20150623. https://doi.org/10.1098/rsbl.2015.0623

Benjamini, Y., & Hochberg, Y. (2000). On the adaptive control of the false discovery rate in multiple testing with independent statistics. *Journal of Educational and Behavioral Statistics*, *25*(1), 60–83.

Bidani, A., Nouri-Ellouz, O., Lakhoua, L., Sihachakr, D., Cheniclet, C., Mahjoub, A., Drira, N., & Gargouri-Bouzid, R. (2007). Interspecific potato somatic hybrids between *Solanum berthaultii* and *Solanum tuberosum* L. showed recombinant plastome and improved tolerance to salinity. *Plant Cell, Tissue and Organ Culture*, *91*(3), 179–189. https://doi.org/10.1007/s11240-007-9284-6

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *btu170*, https://doi.org/10.1093/bioinformatics/btu170

Burke, E. M., & Pough, F. H. (1976). The role of fatigue in temperature resistance of salamanders. *Journal of Thermal Biology*, *1*(3), 163–167. https://doi.org/10.1016/0306-4565(76)90008-5

California Department of Fish Game (2010). A status review of the California tiger salamander (*Ambystoma californiense*). In *Report to the Fish and Game Commission, State of California. Nongame Wildlife Program Report*, *4*.

Campbell-Staton, S. C., Winchell, K. M., Rochette, N. C., Fredette, J., Maayan, I., Schweizer, R. M., & Catchen, J. (2020). Parallel selection on thermal physiology facilitates repeated adaptation of city lizards to urban heat islands. *Nature Ecology & Evolution*, *4*(4), 652–658. https://doi.org/10.1038/s41559-020-1131-8

Carretero, M. A. (2012). Measuring body temperatures in small lacertids: Infrared vs. contact thermometers. *Basic and Applied Herpetology*, *26*, 99–105.

Chahota, R. K., Kishore, N., Dhiman, K. C., Sharma, T. R., & Sharma, S. K. (2007). Predicting transgressive segregants in early generation using single seed descent method-derived micro-macrosperma genepool of lentil (Lens culinaris Medikus). *Euphytica*, *156*(3), 305–310. https://doi.org/10.1007/s10681-007-9359-9

Chan, W. Y., Chung, J., Peplow, L. M., Hoffmann, A. A., & van Oppen, M. J. H. (2020). Maternal effects in gene expression of interspecific coral hybrids. *Molecular Ecology*, 1–11. https://doi.org/10.1111/mec.15727

Comai, L., Madlung, A., Josefsson, C., & Tyagi, A. (2003). Do the different parental 'heteromes' cause genomic shock in newly formed allopolyploids? *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *358*(1434), 1149–1155.

Comings, D. E., & MacMurray, J. P. (2000). Molecular Heterosis: A Review. *Molecular Genetics and Metabolism*, *71*(1), 19–31. https://doi.org/10.1006/mgme.2000.3015

Cooper, R. D., & Shaffer, B. H. (2019). Allele specific expression and gene regulation explain transgressive thermal tolerance in non-native hybrids of the endangered California tiger salamander (*Ambystoma californiense*). *NCBI Gene Expression Omnibus, GSE137607*. Retrieved from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE137607

Cotroneo, M. S., Merry, G. M., Haag, J. D., Lan, H., Shepel, L. A., & Gould, M. N. (2006). The Mcs7 quantitative trait locus is associated with an increased susceptibility to mammary cancer in congenic rats and an allele-specific imbalance. *Oncogene*, *25*(36), 5011. https://doi.org/10.1038/sj.onc.1209506

Cubillos, F. A., Stegle, O., Grondin, C., Canut, M., Tisné, S., Gy, I., & Loudet, O. (2014). Extensive *cis* -regulatory variation robust to environmental perturbation in *Arabidopsis*. *The Plant Cell Online*, *26*(11), 4298–4310. https://doi.org/10.1105/tpc.114.130310

Degner, J. F., Marioni, J. C., Pai, A. A., Pickrell, J. K., Nkadori, E., Gilad, Y., & Pritchard, J. K. (2009). Effect of read-mapping biases on

detecting allele-specific expression from RNA-sequencing data. *Bioinformatics*, *25*(24), 3207–3212. https://doi.org/10.1093/bioinformatics/btp579

Delmas, V., Baudry, E., Girondot, M., & Prevot-julliard, A.-C. (2007). The righting response as a fitness index in freshwater turtles. *Biological Journal of the Linnean Society*, *91*(1), 99–109.

Dittrich-Reed, D. R., & Fitzpatrick, B. M. (2013). Transgressive hybrids as hopeful monsters. *Evolutionary Biology*, *40*(2), 310–315. https://doi.org/10.1007/s11692-012-9209-0

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Edgar, R., Domrachev, M., & Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research*, *30*(1), 207–210. https://doi.org/10.1093/nar/30.1.207

Edsgärd, D., Iglesias, M. J., Reilly, S.-J., Hamsten, A., Tornvall, P., Odeberg, J., & Emanuelsson, O. (2016). GeneiASE: Detection of condition-dependent and static allele-specific expression from RNA-seq data without haplotype information. *Scientific Reports*, *6*, 21134. https://doi.org/10.1038/srep21134.

Ellstrand, N. C., & Rieseberg, L. H. (2016). When gene flow really matters: Gene flow in applied evolutionary biology. *Evolutionary Applications*, *9*(7), 833–836. https://doi.org/10.1111/eva.12402

Fitzpatrick, B. M., Johnson, J. R., Kump, D. K., Shaffer, H. B., Smith, J. J., & Voss, S. R. (2009). Rapid fixation of non-native alleles revealed by genome-wide SNP analysis of hybrid tiger salamanders. *BMC Evolutionary Biology*, *9*, 176. https://doi.org/10.1186/1471-2148-9-176

Fitzpatrick, B. M., & Shaffer, H. B. (2007a). Introduction history and habitat variation explain the landscape genetics of hybrid tiger salamanders. *Ecological Applications*, *17*(2), 598–608. https://doi.org/10.1890/06-0369

Fitzpatrick, B. M., & Shaffer, H. B. (2007b). Hybrid vigor between native and introduced salamanders raises new challenges for conservation. *Proceedings of the National Academy of Sciences*, *104*(40), 15793–15798. https://doi.org/10.1073/pnas.0704791104

Fu, D., Xiao, M., Hayward, A., Fu, Y., Liu, G., Jiang, G., & Zhang, H. (2014). Utilization of crop heterosis: A review. *Euphytica*, *197*(2), 161–173. https://doi.org/10.1007/s10681-014-1103-7

Fujimoto, R., Taylor, J. M., Shirasawa, S., Peacock, W. J., & Dennis, E. S. (2012). Heterosis of Arabidopsis hybrids between C24 and Col is associated with increased photosynthesis capacity. *Proceedings of the National Academy of Sciences*, *109*(18), 7109–7114. https://doi.org/10.1073/pnas.1204464109

Gan, X., Stegle, O., Behr, J., Steffen, J. G., Drewe, P., Hildebrand, K. L., Lyngsoe, R., Schultheiss, S. J., Osborne, E. J., Sreedharan, V. T., Kahles, A., Bohnert, R., Jean, G., Derwent, P., Kersey, P., Belfield, E. J., Harberd, N. P., Kemen, E., Toomajian, C., … Mott, R. (2011). Multiple reference genomes and transcriptomes for Arabidopsis thaliana. *Nature*, *477*(7365), 419–423. https://doi.org/10.1038/nature10414

Gleason, L. U., & Burton, R. S. (2015). RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*. *Molecular Ecology*, *24*(3), 610–627. https://doi.org/10.1111/mec.13047

Glenn, T. C., Nilsen, R., Kieran, T. J., Finger, J. W., Pierson, T. W., Bentley, K. E., Hoffberg, S., Louha, S., Garcia-De-Leon, F. J., del Portilla, M. A. R., Reed, K., Anderson, J. L., Meece, J. K., Aggery, S., Rekaya, R., Alabady, M., Belanger, M., Winker, K., & Faircloth, B. C. (2019). Adapterama I: Universal stubs and primers for thousands of dual-indexed Illumina libraries (iTru & iNext). *PeerJ*, *7*, 049114. https://doi.org/10.1101/049114

Gómez, J. M., González-Megías, A., Lorite, J., Abdelaziz, M., & Perfectti, F. (2015). The silent extinction: Climate change and the potential hybridization-mediated extinction of endemic high-mountain plants. *Biodiversity and Conservation*, *24*(8), 1843–1857. https://doi.org/10.1007/s10531-015-0909-5

Goncalves, A., Leigh-Brown, S., Thybert, D., Stefflova, K., Turro, E., Flicek, P., Brazma, A., Odom, D. T., & Marioni, J. C. (2012). Extensive compensatory cis-trans regulation in the evolution of mouse gene expression. *Genome Research*, *22*(12), 2376–2384. https://doi.org/10.1101/gr.142281.112

Groszmann, M., Greaves, I. K., Albertyn, Z. I., Scofield, G. N., Peacock, W. J., & Dennis, E. S. (2011). Changes in 24-nt siRNA levels in Arabidopsis hybrids suggest an epigenetic contribution to hybrid vigor. *Proceedings of the National Academy of Sciences*, *108*(6), 2617–2622. https://doi.org/10.1073/pnas.1019217108

Guindon, M. F., Martin, E., Cravero, V., & Cointry, E. (2019). Transgressive segregation, heterosis and heritability for yield-related traits in a segregating population of Pisum sativum L. *Experimental Agriculture*, *55*(4), 610–620. https://doi.org/10.1017/S0014479718000224

Gurevitch, J., & Padilla, D. K. (2004). Are invasive species a major cause of extinctions? *Trends in Ecology & Evolution*, *19*(9), 470–474. https://doi.org/10.1016/j.tree.2004.07.005

Gvozdík, L. (2011). Plasticity of preferred body temperatures as means of coping with climate change? *Biology Letters*, *8*, 262–265. https://doi.org/10.1098/rsbl.2011.0960

Hamilton, J. A., & Miller, J. M. (2016). Adaptive introgression as a resource for management and genetic conservation in a changing climate. *Conservation Biology*, *30*(1), 33–41. https://doi.org/10.1111/cobi.12574

He, F., Arce, A. L., Schmitz, G., Koornneef, M., Novikova, P., Beyer, A., & de Meaux, J. (2016). The footprint of polygenic adaptation on stress-responsive cis-regulatory divergence in the arabidopsis genus. *Molecular Biology and Evolution*, *33*(8), 2088–2101. https://doi.org/10.1093/molbev/msw096

He, G., Luo, X., Tian, F., Li, K., Zhu, Z., Su, W., Qian, X., Fu, Y., Wang, X., Sun, C., & Yang, J. (2006). Haplotype variation in structure and expression of a gene cluster associated with a quantitative trait locus for improved yield in rice. *Genome Research*, *16*(5), 618–626. https://doi.org/10.1101/gr.4814006

Hedrick, P. W. (2012). What is the evidence for heterozygote advantage selection? *Trends in Ecology & Evolution*, *27*(12), 698–704. https://doi.org/10.1016/j.tree.2012.08.012

Holland, D. C., Hayes, M. P., & McMillan, E. (1990). Late summer movement and mass mortality in the california tiger salamander (*Ambystoma californiense*). *The Southwestern Naturalist*, *35*(2), 217–220. https://doi.org/10.2307/3671546

Huey, R. B., & Bennett, A. F. (1987). Phylogenetic studies of coadaptation: Preferred temperatures versus optimal performance temperatures of lizards. *Evolution*, *41*(5), 1098–1115. https://doi.org/10.1111/j.1558-5646.1987.tb05879.x

Huey, R. B., Kearney, M. R., Krockenberger, A., Holtum, J. A. M., Jess, M., & Williams, S. E. (2012). Predicting organismal vulnerability to climate warming: Roles of behaviour, physiology and adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1596), 1665–1679. https://doi.org/10.1098/rstb.2012.0005

Jain, A., Joshi, G., Chauhan, C., & Das, S. (2018). Abiotic stress response in plants: A Cis-regulatory perspective. In S. M. Zargar, & M. Y. Zargar (Eds.), *Abiotic stress-mediated sensing and signaling in plants: An omics perspective* (pp. 183–205). Springer. https://doi.org/10.1007/978-981-10-7479-0_6

Jiang, Y., Schmidt, R. H., Zhao, Y., & Reif, J. C. (2017). A quantitative genetic framework highlights the role of epistatic effects for grain-yield heterosis in bread wheat. *Nature Genetics*, *49*(12), 1741–1746. https://doi.org/10.1038/ng.3974

Johnson, J. R., Fitzpatrick, B. M., & Shaffer, H. B. (2010). Retention of low-fitness genotypes over six decades of admixture between native and introduced tiger salamanders. *BMC Evolutionary Biology*, *10*, 147. https://doi.org/10.1186/1471-2148-10-147

Johnson, J. R., Johnson, B. B., & Bradley Shaffer, H. (2010). Genotype and temperature affect locomotor performance in a tiger salamander hybrid swarm. *Functional Ecology*, *24*(5), 1073–1080. https://doi.org/10.1111/j.1365-2435.2010.01723.x

Johnson, J. R., Ryan, M. E., Micheletti, S. J., & Shaffer, H. B. (2013). Short pond hydroperiod decreases fitness of nonnative hybrid salamanders in California. *Animal Conservation*, *16*(5), 556–565. https://doi.org/10.1111/acv.12029

Johnson, J. R., Thomson, R. C., Micheletti, S. J., & Shaffer, H. B. (2011). The origin of tiger salamander (*Ambystoma tigrinum*) populations in California, Oregon, and Nevada: Introductions or relicts? *Conservation Genetics*, *12*(2), 355–370. https://doi.org/10.1007/s10592-010-0144-2

Jones, O. R., & Wang, J. (2010). COLONY: A program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, *10*(3), 551–555. https://doi.org/10.1111/j.1755-0998.2009.02787.x

Kang, E. Y., Martin, L. J., Mangul, S., Isvilanonda, W., Zou, J., Ben-David, E., Han, B., Lusis, A. J., Shifman, S., & Eskin, E. (2016). Discovering single nucleotide polymorphisms regulating human gene expression using allele specific expression from RNA-seq data. *Genetics*, *204*(3), 1057–1064. https://doi.org/10.1534/genetics.115.177246

Keane, T. M., Goodstadt, L., Danecek, P., White, M. A., Wong, K., Yalcin, B., Heger, A., Agam, A., Slater, G., Goodson, M., Furlotte, N. A., Eskin, E., Nellåker, C., Whitley, H., Cleak, J., Janowitz, D., Hernandez-Pliego, P., Edwards, A., Belgard, T. G., … Adams, D. J. (2011). Mouse genomic variation and its effect on phenotypes and gene regulation. *Nature*, *477*(7364), 289–294. https://doi.org/10.1038/nature10413

Khan, M. R. A., Mahbub, M. M., Reza, M. A., Shirazy, B. J., & Mahmud, F. (2016). Selection of field pea (*Pisum sativum* L.) genotypes through multivariate analysis. *Scientia Agriculturae*, *16*(3), 98–103.

Kita, R., Venkataram, S., Zhou, Y., & Fraser, H. B. (2017). High-resolution mapping of cis-regulatory variation in budding yeast. *Proceedings of the National Academy of Sciences*, *114*(50), E10736–E10744. https://doi.org/10.1073/pnas.1717421114

Kovach, R. P., Luikart, G., Lowe, W. H., Boyer, M. C., & Muhlfeld, C. C. (2016). Risk and efficacy of human-enabled interspecific hybridization for climate-change adaptation: Response to Hamilton and Miller (2016). *Conservation Biology*, *30*(2), 428–430. https://doi.org/10.1111/cobi.12678

Kumar, M., Jeberson, M. S., Singh, N. B., & Sharma, R. (2017). Genetic analysis of seed yield and its contributing traits and pattern of their inheritance in fieldpea (*Pisum sativum* L). *International Journal of Current Microbiology and Applied Sciences*, *6*(6), 172–181.

Landau, W., Niemi, J., & Nettleton, D. (2019). Fully bayesian analysis of RNA-seq counts for the detection of gene expression heterosis. *Journal of the American Statistical Association*, *114*(526), 610–621. https://doi.org/10.1080/01621459.2018.1497496

Lannoo, M. J. (2005). *Amphibian declines: The conservation status of United States species*. University of California Press.

Lauss, K., Wardenaar, R., Oka, R., van Hulten, M. H. A., Guryev, V., Keurentjes, J. J. B., Stam, M., & Johannes, F. (2018). Parental DNA methylation states are associated with heterosis in epigenetic hybrids. *Plant Physiology*, *176*(2), 1627–1645. https://doi.org/10.1104/pp.17.01054

Layne, J. R., & Claussen, D. L. (1982). The time courses of CTMax and CTMin acclimation in the salamander *Desmognathus fuscus*. *Journal of Thermal Biology*, *7*(3), 139–141. https://doi.org/10.1016/0306-4565(82)90002-X

Liu, J., Li, M., Zhang, Q., Wei, X., & Huang, X. (2020). Exploring the molecular basis of heterosis for plant breeding. *Journal of Integrative Plant Biology*, *62*(3), 287–298. https://doi.org/10.1111/jipb.12804

Logan, C. A., & Buckley, B. A. (2015). Transcriptomic responses to environmental temperature in eurythermal and stenothermal fishes. *Journal of Experimental Biology*, *218*(12), 1915–1924. https://doi.org/10.1242/jeb.114397

López-Maury, L., Marguerat, S., & Bähler, J. (2008). Tuning gene expression to changing environments: From rapid responses to evolutionary adaptation. *Nature Reviews Genetics*, *9*(8), 583–593. https://doi.org/10.1038/nrg2398

Loredo, I., Van Vuren, D., & Morrison, M. L. (1996). Habitat use and migration behavior of the California tiger salamander. *Journal of Herpetology*, *30*(2), 282–285. https://doi.org/10.2307/1565527

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 1–21. https://doi.org/10.1186/s13059-014-0550-8

Lutterschmidt, W. I. (1997). *Comparative physiology and macroecology of thermal tolerance in ectothermic vertebrates with correlates of phylogeny and biogeography*. [Thesis]. Retrieved from https://shareok.org/handle/11244/5569

Lutterschmidt, W. I., & Hutchison, V. H. (1997). The critical thermal maximum: Data to support the onset of spasms as the definitive end point. *Canadian Journal of Zoology*, *75*(10), 1553–1560. https://doi.org/10.1139/z97-782

Mallet, J. (2005). Hybridization as an invasion of the genome. *Trends in Ecology & Evolution*, *20*(5), 229–237. https://doi.org/10.1016/j.tree.2005.02.010

Markle, T. (2015). *Ecology and evolution of geographic range size variation in North American Plethodontid salamanders: Perspectives from thermal physiology*. Retrieved from http://conservancy.umn.edu/handle/11299/175251

McManus, C. J., Coolon, J. D., O'Duff, M., Eipper-Mains, J., Graveley, B. R., & Wittkopp, P. J. (2010). Regulatory divergence in Drosophila revealed by mRNA-seq. *Genome Research*, https://doi.org/10.1101/gr.102491.109

Michalak, P., & Noor, M. A. (2003). Genome-wide patterns of expression in Drosophila pure species and hybrid males. *Molecular Biology and Evolution*, *20*(7), 1070–1076.

Miller, M., Song, Q., Shi, X., Juenger, T. E., & Chen, Z. J. (2015). Natural variation in timing of stress-responsive gene expression predicts heterosis in intraspecific hybrids of Arabidopsis. *Nature Communications*, *6*(1), 7453. https://doi.org/10.1038/ncomms8453

Nowoshilow, S., Schloissnig, S., Fei, J.-F., Dahl, A., Pang, A. W. C., Pippel, M., Winkler, S., Hastie, A. R., Young, G., Roscito, J. G., Falcon, F., Knapp, D., Powell, S., Cruz, A., Cao, H., Habermann, B., Hiller, M., Tanaka, E. M., & Myers, E. W. (2018). The axolotl genome and the evolution of key tissue formation regulators. *Nature*, *554*(7690), 50–55. https://doi.org/10.1038/nature25458

O'Neill, E. M., Schwartz, R., Bullock, C. T., Williams, J. S., Shaffer, H. B., Aguilar-Miguel, X., Parra-Olea, G., & Weisrock, D. W. (2013). Parallel tagged amplicon sequencing reveals major lineages and phylogenetic structure in the North American tiger salamander (*Ambystoma tigrinum*) species complex. *Molecular Ecology*, *22*(1), 111–129. https://doi.org/10.1111/mec.12049

Perry, G. M. L., Danzmann, R. G., Ferguson, M. M., & Gibson, J. P. (2001). Quantitative trait loci for upper thermal tolerance inoutbred strains of rainbow trout (*Oncorhynchus mykiss*). *Heredity*, *86*(3), 333–341. https://doi.org/10.1046/j.1365-2540.2001.00838.x

Petranka, J. W. (1998). *Salamanders of the United States and Canada*. Smithsonian Institution Press.

Pfennig, K. S., Kelly, A. L., & Pierce, A. A. (2016). Hybridization as a facilitator of species range expansion. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1839), 20161329. https://doi.org/10.1098/rspb.2016.1329

Pickrell, J. K., Marioni, J. C., Pai, A. A., Degner, J. F., Engelhardt, B. E., Nkadori, E., Veyrieras, J.-B., Stephens, M., Gilad, Y., & Pritchard, J. K. (2010). Understanding mechanisms underlying human gene expression variation with RNA sequencing. *Nature*, *464*(7289), 768–772. https://doi.org/10.1038/nature08872

Pierce, D. W., Kalansky, J. F., & Cayan, D. R. (2018). *Climate, drought, and sea level rise scenarios for California's Fourth Climate Change Assessment*. Scripps Institution of Oceanography). California Energy Commission.

Pounds, J. A. (2001). Climate and amphibian declines. *Nature*, *410*(6829), 639–640. https://doi.org/10.1038/35070683

Price, A. L., Helgason, A., Thorleifsson, G., McCarroll, S. A., Kong, A., & Stefansson, K. (2011). Single-tissue and cross-tissue heritability of gene expression via identity-by-descent in related or unrelated individuals. *PLOS Genetics*, *7*(2), e1001317. https://doi.org/10.1371/journal.pgen.1001317

R Core Team (2013). *R: A language and environment for statistical computing*, Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/

Ranz, J. M., Namgyal, K., Gibson, G., & Hartl, D. L. (2004). Anomalies in the expression profile of interspecific hybrids of Drosophila melanogaster and Drosophila simulans. *Genome Research*, *14*(3), 373–379.

Rhymer, J. M., & Simberloff, D. (1996). Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics*, *27*, 83–109.

Rieseberg, L. H., Archer, M. A., & Wayne, R. K. (1999). Transgressive segregation, adaptation and speciation. *Heredity*, *83*(4), 363–372. https://doi.org/10.1046/j.1365-2540.1999.00617.x.

Riley, S. P. D., Shaffer, H. B., Voss, S. R., & Fitzpatrick, B. M. (2003). Hybridization between a rare, native tiger salamander (*Ambystoma californiense*) and its introduced congener. *Ecological Applications*, *13*(5), 1263–1275. https://doi.org/10.1890/02-5023

Rowley, J. J., & Alford, R. A. (2007). Non-contact infrared thermometers can accurately measure amphibian body temperatures. *Herpetological Review*, *38*, 308–316.

Ryan, M. E., Johnson, J. R., & Fitzpatrick, B. M. (2009). Invasive hybrid tiger salamander genotypes impact native amphibians. *Proceedings of the National Academy of Sciences*, *106*(27), 11166–11171. https://doi.org/10.1073/pnas.0902252106

Salavati, M., Bush, S. J., Palma-Vera, S., McCulloch, M. E. B., Hume, D. A., & Clark, E. L. (2019). Elimination of reference mapping bias reveals robust immune related allele-specific expression in crossbred sheep. *Frontiers in Genetics*, *10*, https://doi.org/10.3389/fgene.2019.00863

Sanabria, E. A., Quiroga, L. B., & Martino, A. L. (2012). Seasonal changes in the thermal tolerances of the toad *Rhinella arenarum* (Bufonidae) in the Monte Desert of Argentina. *Journal of Thermal Biology*, *37*(6), 409–412.

Sarawat, P., Stoddard, F. L., & Marshall, D. R. (1993). Derivation of superior F5 lines from heterotic hybrids in pea. *Euphytica*, *73*(3), 265–272. https://doi.org/10.1007/BF00036705

Schnable, P. S., & Springer, N. M. (2013). Progress toward understanding heterosis in crop plants. *Annual Review of Plant Biology*, *64*(1), 71–88. https://doi.org/10.1146/annurev-arplant-042110-103827

Searcy, C. A., Gabbai-Saldate, E., & Bradley Shaffer, H. (2013). Microhabitat use and migration distance of an endangered grassland amphibian. *Biological Conservation*, *158*, 80–87. https://doi.org/10.1016/j.biocon.2012.08.033

Searcy, C. A., Gray, L. N., Trenham, P. C., & Shaffer, H. B. (2014). Delayed life history effects, multilevel selection, and evolutionary trade-offs in the California tiger salamander. *Ecology*, *95*(1), 68–77. https://doi.org/10.1890/13-0120.1

Searcy, C. A., Rollins, H. B., & Shaffer, H. B. (2016). Ecological equivalency as a tool for endangered species management. *Ecological Applications*, *26*(1), 94–103. https://doi.org/10.1890/14-1674

Searcy, C. A., & Shaffer, H. B. (2016). Do ecological niche models accurately identify climatic determinants of species ranges? *The American Naturalist*, *187*(4), 423–435. https://doi.org/10.1086/685387

Seyednasrollah, F., Laiho, A., & Elo, L. L. (2015). Comparison of software packages for detecting differential expression in RNA-seq studies. *Briefings in Bioinformatics*, *16*(1), 59–70. https://doi.org/10.1093/bib/bbt086

Shaffer, H. B., & McKnight, M. L. (1996). The polytypic species revisited: Genetic differentiation and molecular phylogenetics of the tiger salamander *Ambystoma tigrinum* (amphibia: Caudata) Complex. *Evolution*, *50*(1), 417–433. https://doi.org/10.1111/j.1558-5646.1996.tb04503.x

Shaffer, H. B., Pauly, G. B., Oliver, J. C., & Trenham, P. C. (2004). The molecular phylogenetics of endangerment: Cryptic variation and historical phylogeography of the California tiger salamander. *Ambystoma Californiense*. *Molecular Ecology*, *13*(10), 3033–3049. https://doi.org/10.1111/j.1365-294X.2004.02317.x

Shen, Y., Sun, S., Hua, S., Shen, E., Ye, C.-Y., Cai, D., Timko, M. P., Zhu, Q.-H., & Fan, L. (2017). Analysis of transcriptional and epigenetic changes in hybrid vigor of allopolyploid Brassica napus uncovers key roles for small RNAs. *The Plant Journal*, *91*(5), 874–893. https://doi.org/10.1111/tpj.13605.

Shiel, B. P., Hall, N. E., Cooke, I. R., Robinson, N. A., & Strugnell, J. M. (2015). De novo characterisation of the greenlip abalone transcriptome (*Haliotis laevigata*) with a focus on the heat shock protein 70 (HSP70) family. *Marine Biotechnology*, *17*(1), 23–32. https://doi.org/10.1007/s10126-014-9591-y

Signor, S. A., & Nuzhdin, S. V. (2018). The evolution of gene expression in cis and trans. *Trends in Genetics*, *34*(7), 532–544. https://doi.org/10.1016/j.tig.2018.03.007

Spotila, J. R. (1972). Role of temperature and water in the ecology of lungless salamanders. *Ecological Monographs*, *42*(1), 95–125.

Srikanth, K., Kumar, H., Park, W., Byun, M., Lim, D., Kemp, S., te Pas, M. F. W., Kim, J.-M., & Park, J.-E. (2019). Cardiac and skeletal muscle transcriptome response to heat stress in Kenyan chicken ecotypes adapted to low and high altitudes reveal differences in thermal tolerance and stress response. *Frontiers in Genetics*, *10*, 993. https://doi.org/10.3389/fgene.2019.00993

Stebbins, R. C. (2003). *A field guide to western reptiles and amphibians*. Houghton Mifflin Harcourt. Retrieved from https://books.google.com/books?hl=en&lr=&id=9JznHN2VFzkC&oi=fnd&pg=PP15&dq=stebbins+herpetology&ots=-8BsHfpRyR&sig=u5OrH0-_QTJAK32pO7SQWTP2I5w

Stelkens, R., & Seehausen, O. (2009). Genetic distance between species predicts novel trait expression in their hybrids. *Evolution*, *63*(4), 884–897. https://doi.org/10.1111/j.1558-5646.2008.00599.x

Stevenson, K. R., Coolon, J. D., & Wittkopp, P. J. (2013). Sources of bias in measures of allele-specific expression derived from RNA-seq data aligned to a single reference genome. *BMC Genomics*, *14*, 536. https://doi.org/10.1186/1471-2164-14-536

Sunday, J. M., Bates, A. E., & Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proceedings of the Royal Society of London B. Biological Sciences*, *278*(1713), 1823–1830. https://doi.org/10.1098/rspb.2010.1295

Tan, E., Kinoshita, S., Suzuki, Y., Ineno, T., Tamaki, K., Kera, A., Muto, K., Yada, T., Kitamura, S., Asakawa, S., & Watabe, S. (2016). Different gene expression profiles between normal and thermally selected strains of rainbow trout, *Oncorhynchus mykiss*, as revealed by comprehensive transcriptome analysis. *Gene*, *576*(2, Part 1), 637–643. https://doi.org/10.1016/j.gene.2015.10.028

Tedeschi, J. N., Kennington, W. J., Tomkins, J. L., Berry, O., Whiting, S., Meekan, M. G., & Mitchell, N. J. (2016). Heritable variation in heat shock gene expression: A potential mechanism for adaptation to thermal stress in embryos of sea turtles. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1822), 20152320. https://doi.org/10.1098/rspb.2015.2320

Tehranchi, A., Hie, B., Dacre, M., Kaplow, I., Pettie, K., Combs, P., & Fraser, H. B. (2019). Fine-mapping cis-regulatory variants in diverse human populations. *eLife*, *8*, e39595. https://doi.org/10.7554/eLife.39595

Todesco, M., Pascual, M. A., Owens, G. L., Ostevik, K. L., Moyers, B. T., Hübner, S., Heredia, S. M., Hahn, M. A., Caseys, C., Bock, D. G., & Rieseberg, L. H. (2016). Hybridization and extinction. *Evolutionary*

Applications, *9*, 892–908. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/eva.12367/pdf

Tomalty, K. M. H., Meek, M. H., Stephens, M. R., Rincón, G., Fangue, N. A., May, B. P., & Baerwald, M. R. (2015). Transcriptional response to acute thermal exposure in juvenile chinook salmon determined by RNAseq. *G3: Genes|genomes|genetics*, *5*(7), 1335–1349. https://doi.org/10.1534/g3.115.017699

Tracy, C. R., Christian, K. A., & Tracy, C. R. (2010). Not just small, wet, and cold: Effects of body size and skin resistance on thermoregulation and arboreality of frogs. *Ecology*, *91*(5), 1477–1484. https://doi.org/10.1890/09-0839.1

Trenham, P. C., Bradley Shaffer, H., Koenig, W. D., & Stromberg, M. R. (2000). Life history and demographic variation in the california tiger salamander (*Ambystoma californiense*). *Copeia*, *2000*(2), 365–377. 10.1643/0045-8511(2000)000[0365:LHADVI]2.0.CO;2

U.S. Fish and Wildlife Service (2003). Endangered and threatened wildlife and plants; Listing of the Centra California Distinct Population Segment of the California tiger salamander; Reclassification of the Sonoma County and Santa Barbara County Distinct Populations from Endangered to Threatened. *Special Rule. Federal Register*, *68*, 28648.

U.S. Fish and Wildlife Service (2004). Endangered and threatened wildlife and plants; determination of threatened status for the California tiger salamander; and special rule exemption for existing routine ranching activities; final rule. *Federal Register*, *69*(149), 47212–47248.

van de Geijn, B., McVicker, G., Gilad, Y., & Pritchard, J. K. (2015). WASP: Allele-specific software for robust molecular quantitative trait locus discovery. *Nature Methods*, *12*(11), 1061–1063. https://doi.org/10.1038/nmeth.3582

Wang, J., Tian, L., Madlung, A., Lee, H.-S., Chen, M., Lee, J. J., Watson, B., Kagochi, T., Comai, L., & Chen, Z. J. (2004). Stochastic and epigenetic changes of gene expression in Arabidopsis polyploids. *Genetics*, *167*(4), 1961–1973.

Wayne, R. K., & Shaffer, H. B. (2016). Hybridization and endangered species protection in the molecular era. *Molecular Ecology*, *25*(11), 2680–2689. https://doi.org/10.1111/mec.13642

Wheeler, H. E., Shah, K. P., Brenner, J., Garcia, T., Aquino-Michaels, K., Cox, N. J., Nicolae, D. L., & Im, H. K. (2016). Survey of the heritability and sparse architecture of gene expression traits across human tissues. *PLOS Genetics*, *12*(11), e1006423. https://doi.org/10.1371/journal.pgen.1006423

Wray, G. A. (2007). The evolutionary significance of *cis*-regulatory mutations. *Nature Reviews Genetics*, *8*(3), 206–216. https://doi.org/10.1038/nrg2063

Yang, M., Wang, X., Ren, D., Huang, H., Xu, M., He, G., & Deng, X. W. (2017). Genomic architecture of biomass heterosis in Arabidopsis. *Proceedings of the National Academy of Sciences*, *114*(30), 8101–8106. https://doi.org/10.1073/pnas.1705423114

Young, V. K. H., & Gifford, M. E. (2013). Limited capacity for acclimation of thermal physiology in a salamander, *Desmognathus brimleyorum*. *Journal of Comparative Physiology B*, *183*(3), 409–418. https://doi.org/10.1007/s00360-012-0717-0

Youssef, M. K., Adolph, S. C., & Richmond, J. Q. (2008). Evolutionarily conserved thermal biology across continents: The North American lizard *Plestiodon gilberti* (Scincidae) compared to Asian Plestiodon. *Journal of Thermal Biology*, *33*(5), 308–312. https://doi.org/10.1016/j.jtherbio.2008.02.007

Zhang, X., & Borevitz, J. O. (2009). Global analysis of allele-specific expression in arabidopsis thaliana. *Genetics*, *182*(4), 943–954. https://doi.org/10.1534/genetics.109.103499

Zhuo, Z., Lamont, S. J., & Abasht, B. (2017). RNA-seq analyses identify frequent allele specific expression and no evidence of genomic imprinting in specific embryonic tissues of chicken. *Scientific Reports*, *7*(1), 11944. https://doi.org/10.1038/s41598-017-12179-9

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Cooper RD, Shaffer HB. Allele-specific expression and gene regulation help explain transgressive thermal tolerance in non-native hybrids of the endangered California tiger salamander (*Ambystoma californiense*). *Mol Ecol*. 2021;30:987–1004. https://doi.org/10.1111/mec.15779

Chapter 2

Title:

Hydroperiod management may reduce, but not eliminate, non-native hybrid advantage over the endangered California tiger salamander (*Ambystoma californiense*).

Abstract:

The introduction of invasive species is one of the greatest threats to biodiversity worldwide. Reclusive life histories and cryptic species differentiation makes identifying non-native species difficult and may render eradication of non-native taxa inviable for some systems. This intractability is compounded when invasive species hybridize with native species, confounding the ability to identify pure natives while threatening them with genomic extinction. Under these circumstances, it is necessary to evaluate alternative management strategies that may increase selection for native genotypes to limit or reverse the spread of non-native alleles through the population. Here I evaluate the efficacy of hydroperiod management for improving the fitness of the endangered California tiger salamander ("CTS", *Ambystoma californiense*), which is besieged by non-native introgression from the introduced barred tiger salamander ("BTS", *Ambystoma mavortium*). Fourteen large (30-foot diameter), naturalistic ponds were constructed *in situ* with a range of hydroperiods and stocked with larval salamanders to evaluate larval survival and mass at metamorphosis for hybrid and native larvae. Longer hydroperiod ponds appear to favor certain larval source ponds and family groups, reducing the diversity of surviving larvae through potential group or kin level selection. I confirm that longer pond duration exponentially increases the mass and survival of both hybrids and natives. There were

no hydroperiod treatments in which native CTS outperformed hybrids in survival or mass, however shorter hydroperiods did significantly reduce hybrid advantage. Using the software BayEnv, I identified 58 genes that may experience hydroperiod-mediated selection. These genes were functionally enriched for ontology terms dealing with growth and metabolism, likely driven by the rapid growth necessary to develop in short hydroperiod ponds. Together these results provide insight into the ecological and molecular consequences of hydroperiod management. It appears that shortening pond hydroperiod will not select for native genotypes, however it may reduce hybrid advantage sufficiently to slow the spread of non-native genes, which may be an important component of hybrid management.

Introduction:

The introduction and establishment of invasive taxa represents one of the most severe and challenging conservation concerns today (Gurevitch & Padilla 2004). Anthropogenic forces have redistributed organisms into novel environments worldwide. Though most introduced populations do not persist, the few that do can cause immeasurable harm to local ecosystems (Alexander et al. 2014). Although these impacts can be seen at any trophic level, it is exceptionally pronounced in apex predators (David et al. 2017). Apex predators often play a major role in ecosystem function through top-down control, limiting or releasing the population sizes of lower trophic levels (Ritchie & Johnson 2009). Minor shifts in prey preference or changes in the total biomass consumed can produce cascading effects on the rest of the ecosystem (Rogers et al. 2017; Feit et al. 2020). Therefore, invasive apex predators may disproportionately disrupt ecosystem balance, and irrevocably alter native communities. For this reason, government agencies and land managers have relied on wholesale eradications to remove

invasive species (Lampert & Liebhold 2021). This can be an incredibly laborious task, involving millions of dollars and monumental human effort to manually eradicate unwanted species (Panetta 2015). Though this strategy is sometimes effective (Hulme 2020), it is not always the optimal choice (Lampert et al. 2014; Liebhold & Kean 2019). Some species are incredibly difficult to find or identify, making eradication ineffective. Furthermore, some invasive species may closely resemble native taxa, making eradication by non-experts a potentially detrimental endeavor (Morais & Reichard 2018), especially if the native is rare or endangered.

In addition to these complications, hybridization between native and non-native species can present new logistic and ethical issues that must be considered. Non-native/native species hybridization occurs more frequently as humans bring into contact taxa that have been geographically isolated for millennia (Mallet 2005). Here I define non-native hybridization as successful interbreeding between a native and non-native species in the wild. These related species may lack the genetic or behavioral mechanisms that prevent successful reproduction. Although hybridization is thought of as rare, new studies are demonstrating the rapid increase in non-native hybridization and the existential threat this poses to native biodiversity (Allendorf et al. 2001; Todesco et al. 2016). These invasions occur on a genomic level, and are therefore difficult to identify and prevent. Non-native hybrids are often difficult to distinguish from "pure native" individuals, making eradication imprecise, which can negatively impact the native population (Fitzpatrick et al. 2015). If left unchecked, hybridization may progress to such a point that few pure native individuals are left on the landscape (Allendorf et al. 2010), leaving limited opportunity for recolonization after hybrid eradication. Furthermore, if hybrids constitute a large fraction of the current population and are eradicated, the disruption to the native ecosystem may

outweigh the impact of hybrid individuals. In these circumstances, it is critical to investigate alternative methods for non-native control, before resorting to potentially harmful eradication.

Here I evaluate one potential method to control non-native hybridization in the California tiger salamander. The California tiger salamander (*Ambystoma californiense*; hereafter "CTS") is a moderately large amphibian that is endemic to California (Stebbins 2003). As aquatic larvae, CTS are important apex predators that help shape vernal pool communities (Ryan et al. 2009a; Searcy et al. 2016; Messerman et al. 2021). This endangered species is protected at both the Federal and State level due to population declines throughout its range (U.S. Fish and Wildlife Service 2004; California Department of Fish Game 2010). These declines are predominantly due to habitat loss, as much of its native range has been converted to agricultural fields and residential developments (Davidson et al. 2002a). However, one of the most challenging issues impeding CTS recovery is hybridization with introduced populations of the non-native barred tiger salamander (*Ambystoma mavortium*, hereafter "BTS"). This congener was intentionally introduced from its native range in northern Texas into the Salinas Valley (Monterey County, California) between 1950 and 1960, where it was raised and sold as fishing bait (Riley et al. 2003a). CTS and BTS readily hybridize and in the time since this introduction, the range of BTS and BTS-CTS hybrids has expanded, filling much of the Salinas Valley with a hybrid swarm (Figure 2.1).

Several studies suggest that hybrids enjoy superior fitness throughout the hybrid zone. Fitzpatrick et al. (2007a) modeled the dynamics of hybrid zone expansion and found that non-native alleles reach fixation much faster than is expected based on model of neutral diffusion. Other studies document hybrid superiority in thermal tolerance (Cooper & Shaffer 2021), locomotor performance (Johnson et al., 2010), and water-quality tolerance (Ryan et al. 2013).

This evidence suggests that eventually hybrid salamanders may completely replace native CTS in this region, resulting in genome-level extinction (Mallet 2005). Established non-native populations in the two other distinct population segments ("DPS") of CTS in Sonoma and Santa Barbara Counties (Johnson et al. 2011) also suggests that the issue of hybridization may affect the entire CTS range, and further emphasizes the need for effective management strategies.

CTS-BTS hybrids (hereafter "hybrids") have a disruptive effect on the vernal pool communities that they inhabit through trophic cascades. Although adult CTS are terrestrial, their larval stage is aquatic, developing in temporary breeding ponds. These larvae are predacious and grow to become the apex predator in vernal pool environments (Holomuzki et al. 1994). These voracious larvae consume large quantities of vertebrate and invertebrate prey to fuel their rapid growth and development (Whiteman et al. 1996), exerting strong top-down effects in the vernal pool trophic web. Using small artificial approximations of temporary ponds (300 gallon semi-natural "mesocosms"), two published studies have shown that hybrid tiger salamanders alter the community assemblage in vernal pool ecosystems. Ryan et al. (2009) demonstrated that hybrids drastically reduce the abundance of Pacific chorus frogs (*Hyliola regilla*), and the California newt (*Taricha torosa*) compared to pure native CTS larvae. The same study also concluded that increased hybrid predation would impact other endangered amphibians such as California red-legged frogs (*Rana draytonii*), and the Santa Cruz long-toed salamander (*Ambystoma macrodactylum croceum*), though these species were not included in the study. Searcy et al. (2016) expanded on this result by examining shifts in the entire vernal pool community, including six taxa that were consistently observed in naturally occurring CTS ponds. The authors concluded that hybrids have a disruptive effect on the trophic community and therefore do not function as ecological surrogates for native CTS. The negative impacts of hybrid tiger

salamanders on CTS and other sensitive vernal pool taxa emphasizes the need for managers to find a way to combat this pervasive conservation issue.

Effective management of hybrids is complicated by the life history of these species. Given the negative effects of hybrids on the landscape, eradication would appear to be an ideal solution. However, CTS and hybrids are reclusive, spending the majority of their adult lives hidden underground in rodent burrows, emerging only an average of two times in their life to breed (Trenham et al. 2000b; Trenham 2001; Trenham & Shaffer 2005a). This reclusiveness coupled with their 10 to 12 year life span, makes eradication extremely difficult, requiring one to capture individuals over a span of 10-15 years to ensure all hybrids are removed. Even if this effort was initiated, it is impossible to know the genotype of a given tiger salamander without conducting expensive genomic analyses that take 4-6 months to complete (McCartney-Melstad et al. 2016; Cooper & Shaffer 2021). It is therefore likely that field technicians would erroneously remove endangered native CTS, or fail to remove hybrids, making successful eradication unlikely. Furthermore, the average migration distance of post-metamorphic and adult CTS is 556m, with 5% of salamanders dispersing more than 1.8km (Searcy et al. 2013). This dispersal capability significantly increases the area that must be managed to ensure that all hybrid migrants are removed. It is therefore prudent to investigate other, more effective management strategies to reduce the success of non-native hybrids on the landscape.

An alternative management strategy could be to modify or restore the natural habitat to remove the apparent hybrid advantage (Wayne & Shaffer 2016). Fitzpatrick and Shaffer (2007a) found a strong positive correlation between non-native allele frequency and artificial ponds with unnaturally long periods of inundation (the amount of time a pond holds water, “hydroperiod”). This observation fits with our understanding of the evolutionary pressures that native CTS

experienced. CTS split from their most recent common ancestor with BTS several million years ago (Shaffer & McKnight 1996), and in this time it has been subjected to the climatic conditions of California. Sparse rain and high summer temperatures dictated CTS survival, which favors rapid development in their natal ponds to complete metamorphosis before ponds dry. Failure to escape would result in mass larval mortality, representing a strong selective force (McMenamin et al. 2008). In contrast, BTS likely experience less desiccation pressure in their native range, which receives an additional 5-10 inches of annual precipitation ("1981-2010 Normals"; www.ncdc.noaa.gov/). The resulting longer hydroperiods enable BTS to remain in ponds longer and exploit those prey-rich environments to achieve a greater size at metamorphosis, a significant determinant of lifetime fitness in tiger salamanders (Searcy et al., 2015; Semlitsch et al., 1988). Historically, this pattern would result in relatively greater fitness for native CTS in California. However, human modification of the landscape has drastically altered this environmental paradigm. A large proportion of the native CTS range has been converted to agriculture and ranching, both requiring substantial water to irrigate crops and hydrate cattle into the hot, rain-free summer months (King 1998). Landowners circumvented the ephemerality of California's water regime by either excavating naturally occurring vernal pools to be deeper, or damming natural waterways (Zacharias & Zamparas 2010). This results in larger ponds that have a much longer hydroperiod than an unmodified pond in the same location. I believe that this extensive landscape modification has favored hybrids that are able to disproportionally benefit from the artificially longer pond hydroperiod. Johnson et al. (2013) tested this hypothesis using mesocosms that dried at different rates to simulate a range of hydroperiod regimes. In that study, hybrids enjoyed greater survival and mass at metamorphosis in the long duration mesocosms, as expected. Native CTS appeared to fare better than hybrids in the short duration ponds, although

this result was less dramatic. This appears to confirm the prediction that hybrids have an advantage in artificially long pond hydroperiods. Although this result is promising, it was conducted in an artificial environment without the normal compliment of vernal pool inhabitants.

Here I seek to understand the effects of pond hydroperiod on non-native tiger salamander success in the field. I constructed large, naturalistic ponds at the edge of the hybrid zone to test whether longer hydroperiod ponds favor hybrid genotypes *in situ*. I inoculated each pond with controlled proportions of native and hybrid larvae, then evaluated their relative success when they completed metamorphosis and emerged from the ponds. First, I compared the relative survival of larvae from different source populations and familial groups to identify potential selection resulting from the hydroperiod treatment. Second, I used survival and mass at metamorphosis to quantify individual success and fitness, testing the prediction that longer hydroperiod favors hybrid over native individuals. Lastly, I scanned for loci that exhibited evidence of strong selection resulting from the hydroperiod gradient. With these data, I evaluate the potential benefits of managing pond hydroperiod in the field to minimize the success of non-native genotypes across the landscape.

Methods:

Pond Construction

To test the effects of hydroperiod variation on the success of non-native hybrid genotypes, I constructed 14 naturalistic ponds on the Fort Ord National Monument in Monterey County (CA, USA) during September and October 2018. I designed 7 ponds in each of two sizes, large (Diameter = 9.1m, Max Depth = 69cm) and small (Diameter = 7.9m, Max Depth = 60cm), both with 15% slope. These two pond sizes were used to coarsely differentiate pond hydroperiod

based on calculations using average evapotranspiration and precipitation for the site following established protocols (Biebighauser 2011). Therefore, pond size is not directly considered in downstream analyses, since the hydroperiod treatment is more precise, and highly correlated with pond size. I constructed 4 additional small ponds to function as reservoirs of water. Ponds filled naturally with rainfall, and throughout the experiment I used a large pump to add (from the reservoir ponds) or remove water in each pond to achieve a range of hydroperiods from 80 to 115 days. The hydroperiod range was 85 to 115 days in 2019 and due to less overall precipitation in 2020 the range was shifted five days earlier, spanning 80 to 110 days. Within each year there were 7 hydroperiod levels (7 experimental ponds) where ponds dried at an interval of approximately 5 days. Across years this yielded 8 hydroperiod levels due to the 5-day shift in 2020. The range of hydroperiods were selected based on results from the mesocosm hydroperiod study (Johnson et al. 2013), which demonstrated a significant shift in native/non-native fitness between 90 and 120 days. I used established methods to install drift fencing with pitfall traps around each pond and around each pond complex to collect post-metamorphic salamanders as they exited the ponds (Searcy et al., 2014; Trenham & Shaffer, 2005b). In brief, drift fences were constructed using partially buried, 0.3m tall shade cloth that completely encircles each pond approximately 1m from the edge of the constructed basin. An additional line of drift fencing surrounded the entire site to ensure no hybrid salamanders escaped. Pitfall traps consisted of 1-gallon buckets buried so that they were flush with the surface of the ground and spaced every 10 meters on both sides of the drift fence. Bucket lids were modified by attaching wooden feet to the top of the lid so that the lid could be positioned over the open trap to provide shade and cover to prevent desiccation. These lids could be flipped over and used to close the traps when not in use.

Once ponds filled with natural precipitation, I inoculated experimental ponds with plankton from nearby natural ponds in November 2018 when they first filled with rainfall. After this initial inoculation, ponds were naturally colonized by other vertebrate and invertebrate prey. Although there was likely natural variation in prey density between ponds, the close proximity of ponds (less than approximately 10 feet apart), and the randomized distribution of hydroperiod treatments throughout the site reduced the risk of consistent bias. Furthermore, surveys of macro-invertebrate and vertebrate communities confirmed the presence of prey at relatively equal abundance across all experimental ponds (Cooper et al. unpublished data).

CTS and Hybrid Larvae:

I collected pure CTS and hybrid larvae from source ponds around the Salinas Valley. Source ponds were selected based on observed larval abundance and previously measured non-native allele frequencies (McCartney-Melstad et al. unpublished data). I attempted to select ponds that would provide a wide range of native and non-native genotypes to increase the genetic variation in the experimental ponds. However, options were limited by the unpredictable breeding patters of CTS. Ultimately, I selected five ponds in year 1 (2019) and five different ponds in year 2 (2020) for a total of 10 unique source ponds in the study (Figure 2.1). Previous experience indicated that larvae must be approximately 15 mm snout-vent length (SVL) to be large enough to be caught and moved without being harmed.

Each collection year, I collected ~ 15 mm SVL individuals randomly using a 3m wide, 1/8-inch (3.18 mm) mesh seine. Upon capture, I sorted individuals into large and small size classes. Larvae were immediately transported to the experimental ponds in their natural pond water, allowed to acclimate to the experimental pond conditions for 1 hour during which time

they were able to freely swim out of the transportation bucket into the pond. All ponds within a specific larval treatment received the same number of larvae of each size class from each source pond. This balanced distribution maximized the probability that each pond started with equivalent allele frequencies. At the same time that larvae were introduced into experimental ponds, I collected a representative sample of 40-60 larvae comprised of the same proportion of large and small size classes. These larvae were immediately stored in 95% ethanol and used in subsequent genomic analyses to assign source pond information to the larvae that survived to metamorphosis. Although sampling the true founder individuals would have been ideal, this method was not feasible for several reasons. First, the size of the tissue sample required for the target-capture protocol is too large to excise from the founder larvae without causing serious injury or death (McCartney-Melstad et al. 2016). Second, tissue sampling injuries would have disproportionately disadvantaged smaller larvae, potentially favoring large larvae. Third, sequencing each of the 2,730 larvae included in this study was prohibited by financial constraints, necessitating a representative sampling design. Therefore, sequencing a randomly drawn sample from the pool of founders, balanced across the observed size distribution, represents the most rigorous method available.

Each experimental pond received a specific ratio of native to hybrid larvae. This larval treatment consisted of three levels across the two-year experiment, low-hybrid (60 native and 60 hybrids), medium-hybrid (15 native and 60 hybrids) and all-hybrid (0 native, 120 hybrids). Year 1 included one low- and one high-hybrid treatments, year 2 included two medium-hybrid treatments. An all-native treatment was not included in the study to reduce the impact on an already imperiled wild CTS population. Each larval treatment level had 7 ponds that spanned the range of hydroperiods (80-115 days). The low- and high-hybrid treatments received 120 total

larvae, which is approximately 6.7 larvae per cubic meter of maximum pond volume. This density was chosen to replicate the previous hydroperiod mesocosm study (Johnson et al. 2013) which used 6.6 larvae/m^3. The medium-hybrid treatment received fewer total larvae across all experimental ponds (75 larvae, or approximately 4.2 larvae/m^3) due to exceptionally low breeding in wild source ponds. Given the low numbers of breeding tiger salamanders during both sampling years, the total number of larvae used in the experiment was reduced to minimize the impact on the native CTS. I account for these differences by introducing larval treatment as a random effect in all applicable analyses. The larval densities used in this experiment are within the range of natural CTS densities, which a previous study estimated to be between 3.5 and 7.0 larvae/m^3 (Searcy et al. 2016).

Larvae developed until they completed metamorphosis and naturally migrated out of the ponds. These post-metamorphic salamanders (hereafter "metamorphs") were intercepted by the drift fence surrounding each pond and directed into a pitfall trap bucket. When traps were active (open) they were checked each morning prior to local sunrise. At the time of capture, I collected 1) time and date; 2) bucket location; 3) total length (mm); 4) snout-to-vent length (mm); 5) Mass (g); and 6) genetic tissue (1 cm of tail tip). Length was measured to the nearest millimeter using a standard ruler. Mass was measured using a digital scale (0.01g precision). Genetic tissue was collected from the tip of the tail using surgical scissors and stored in a 2mL vial of 95% ethyl alcohol. All metamorphs were euthanized using a 5g/L solution of tricaine methanesulfonate ("MS-222", Leary et al. 2013) and preserved in the UCLA HBS museum.

Molecular Methods:

Genomic DNA was extracted from larval and metamorph tissue using a modified salt extraction protocol (Sambrook & Russel 2001). DNA was diluted to 100 ng/μL (10,000 ng total) and sheared to approximately 500 bp using a BioRuptor (Diagenode, Denville, NJ). I performed a double-sided size selection using SPRI beads (Bronner et al. 2013) to obtain an average fragment size of 400 bp, and recovered approximately 1,000ng of DNA to use in library preparations. I used Kapa LTP library preparation kits (Kapa Biosystems, Wilmington, MA) to perform standard Illumina library preparations (end repair, A-tailing, and adapter ligation). Sample libraries were then dual-indexed using 8-bp indices that were incorporated using PCR (adapters from Travis Glenn, University of Georgia). Following library preparation, 16 sample-libraries were pooled together (4,000 ng total in 7uL in 10mM Tris-HCl, pH 8) for sequence capture reactions, targeting 5,237 genes with a CTS-specific protocol (McCartney-Melstad et al. 2016). I followed a modified MYBAITS protocol (version 2.3.1) with our own species-specific repetitive DNA blocker c_0t-1 (30,000 ng in 5uL in 10mM Tris-HCl, pH 8) for use in the capture reactions. Libraries were hybridized to probes for 30 hours, subjected to three high-stringency wash steps, and PCR-amplified to enriched for target DNA. Each pool was split each into 4 replicate reactions to help reduce PCR bias (Barnard et al. 1998), and capture pools were then combined into two final pools (2019 and 2020 samples). Each pool was sequenced on a single Illumina NovaSeq S4 150-bp paired-end lane at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley.

Bioinformatics:

Adapter sequences and low quality bases were trimmed from raw sequences using TRIMMOMATIC version 0.35 (Bolger et al. 2014). I used BWA-MEM version 0.7.16a to map

trimmed reads to the recently published Mexican Axolotl (*Ambystoma mexicanum*) genome (Nowoshilow et al. 2018; Smith et al. 2019), which is a close relative of CTS and BTS (Shaffer & McKnight 1996; Everson et al. 2021)). I then followed the GATK (version 4.0) best practices pipeline (Auwera et al. 2013) for calling variants across all samples. First, Illumina adapters and duplicate reads were marked using PICARD. I used GATK to recalibrate base map-quality scores in known variant sites using a variant database from previous CTS studies (McCartney-Melstad et al., 2016; McCartney-Melstad et al. unpublished data). I used GATK HAPLOTYPECALLER to call haplotypes over genomic regions that matched our 5,237-gene target regions (option "-L" with a BED file of target regions with a 300bp buffer). These individual GVCF files were then combined into one multi-sample GVCF using GATK COMBINEGVCFS. I then called genotypes using GATK GENOTYPEGVCFS. I used GATK VARIANTFILTRATION to remove loci in the VCF that failed any of the following conditions: QualityByDepth (QD) < 2, MappingQuality (MQ) < 40, FisherStrand (FS) > 60, MQRankSum < -12.5, ReadPosRankSum < -8.0, QUAL < 30 (For description see URL: https://gatk.broadinstitute.org/hc/en-us/articles/360035890471-Hard-filtering-germline-short-variants). I used VCFTOOLS (Danecek et al. 2011) to remove individual genotype calls with quality less than 20 ("--minGQ 20") and depth less than 8 ("--minDP 8"). I also used VCFTOOLS to filter loci that met the following conditions: were not bi-allelic ("--min-alleles 2 --max-alleles 2"), were missing data across more than 50% of individuals ("--max-missing 0.5"), had a minor allele frequency less than 10% ("--maf 0.1"). For some downstream analyses I used the "prune" plugin in BCFTOOLS (Danecek et al. 2021) to filter loci that were physically linked with an r^2 greater than 0.8 within a 1000bp sliding window ("-m 0.80 -w 1000").

Hybrid Index Score:

The Hybrid Index Score ("HIS"; Johnson et al. 2010b) was estimated for each source pond larva and each surviving metamorph with sufficient read depth. To calculate the HIS I used a reference panel comprised of 150 confirmed native CTS that span the entire known range and 30 non-native BTS individuals from the same source population in Texas that was introduced to California. These reference individuals were used to find diagnostic loci that were fixed-different between the two species (Cooper & Shaffer 2021). To identify diagnostic loci, I generated VCF files for the pure CTS and pure BTS separately, then filtered these files using VCFTOOLS ("--max-maf 0.001 –max-alleles 2") to identify loci that were monomorphic in each group. I then used custom R scripts to find loci that were fixed for different alleles in the CTS and BTS reference groups. This yielded a list of diagnostic loci with information about the species-specific origin of each allele. This list of loci was used to subset the main sample VCF such that only diagnostic loci were included. Loci were then filtered using a strict 95% threshold for linkage disequilibrium using the "prune" plugin in BCFTOOLS (Danecek et al. 2021) with a 1000bp sliding window ("-m 0.95 -w 1000"). This reduced the likelihood of counting two physically linked diagnostic loci, which would not represent independent data. For each individual, I then calculated the HIS as the proportion of BTS derived divided by the total number of non-missing alleles scored in that individual.

Assigning Source Populations:

To test for the effect of larval source pond on survival, I assigned metamorphs to their original source pond using Discriminant Analysis of Principal Component (DAPC) methods. First, for each year of the experiment I used the representative sample of source pond larvae to construct a DAPC using the package ADEGENET in R version 4.0.4 (R. Core Team 2013). This

model determines which principal component axes (eigenvectors) of genetic variation best discriminate between the pre-assigned source populations. If two or more populations were not easily distinguishable in the first DAPC, I ran additional subset DAPC models with only the overlapping populations and metamorphs. I then used these DAPC models to predict the pond of origin of all metamorphs that emerged from the experimental ponds. Any individuals that could not be assigned to a single source pond were dropped from the analysis.

Assigning Family Groups:

The most likely sibling cohorts were identified from the source pond and metamorph groups using the program Colony 2 (Jones & Wang 2010). Colony 2 was run separately for each source pond, comprised of source pond exemplars and the metamorphs assigned to the source pond using DAPC. Adult salamanders that were incidentally caught during the larvae collection phase were included as potential parents in each source pond. I analyzed the "BestCluster" output files from Colony to determine probable family groups within each pond group using custom R scripts, and used these cohort assignments for downstream analyses.

Differential Group Survival:

I investigated the possibility that hydroperiod drives differential survival among source ponds or family groups. A Chi-Squared statistic ("χ^2"; defined as the sum of (observed-expected)^2 / expected) was calculated for each experimental pond to quantify the degree of dissimilarity between the input versus output group distribution. I performed this analysis for source pond and family group distributions. For each, I used the known input proportions as "expected" values. I calculated "observed" proportions in the metamorphs from the

computational assignments to either family group (colony) or source pond (DAPC). Then Linear Mixed Model (LMM) regression was performed using the LME4 version 1.1-26 package in R to test whether larval survival through metamorphosis was associated with source ponds or families. This model included the log-transformed χ^2 statistic as the response variable with hydroperiod as the predictor and larval treatment as a random effect.

Larval Treatment:

The proportion of larvae that survived in each pond was compared across the three larval treatments (low-hybrid, medium-hybrid and all-hybrid larval combinations). The means of these three groups were compared using a generalized linear model (GLM) with proportion of all larvae that survived as the dependent variable and larval period and hydroperiod as the independent variables.

Larval Survival:

To investigate the effect of hydroperiod on survival I compared the proportion of hybrid and native individuals that survived from each pond, estimated as the number of metamorphs that emerged divided by the number of larvae that were added. The overall proportion (all individuals; all-larvae-in:all-metamorphs-out), the CTS proportion (HIS < 0.10; CTS-larvae-in:CTS-metamorphs-out), and the Hybrid proportion (HIS ≥ 0.1; Hybrid-larvae-in:Hybrid-metamorphs-out) were calculated for each experimental pond. This pond-specific proportion was used as the response variable in a quasibinomial logistic GLM with hydroperiod as the independent variable. The significance of this was evaluated using a likelihood ratio test (LRT) to compare the model with hydroperiod to a simpler nested model that does not include

hydroperiod. The interaction between genotype and hydroperiod was then visualized by subtracting the GLM marginal effects at the mean for natives from the model predictions for hybrids. This yielded a single curve that describes the additional survival probability of hybrid genotypes for each hydroperiod, and used this relationship to evaluate the hybrid advantage across the hydroperiod treatments.

Metamorph Size:

Linear mixed models (LMMs) were used in R to investigate the effect of hydroperiod and HIS on metamorph mass. I built two models, a linear model with HIS and hydroperiod, and a quadratic model with HIS, hydroperiod, and hydroperiod-squared ($hydroperiod^2$), and compared the fit of the two models using a likelihood ratio test. Similarly, the effect of hydroperiod and HIS on metamorph SVL was tested using a LMM with larval treatment as a random effect. A LRT was then used to compare this model to the simpler model which includes only HIS. To evaluate the effect of hydroperiod on metamorph mass and number together, I calculated a standardized "total mass" that is corrected by the number of input larvae. This "mass per input larvae' enables the comparison of hybrid and native success across the three larval treatment groups, representing the total mass of genotype-specific metamorphs that would result from the same reproductive effort (i.e., a single clutch from one female). This standardized measure of total mass was included as the response variable in an LMM with hydroperiod as the only predictor and larval treatment as a random effect.

Loci Under Selection:

The program BayEnv (Coop et al. 2010) was used to scan the sequenced target regions for loci that experienced differential selection across the hydroperiod treatments. I analyzed metamorphs from each of the three larval treatment groups separately, using hydroperiod as a continuous predictor. BayEnv calculated a Bayes Factor for each locus after controlling for the underlying variance-covariance that results from uneven sample sizes and shared population history (Günther & Coop 2013). These Bayes factors are used in Bayesian model selection and can be interpreted similarly to the frequentist likelihood ratio (Berger & Pericchi 2015). Following Jeffery's scale of evidence for Bayes factors, I selected loci with a Bayes factor of 10 or greater, which suggests "very strong" evidence for selection (Kass & Raftery 1995). I then analyzed the raw allele frequencies of these significant loci to determine the direction of selection. I built generalized linear models with the frequency of reference alleles (# reference / (# reference + # alternate)) as the dependent variable and hydroperiod as the independent predictor. I extracted the model coefficients to determine the direction (+/-) and magnitude (absolute value) of the shift in allele frequency. I also used the frequency of reference and alternate alleles in the reference panel of 150 pure CTS and 30 pure non-native BTS to identify alleles that were predominantly associated with CTS or BTS. I tested for differences in the number of loci that experienced a CTS versus BTS biased shift in allele frequencies using a 1-sample proportions test. Finally, I constructed a linear mixed model to test the relative strength of the allele frequency shift in CTS versus BTS associated alleles. This model included the absolute value of the marginal effect of allele frequency shift as the response variable and the genotype of the preferred allele as the predictor, with larval treatment as the random effect.

Functional enrichment among genes that experienced hydroperiod-mediated selection was analyzed using the web server "g:Profiler" (Raudvere et al. 2019) to identify gene ontology

terms that were overrepresented in the BayEnv list. This list of genes was compared to the closest model organism, the African clawed-frog (*Xenopus tropicalis*) in addition to the best studied system, humans (*Homo sapiens*). I report the gene ontology terms that had a p-value of less than 0.05 after applying the software's custom "g:SCS" correction for multiple tests.

Results:

Pond and Metamorph Results:

The experimental ponds successfully held water long enough to achieve the desired range of hydroperiods (Figure 2.2). In 2019 ponds held water for 85 to 115 days (5-day intervals) and in 2020 ponds held water between 80 and 110 days (5-day intervals). Across years 249 living tiger salamanders were recovered. Twenty-one were found in the dried pond basin and had failed to complete metamorphosis; these animals were sampled, but excluded from all analyses, since they would have died from heat and desiccation that same day in the wild. The remaining 228 successful metamorphs constitute an across-year survival rate of 8.4% out of the 2,730 larvae that were included in the experiment (Figure 2.2). Survival rates were similar in 2019 (149 metamorphs out of the 1680 total larvae; 8.9% survival rate) and 2020 (76 metamorphs out of 1,050 initial larvae; 7.2% survival rate).

Sequencing Results:

A total of 485 (median = 58) representative larvae were collected from the 10 source ponds used in the experiment. All source pond larvae and 249 metamorphs were sequenced for a total of 734 individuals. Of these, 27 source pond larvae and 1 metamorph were dropped from HIS analysis due to insufficient depth of sequencing resulting from failed library preparation or

ineffective target-capture. Overall, I generated 1,732 billion bases pairs of sequence data across 5.74 billion read pairs. I identified 255,350 loci that contained single nucleotide polymorphisms (SNP), which yielded a total of 5,919 SNPs after filtering, of which 1,788 SNPs were diagnostic between CTS and BTS.

Source Pond HIS:

Non-native alleles were detected in all larval source ponds that had previously been identified as hybrid (McCartney-Melstad unpublished data; Shaffer et al. 2020). However, within the hybrid ponds the larvae consistently had a greater degree of non-native ancestry than expected (Figure 2.3). The average HIS across hybrid source ponds was 0.90 ± 0.065 (median $\pm$ SD). While this does not capture the range of HIS that we had anticipated, it does accurately represent the current HIS dynamic in the hybrid zone. That is, the current frequency of non-native allele frequencies present in wild hybrid ponds was accurately represented in the experimental ponds, yielding results relevant to the current hybrid swarm. The average HIS of native source ponds was 0.07 ± 0.016. This small degree of HIS is likely due to sample missingness or incomplete lineage sorting compared to the reference panel. Therefore, individuals with HIS < 0.10 were considered native and individuals with HIS ≥ 0.10 were hybrid for downstream analyses.

Larval Treatment:

There was no significant difference in the proportion of larvae that survived across the three larval treatment groups (LRT: $F = 0.387$, $df = 2$, $p = 0.683$). Since there was no detectable

difference in larval survival across larval treatment groups, these levels were combined for subsequent analyses. However, larval treatment was included as a random effect in all relevant mixed models to account for differences in the number of larvae and source pond composition of the treatment groups.

Differential Group Survival:

The model comparing the dissimilarity of group distribution using χ^2 was significant at both source pond and family group level. I found a significant increase in source pond dissimilarity (χ^2) as hydroperiod increases (LMM: estimate = 0.03, CI = (0.006, 0.063), p = 0.02, Figure 2.4A), with larval treatment included as a random effect. This increase in dissimilarity suggests that the surviving larvae in longer duration ponds are not evenly distributed across the initial groups that were added to each pond. This non-random distribution of survivors may indicate group-level selection that becomes more pronounced in long duration ponds. I found a similar, but weaker, change in the distribution of individuals across family groups (LMM: estimate = 0.029, CI = (0.002, 0.056), p = 0.047; Figure 2.4B). A graphical representation of this non-random survivorship can be viewed in Figure 2.5. In this heatmap, the initial frequency of larvae across groups can be seen in panels A and C, while the distribution of survivors can be seen in panels B and D.

Larval Survival:

The proportion of hybrid larvae that survived in each pond significantly increased with longer hydroperiods (GLM: estimate = 0.068, CI = (0.038,0.099), p = 2.16×10^{-4}; Figure 2.6A) and this model was favored over the model that excluded hydroperiod (LRT: F = 20.8, df = 26, p

= 1.15×10^{-4}). Native larval survival did not significantly increase with hydroperiod when all points were considered (GLM: estimate = 0.092, CI = (0.006, 0.198), p = 0.070; Figure 2.6B). However, when an extreme outlier pond was removed the relationship was significant (GLM: estimate = 0.088, CI = (0.030, 0.154), p=0.011; Figure 2.6C). The outlier pond ("2020_F") had a 105-day hydroperiod and exhibited an extraordinarily high degree of native survival. Though this pond may represent an interesting biological phenomenon that favors native salamanders, I do not have sufficient data to confirm this hypothesis. The model with the outlier removed was favored over the model that did not include hydroperiod (LRT: F = 9.31, df = 18, p = 0.007). The difference between model predictions for hybrid and native larval survival was positive and increased non-linearly with hydroperiod (Figure 2.6D), though the interaction term between hydroperiod and genotype was not significant (GLM: estimate = 0.024, CI = (-0.057, 0.113), p = 0.575). These results suggest that hybrids maintain a survival advantage over native CTS across all levels of hydroperiod included in this study.

Metamorph Size:

Overall, metamorph mass was greater in 2020 than in 2019 (LMM: estimate = 4.75, CI = (3.69, 5.84), p = 2×10^{-16}), which was likely a result of the lower initial larval densities used in 2020 due to limited breeding in wild source ponds. Metamorph mass was significantly correlated with HIS (LMM: estimate = 7.68, CI= (6.09, 9.24), p = 2×10^{-16}; Figure 2.7A), hydroperiod (LMM: estimate = 1.30, CI= (0.352,2.27), p = 0.0086; Figure 2.8) and hydroperiod^2 (LMM: estimate = -0.006, CI= (-0.011, -0.002), p = 0.010; Figure 2.8), which was included in the same model to account for non-linearity in mass. This model with the quadratic hydroperiod parameter was favored over a simpler model with only HIS and hydroperiod (LRT: dAIC = 4.86, χ^2 =

6.86, p = 0.009). I show the relationship between metamorph mass and HIS (Figure 2.7) and hydroperiod + hydroperiod^2(Figure 2.8) separately to improve interpretability.

Metamorph SVL also significantly increased with HIS (LMM: estimate = 1.66, CI = (1.38, 1.94), p = 2.0×10^{-16}; Figure 2.7B). The model that included both HIS and hydroperiod was not significantly different from this simpler model which only included HIS (LRT: dAIC = 0.22, χ^2 = 2.23, p = 0.136), and is therefore not preferred.

There was a significant increase in the standardized metamorph mass with respect to hydroperiod (LMM: estimate = 0.062, CI = (0.027, 0.093), p = 9.1×10^{-4}; Figure 2.9A), and this model was favored over a model that did not include hydroperiod as a predictor (LRT: dAIC = 9.24, χ^2 =11.2, p = 8.0×10^{-4}). With all data included, the effect of hydroperiod on native mass per larvae was positive, but not significant (LLM: estimate = 0.033, CI = (-0.013, 0.078), p = 0.175; Figure 2.9B) and this model was not favored over a simple intercept model (LRT: dAIC = 0.097, χ^2 = 2.09, p = 0.148). However, when a single extreme outlier was removed, the relationship was significant (LMM: estimate = 0.014, CI = (0.004, 0.027), p = 0.02; Figure 2.9C), suggesting that the near-significant effect of hydroperiod on native larvae is probably biologically significant. This outlier pond was the same outlier from the larval survival analysis ("2020_F"). This model was favored over a model without the hydroperiod parameter (LRT: dAIC = 4.45, χ^2 = 6.45, p = 0.011).

Loci Under Selection:

Across larval treatments, 86 loci exhibited significant allele frequency shifts resulting from the hydroperiod treatment. I found 22 loci (9 BTS- and 13 CTS-biased shifts in allele frequency) in the "half-hybrid" treatment, 34 loci (12 BTS and 22 CTS) in the "mostly-hybrid"

treatment, and 30 loci (17 BTS and 13 CTS) in the "all-hybrid" treatment that had a Bayes factor greater than 10. A single locus (gene = "SRFBP1") was significant in more than one larval treatment. There was no difference between CTS- versus BTS-biased shifts in allele frequency (Prop Test: $p = 0.33$, prop = 0.56, CI = (0.45, 0.66)). Overall, loci that exhibited a CTS-biased shift in allele frequencies had a greater magnitude of effect than BTS-biased loci (LMER: estimate = 3.40×10^{-3}, CI = (1.4×10^{-3}, 5.2×10^{-3}), $p = 5.1 \times 10^{-4}$). However, it is possible that this result reflects the unequal ratio of CTS to BTS associated alleles in the experiment.

There were no enriched gene ontology ("GO") terms in the list of loci under selection when compared with the *Xenopus tropicalis* annotation library. There was, however, a single significant GO term ("hsa-mir-10b-5p") when compared to the human annotation repository (corrected $p = 3.71 \times 10^{-2}$).

Discussion:

In this study, I used a large-scale field ecological experiment to evaluate the potential to use shortened breeding pond hydroperiod as a promising strategy for reducing the success of BTS/CTS-hybrids in California. Previous research on this system has demonstrated that hybrids on average have a fitness advantage over native tiger salamanders, and field populations achieve overall greater non-native allele frequencies in perennial ponds with artificially long hydroperiods. I created 14 semi-natural ponds that mimic natural pond dynamics and tested the effect of pond duration on native and non-native fitness, measured as survival to, and size at, metamorphosis. I also examined the effect of hydroperiod on within-pond cohort shifts in Hybrid Index Scores (HIS) and allele frequencies. My experiments show that hybrid salamanders have greater fitness across the entire range of experimental hydroperiods, and that the disparity

between hybrid and native salamander success increases with longer pond duration. I found no conditions where native fitness was greater than that of hybrids. However, there is a significant decrease in hybrid advantage in shorter hydroperiods, which may sufficiently slow the spread of non-native alleles to be considered as a management strategy.

Differential Group Survival:

Across the hydroperiod treatment, differential survival shifted the distribution of successful individuals away from the initial proportions of larval source pond and family groups (Figure 2.4). It appears that only a few source ponds/family groups grow to dominate the experimental pond survivors in long treatments (Figure 2.5). This could result from a number of different forces, including kin selection. This theory has been extensively tested in a subspecies of the barred tiger salamander, *Ambystoma mavortium nebulosum* in Arizona. Pfennig et al. (1994) discovered that cannibalistic morphs of these tiger salamanders preferentially consumed unrelated larvae, even suggesting that cannibals could distinguish between different levels of relatedness (i.e. sibling vs cousin). Pfennig et al. (1999) later confirmed that this pattern of selective cannibalism was likely driven by kin selection by testing and rejecting several other competing hypotheses. Although the exact mechanism of this kin selection appears variable during different life stages (Mott et al. 2019), it may explain the reduction in source pond and family diversity as hydroperiod increases. Following the classic Wilbur-Collins model of amphibian larval growth (Wilbur & Collins 1973), some larvae will rapidly achieve a greater body size than other individuals in the pond. If there is a source pond or family group component to which individuals achieve this greater size, then preferential cannibalism for unrelated individuals may drive the success of certain groups in my experimental ponds. Increased pond

duration would allow these larvae to reach an even greater size, improving their feeding performance, facilitating cannibalism of larger conspecifics (Reilly et al. 1992). The extra time spent in the ponds would also allow for more opportunity to consume other CTS larvae. In addition, the reduced abundance of food in the late season would also likely increase cannibalism (Anderson et al. 2013). This type of cannibalism may further reduce the success of native CTS if larger BTS hybrids preferentially consume natives in their natal breeding ponds. This dynamic is likely exacerbated by the fact that cannibalism is rare in purely native CTS populations (Ryan et al. 2009a), which suggests that this large predation pressure would favor non-native hybrids. Despite this effect, I did not see a significant difference in the survival of native CTS in the different hybrid density treatments, although the limited sample size reduces the power of this analysis.

Larval Survival:

Hybrid salamanders appear to enjoy greater survival in ponds with longer hydroperiods. This pattern was evident when analyzing data at the pond level, where the proportion of individuals that survived increased with longer hydroperiod. Based on the model predictions, hybrid survival increased from 3.2% in an 85-day hydroperiod, to 20% survival in 115 days (Figure 2.6A). This increase is both statistically significant and biologically important, given the average larval survival rate of 8.4% observed in this study. In native CTS, when one extreme outlier was included, the relationship was similar to the hybrids (1.3% and 17% survival at 85 and 115 days) although this was not significant (Figure 2.6B), however, this pattern was significant when the outlier was removed. When considering the model omitting the outlier pond, CTS experienced about 0.8% survival at an 85-day hydroperiod and 11% at 115 days, a

nearly 14-fold increase (Figure 2.6C). In both models pure CTS consistently exhibit lower survival probability than hybrids. In all hydroperiods, the difference in model predictions (hybrid – native survival; Figure 2.6D) is always positive, which further confirms that hybrids consistently enjoy greater survival than native CTS across different pond durations, which agrees with previous experimental work. Fitzpatrick & Shaffer (2007b) found that selection for heterozygous individuals culminated in greater survival for hybrid CTS larvae in wild populations. This enhanced hybrid fitness derived from increased heterozygosity may explain why hybrid individuals enjoy greater survival across all hydroperiod treatments. The slope of the hybrid model difference curve increases exponentially, suggesting that hybrids enjoy an increasing advantage in survival with the longer hydroperiods. For example, hybrids enjoy a 2.4% greater survival probability than native CTS at 85 days which grows to 10% at 115 days (Figure 2.6D). This confirms the results from Johnson et al. (2013) which found hybrid survival to be approximately twice that of CTS in longer hydroperiods. This consistent pattern explains why hybrids enjoy an increasing survival advantage in ponds with longer durations. This finding also explains previous field studies that observe greater non-native allele frequencies in artificially enhanced, perennial ponds (Fitzpatrick & Shaffer 2007b; Fitzpatrick et al. 2009). Together these results suggest that longer hydroperiods disproportionately increase the fitness advantage of hybrids in the field, facilitating their rapid and persistent expansion.

Metamorph Size:

Metamorph mass was significantly correlated with HIS and hydroperiod. Metamorphs with greater non-native ancestry are more massive than native CTS (Figure 2.7A). The predicted mass of a native CTS at metamorphosis across experiments was 6.5g, while the predicted mass

of a highly non-native hybrid (HIS = 0.90) was 11.2g. This 1.7-fold increase in mass based on non-native ancestry likely plays a tremendous role in the apparent fitness advantage of adult hybrids in the wild. Previous work has highlighted the critical role of mass at metamorphosis in the survival and lifetime fitness of native CTS (Searcy et al. 2014b). Assuming that this pattern applies to non-native hybrids as well as native CTS, this genotype-linked size difference could be driving selection for and possible fixation of non-native alleles in the hybrid zone.

Mass at metamorphosis was also correlated with pond hydroperiod, though not in a simple monotonic relationship (Figure 2.8A). The significant quadratic term ($hydroperiod^2$) in the preferred model accounted for the apparent drop in metamorph mass in the longest hydroperiod ponds (Figure 2.8) It is likely that this inflection occurs due to the increase in the number of metamorphs that survived, coupled with a potential decrease in prey availability in long duration ponds. These late-stage larvae likely experience increased competition limiting their growth. Previous work on a related congener, *Ambystoma talpoideum*, found that increased pond duration did not have a consistent effect on individual mass at metamorphosis (Semlitsch 1987; Semlitsch & Wilbur 1988). However, longer hydroperiod ponds did produce more individuals that successfully completed metamorphosis. It is therefore possible that *A. talpoideum* exhibited a non-linear relationship between mass and hydroperiod, similar to that reported here, driven by the increase in metamorph survival in long duration ponds. This increase in survival would result in more larval competitors and therefore less prey for each individual. I therefore explored the combined effect of increased metamorph survival and mass using a standardized measure of metamorph biomass.

When I compared total metamorph mass that emerged from each pond (after correcting for the number of larvae that were added) I found that longer hydroperiods produced more

metamorph biomass overall. This pattern was quite strong in hybrids, with a linear slope of 0.06 grams per day of hydroperiod increase (Figure 2.9A). These differences can be estimated from the predicted number of grams of metamorph produced for each additional larvae added using a simplified linear model without random effects. This amounts to a standard mass of 0.25g/larva at an 85-day hydroperiod which increases to about 2g/larva at 115 days. This large, 8-fold increase in total hybrid mass can significantly alter the community ecology, since it yields a much greater biomass of hybrid salamander for the same reproductive investment. Native CTS again had a more complicated relationship. With all data points considered, there was no significant relationship with mass, however this was driven by an extreme outlier (Figure 2.9B). Removing the outlier yielded a consistent positive relationship between hydroperiod and total metamorph mass with a slope of 0.01. From the model, Native CTS are predicted to produce essentially zero metamorph mass at an 85-day hydroperiod, which increases to 0.5g/larva at 115 days (Figure 2.9C). Similar to other results in this study, hybrids consistently outperform native CTS in terms of total biomass; however, hybrid larvae benefit 6x more from each additional day of hydroperiod than native CTS. Although shorter hydroperiod may not specifically select for native CTS, it may reduce hybrid advantage enough to slow the spread of non-native genes, compared to populations with unmanaged pond hydroperiods. The efficacy of this management strategy will be examined in future work (Cooper et al. Ch3).

Outlier pond:

There was a single pond ("2020_F") from 2020 that was identified as a statistical outlier in both the survival and metamorph mass analyses. Although this pond had only a moderately long hydroperiod of 105 days, it yielded a greater native survival rate and produced more

massive metamorphs than ponds in similar conditions. However, this pattern was only true in 2020, not in 2019, suggesting that it is not a consistent characteristic of the pond. One possible explanation is that a greater abundance of prey species naturally colonized this pond in 2020. Enhanced resource abundance could explain the increase in survival and mass observed from the pond (Searcy et al. 2015; Takatsu & Kishida 2020). While all ponds were densely clustered, this pond was relatively close to an ephemeral creek at the southern end of the site, though many ponds across hydroperiod treatments shared this proximity. It is possible that more vertebrate and invertebrate prey chose to lay their eggs in this pond, resulting in a greater prey resource for the developing larvae. It may be that high prey densities could reduce the disparity between hybrid and native survival and mass at metamorphosis seen in the main results. Although this may be an interesting biological phenomenon, I do not have sufficient data to evaluate this hypothesis. Future studies should investigate the effects of varied prey density on hybrid and native larval fitness.

Loci Under Selection:

My analyses identified many loci that experienced shifts in allele frequencies that correlated with hydroperiod. These loci may have conferred an adaptive benefit to the larvae enabling some individuals to survive in extreme hydroperiod treatments. Survival in short hydroperiod ponds is predicated on rapid growth, so that larvae can complete development and transform into terrestrial metamorphs before succumbing to desiccation-related mortality. Alternatively, survival in long hydroperiod ponds may be driven by the ability to escape predation and competition from conspecifics. It is therefore plausible that these opposing factors drive selection for different alleles across the hydroperiod gradient. I found 86 candidate genes

that may have undergone such selection through the course of this experiment. While I follow the BayEnv software's recommended Bayes Factor cutoff of 10, there are likely some false positives in this list of 86 genes. Though this software does not provide q-values or FDR estimates, previous comparisons of similar program found BayEnv to have the greatest power and least error in most modeled scenarios (Villemereuil et al. 2014).

Few studies have investigated loci under hydroperiod-mediated selection in amphibian systems. However, some studies have looked broadly at environmentally-driven selection in wild populations. A recent study on the nine moor frog (*Rana arvalis*) in Sweden identified 153 loci that exhibited a significant correlation with breeding-time (Rödin-Mörch et al. 2021), though it is important to note that this metric only captures the window of time that adults breed in pools rather than my focus of time to metamorphosis. Of these loci, only 53 mapped to annotated genes and proteins. Though none of these reported genes were identified in my results, this study did find several genes related to development and growth, which were also highlighted in the present study as well. Similarly, a study on an Australian frog identified 413 loci under environmentally driven selection, likely driven by rainfall and evaporation (Cummins et al. 2019), though the single annotated gene reported in this study (protein kinase C) was also not identified in my results. Although there is little overlap in the loci under selection across studies, this may result from the relatively poor representation of annotated amphibian genes, coupled with the difficulty of isolating and sequencing complex amphibian genomes (Treangen & Salzberg 2012; McCartney-Melstad et al. 2016). New sequencing technology coupled with expanding genomic resources for difficult amphibian systems should improve the repeatability of these analyses in years to come (Storfer et al. 2009). Future studies should analyze loci under selection in wild ponds that consistently experience a range of hydroperiods (e.g., consistently short hydroperiod

ponds, consistently long, etc.) in order to evaluate the repeatability of loci highlighted in this study.

The 86 outlier genes are functionally enriched for a single Gene Ontology term, "hsa-mir-10b-5p". This term corresponds to a micro RNA ("miRNA") that regulates the expression of a diverse group of genes that stimulate growth. Specifically, this group has been linked to the regulation of lipid metabolism (Zheng et al. 2010). Previous studies on amphibian metamorphosis have shown that lipid metabolism is dynamic; it is initially low to increase fat stores, then metabolism increases to fuel metamorphosis (Sheridan & Kao 1998). In a congener, *Ambystoma opacum*, the accumulation of these fat stores significantly increases post-metamorphic survival (Scott et al. 2007). It may be that variants of the "mir-10b" gene-family confer increased survival in short or long hydroperiod treatments, resulting in consistent selection across larval treatments.

This family of miRNAs have also been shown to promote vascular endothelial growth during the development of blood vessels (Hassel et al. 2012). This function may be critical in drying ponds since oxygen content is often low (Sacerdote & King 2009). It may be critical to promote substantial vasculature in the gills to facilitate the rapid growth required to escape a drying pond. A recent study in the congener *Ambystoma velasci* highlights numerous genes that are differentially expressed during metamorphosis that are responsible for increased vascularization (Palacios-Martinez et al. 2020).

The protein coding gene Serum Response Factor Binding Protein 1 ("SRFB1") was significantly correlated with hydroperiod in more than one larval treatment, suggesting a greater importance for this gene in mediating survival in short vs. long duration ponds. Studies on mice suggest that SRFB1 is a translational regulator that plays a significant role in cardiac aging and

mitochondrial function (Zhang et al. 2004). Expression of this gene has been shown to reduce mitochondrial size and oxygen consumption (Zhang et al. 2016). If this functions in a similar manner in tiger salamanders, it may play a role in modulating the rate of metabolism in different hydroperiod regimes. For example, SRFB1 variants may elevate metabolism in short hydroperiod ponds to ensure adequate growth and development so that larvae may complete metamorphosis before the ponds dry. Conversely, it may be important to reduce the rate of metabolism in long duration ponds if most of the larval vertebrate and invertebrate prey have transformed and left the aquatic habitat. It is important to note that the explicit function of this gene in the CTS system is unknown, therefore future studies should investigate the significance of this gene in salamander metabolism and development through empirical studies including knockout (Wu et al. 2018) or quantitative trait loci experiments (Beavis 1998).

Conclusion:

In this study, I have identified several components of tiger salamander fitness that are affected by pond duration. I found that hybrids have a significant advantage over native CTS with respect to both size and survival, and that both increase dramatically as pond hydroperiod becomes longer. It may therefore be prudent to manage pond hydroperiod to remove a large degree of non-native advantage in ponds within the hybrid zone. This management action may minimize the difference in fitness enough to slow the spread of non-native alleles across the landscape. However, it is likely necessary to combine hydroperiod restoration with other management strategies to ensure the survival of pure native genotypes in the wild.

In Chapter 3, I will assess the impact that hydroperiod management could have on hybrid demographic success and its impact on non-native allele frequencies in hybrid populations.

Using the most recent advances in CTS demographic modeling, I will quantify these key parameters to help managers determine the exact cost and benefit of enacting such measures, while evaluating their potential to slow the spread of non-native genes across the landscape.

CTS serve an important ecological role as apex predators in vernal pool communities. Disruption of this role can, and does (Searcy et al., 2016) have cascading effects on the trophic system, significantly affecting other endemic species. It is therefore critical to implement active measures to prevent further hybridization or extirpation of the species. There are no easy solutions to control the spread of non-native alleles on the landscape; however, managing pond hydroperiod, particularly for key ponds within the hybrid zone, may represent a relatively inexpensive strategy to reduce the relative fitness of hybrid individuals, and therefore slow the spread of non-native alleles.

Chapter 2 Figures

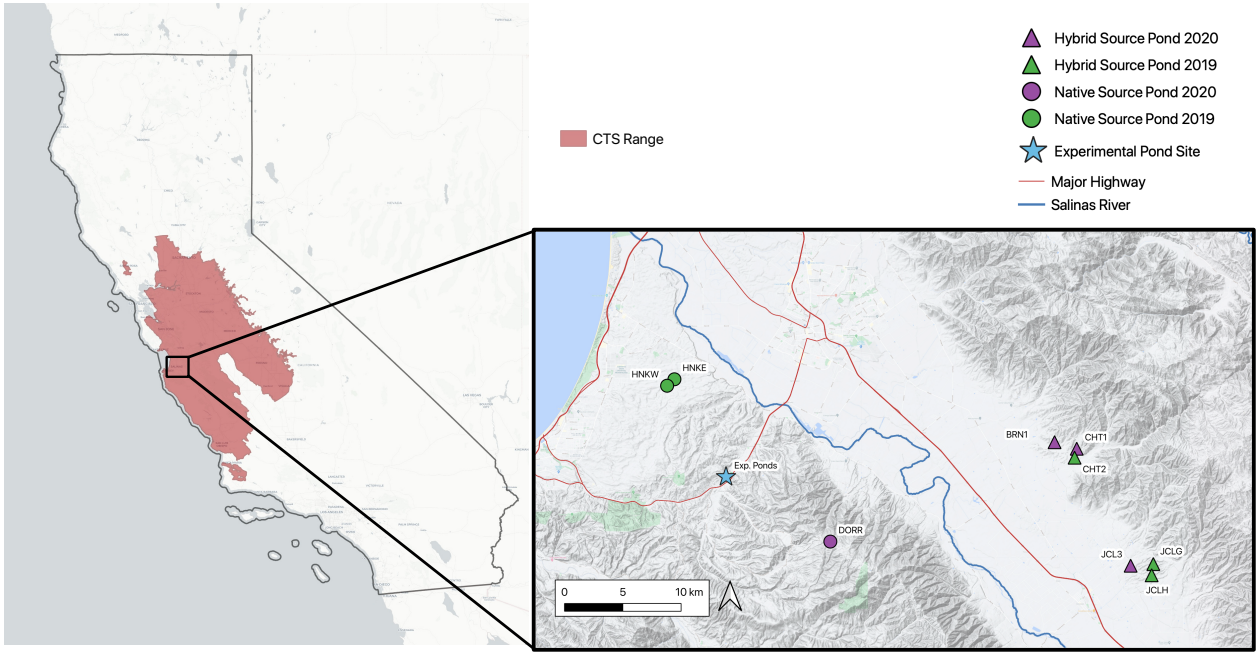

Figure 2.1: Map of California (on left) with the California tiger salamander range highlighted in red. Map insert (on right) shows an expanded view of the Salinas Valley (Monterey County, CA) with points indicating the location of larval source ponds and experimental ponds. Triangles represent hybrid source ponds and circles represent native source ponds. Green points denote larvae that were collected in year 2019 and purple were from 2020. The blue star indicates the location of the constructed ponds used in the hydroperiod experiment. These ponds are located on the southern edge of the Fort Ord National Monument (Monterey County, CA).

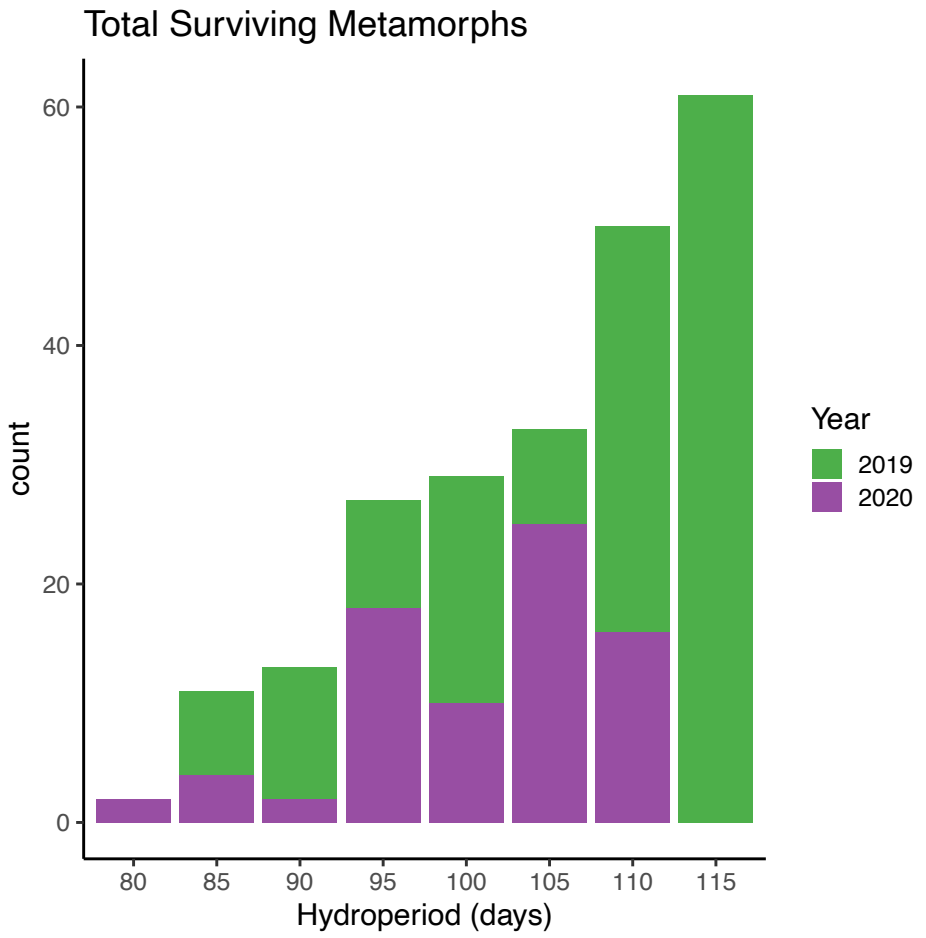

Figure 2.2: The total number of successful metamorphs captured in 2019 and 2020 across hydroperiods.

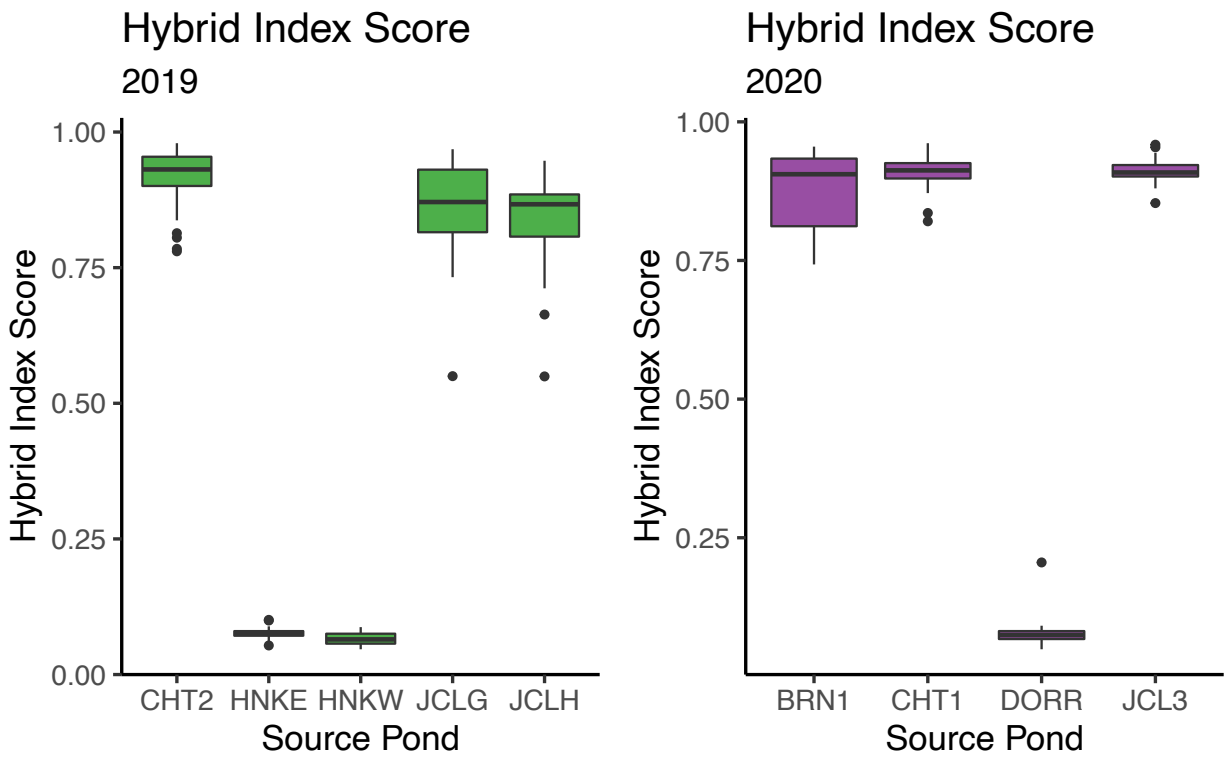

Figure 2.3: This figure demonstrates the Hybrid Index Score ("HIS") of each of the source ponds from which larvae were collected. HIS is scaled from 0 (completely native CTS) to 1 (completely non-native BTS). Green bars are source ponds from 2019 and purple bars are from 2020.

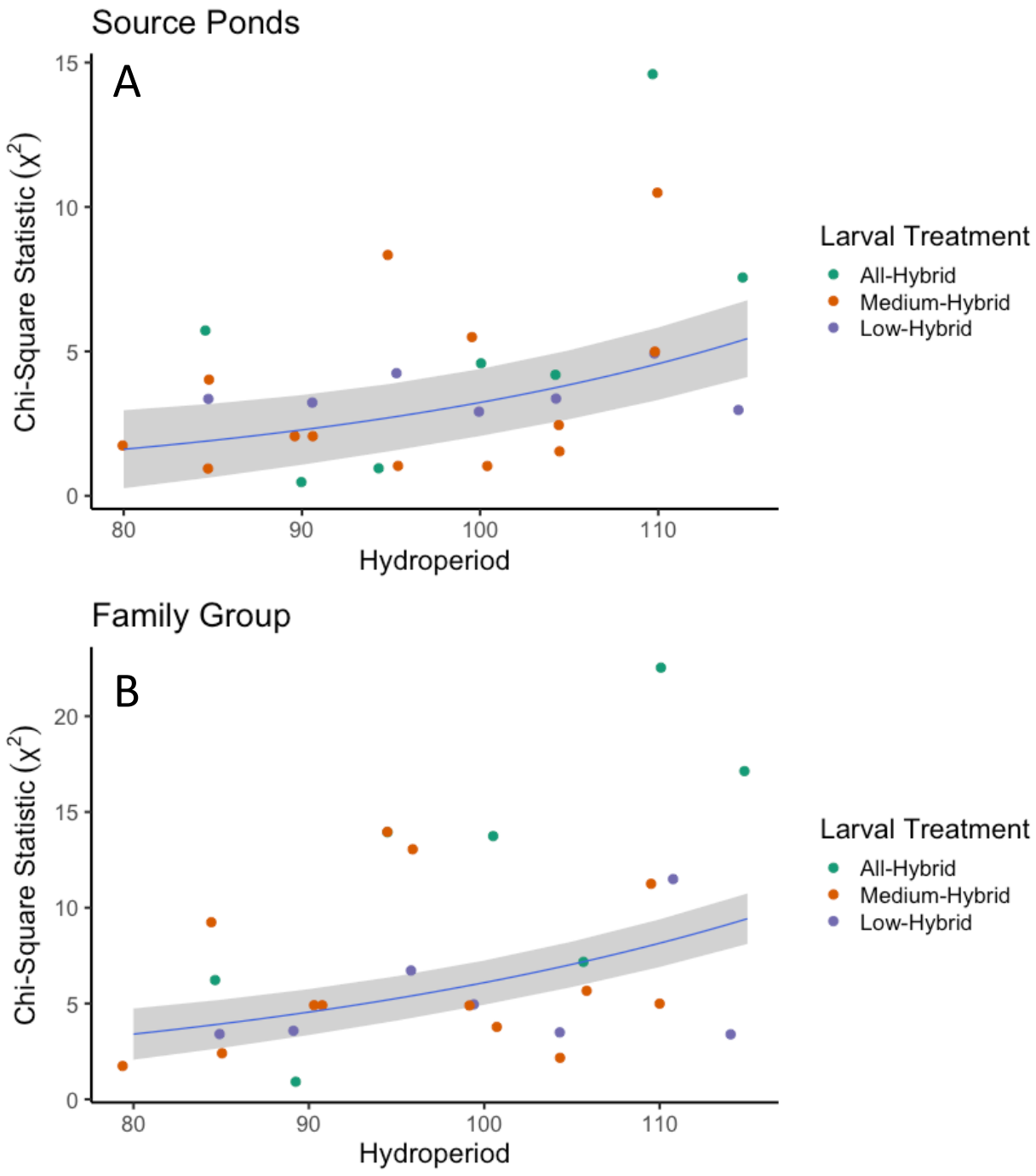

Figure 2.4: This figure shows the relationship between hydroperiod and survivor group distribution. The response is χ^2 which represents the departure from the starting larval group proportions, calculated as the sum of (observed-expected)^2 / expected). Points were jittered on the x-axis to show overlapping values. The panels show the increase in source pond (A) and family group (B) dissimilarity as pond hydroperiod increases. These results suggest that the larvae that survive in longer duration ponds are not evenly distributed across the groups that were added to each pond. This non-random distribution of survivors may indicate group-level selection that becomes more pronounced in long duration ponds.

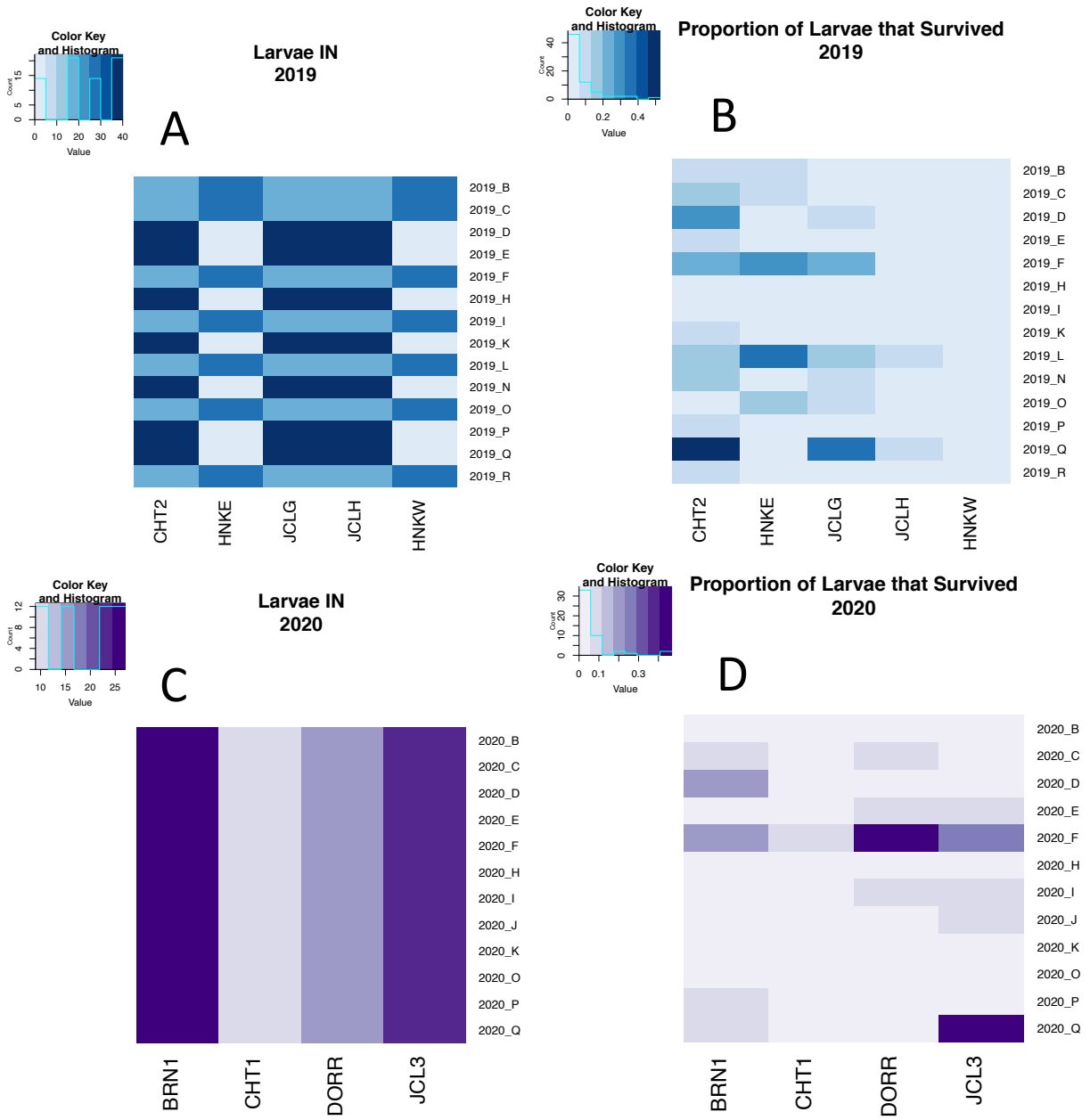

Figure 2.5: This figure shows heatmaps of the number of larvae from each source pond that were added to the experimental ponds (left plots, A and C) and the number of surviving metamorphs that emerged from the ponds (right plots, B and D). This demonstrates the strong dissimilarity between the starting and ending proportions. There is not a single source pond that performs exceptionally well across ponds. However, there appears to be an unequal distribution of surviving metamorphs. It appears that 1-2 source ponds make up the majority of all metamorphs that emerge from an experimental pond, suggesting a strong source pond/family group effect.

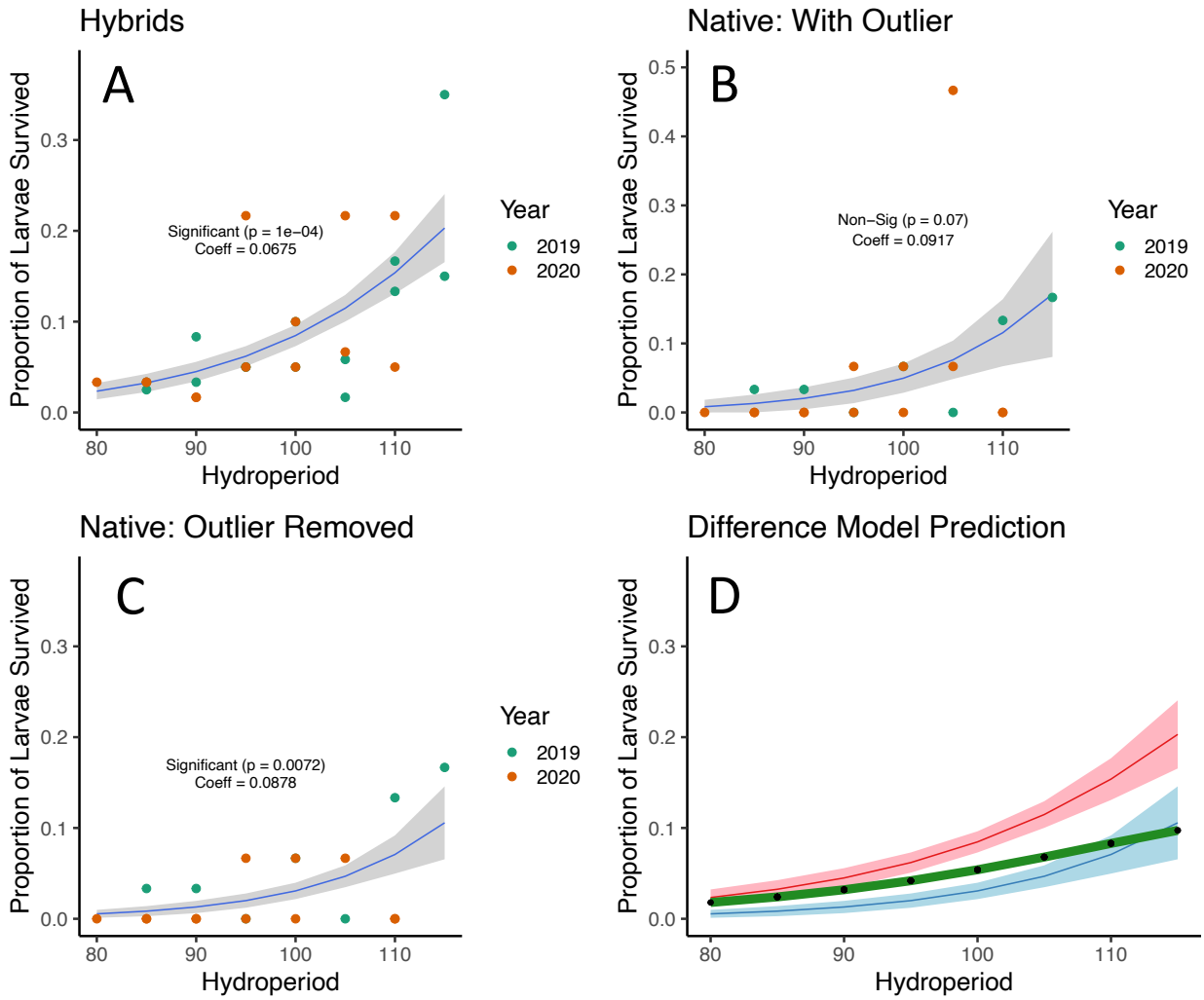

Figure 2.6: These figures show the relationship between larval survival and hydroperiod. Models are fit using a quasi-binomial error distribution in a Generalized Linear Model. The points each represent a single pond and are colored based on the year which was included as a random effect in the model. Model coefficients and p-values are included in the plot. The panels show the model fit for hybrids (A), natives with all data points considered (B), natives with the extreme outlier removed (C). Panel D shows the difference (green line, thickness arbitrary) between the marginal effects of the hybrid (red line) and native (blue line) relationships (hybrid – native).

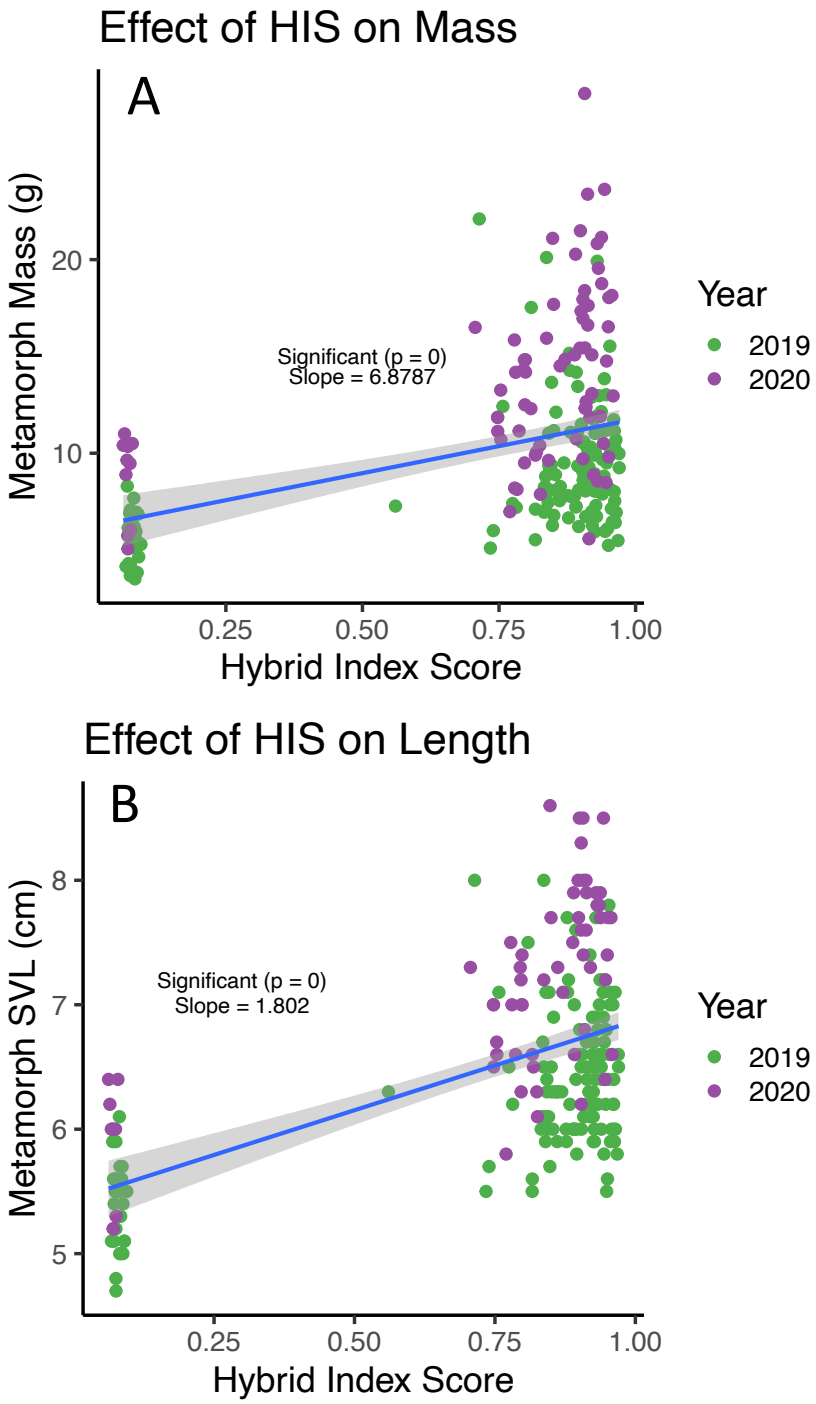

Figure 2.7: The effect of Hybrid Index Score ("HIS") on metamorph mass (A) and Snout-to-Vent Length ("SVL"; B). These figures show the fit of the linear model fit. Points represent a single individual colored by year which was included as the random effect in the linear mixed model.

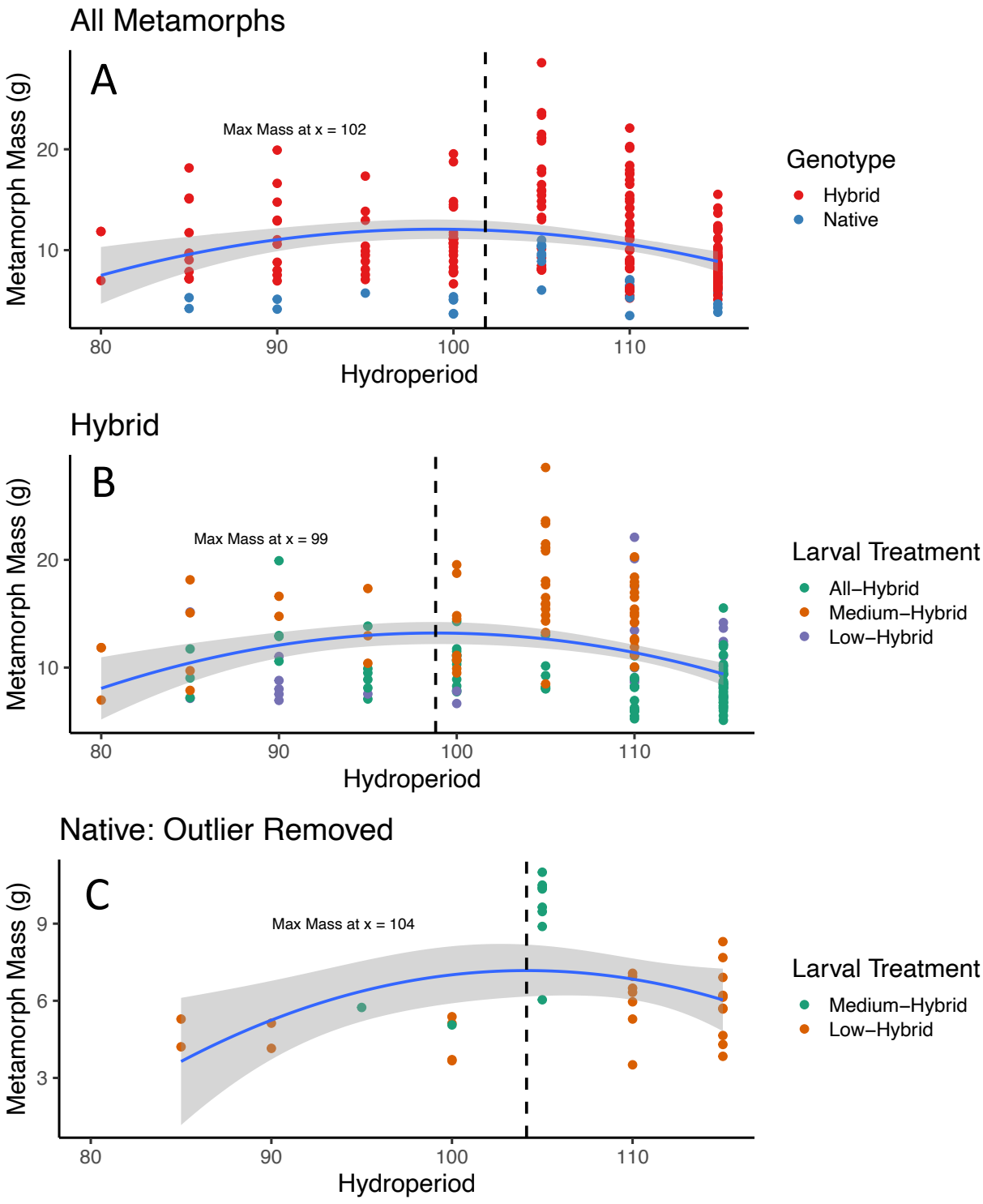

Figure 2.8: Relationship between Mass and Hydroperiod for All (A), Hybrid (B) and Native (C) metamorphs that emerged from the experimental ponds. Each point is a metamorph colored by the larval treatment group, which was included as a random effect in the linear mixed model. The model included a quadratic term, hydroperiod^2, which accounts for the decrease in mass in long hydroperiod treatments.

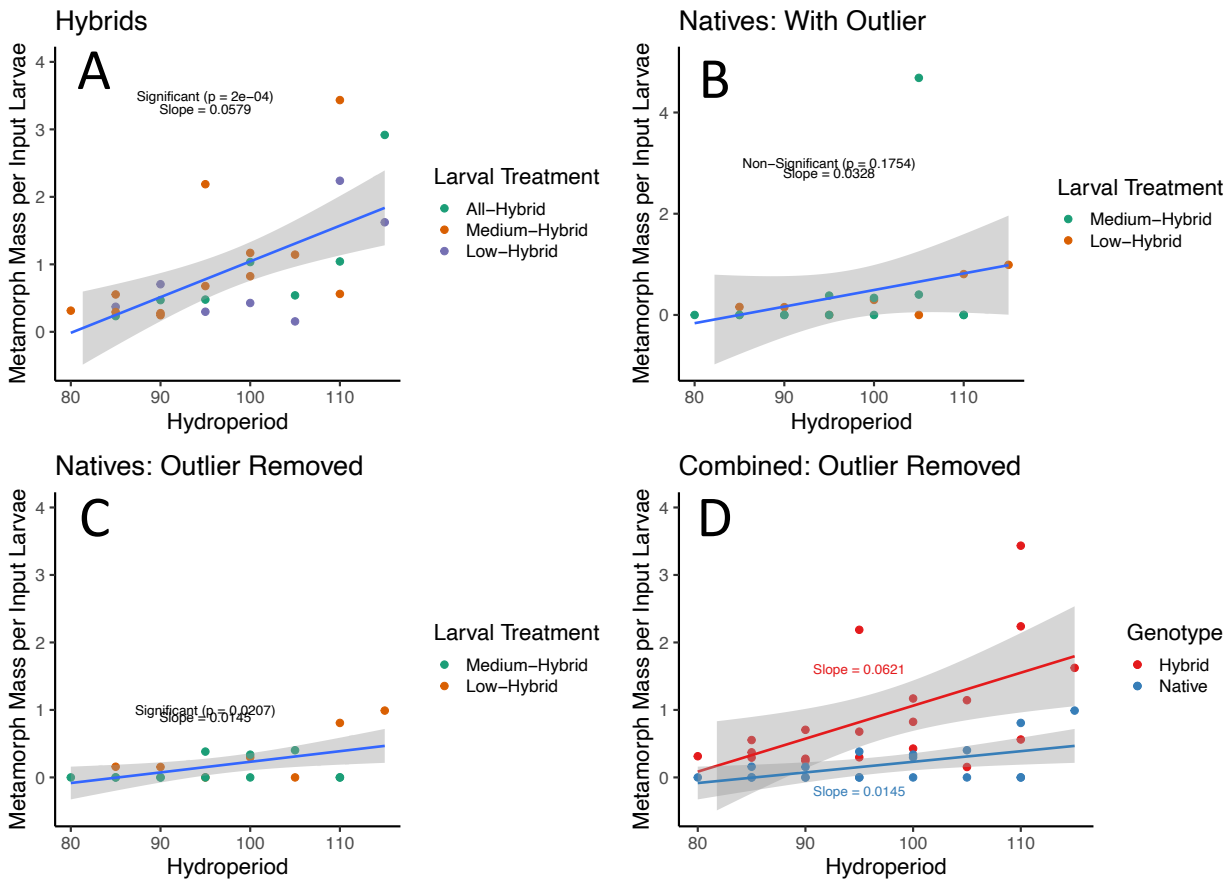

Figure 2.9: This figure shows the relationship between standardized total metamorph mass and hydroperiod. Standardized total mass incorporates both the mass and the number of metamorphs that emerged from each pond. This value is calculated as the sum of all metamorph mass that emerged from a pond, divided by the number of larvae that were added to the pond. This total mass represents the total mass of metamorphs that would be produced from a similar reproductive effort, and is therefore comparable across larval treatments. The panels show the linear fit for hybrids (A), natives with all data points considered (B), natives with the extreme outlier removed (C), and both hybrids and natives plotted together (D). Each point represents the sum of metamorph mass of a specific genotype for a given pond. Points are colored by larval treatment which is included as a random effect in the linear mixed model.

Chapter 3

Title:

Applying individual-based demographic simulations to evaluate hydroperiod management as a strategy to reduce non-native hybridization in the California tiger salamander system (*Ambystoma californiense*).

Abstract:

The introduction of invasive species presents one of the most challenging threats to native biodiversity. This threat is compounded when hybridization occurs between threatened native species and an introduced relative, complicating efforts for species recovery. Non-native hybridization between the endangered California tiger salamander ("CTS", *Ambystoma californiense*) and the introduced barred tiger salamander ("BTS", *Ambystoma mavortium*) presents a difficult problem for conservation practitioners. Reclusive life history and cryptic hybridization make eradication programs difficult to implement. This study seeks to evaluate hydroperiod management as a tool to conserve native CTS populations impacted by hybridization with introduced BTS. Adapting the most extensive and accurate Integral Projection Model ("IPM"; Searcy et al. *in press*) for CTS to an individual-based model enables unparalleled accuracy in evaluating alternative management solutions. Using data from a large-scale field ecological study, I parameterized functions that use breeding pond hydroperiod and individual-specific non-native ancestry to estimate larval survival and mass at metamorphosis. From this adapted IPM model I estimate the intrinsic population growth rate (λ), density-dependent carrying capacity (K), and 100-year population viability (PVA) for a range of demographic

scenarios of varying hydroperiod and hybrid frequency. Together these results are used to assess the validity of hydroperiod management to reduce the success of hybrid tiger salamanders in the field. I suggest the following strategy for reducing non-native tiger salamander success on the landscape. First, native populations around the periphery of the hybrid zone should be managed intensively to bolster them against hybrid immigration. This may include managing ponds to ensure an adequately long hydroperiod in most years to support a robust population. Second, ponds within the hybrid zone should be managed to reduce pond hydroperiods, which will limit population sizes and discourage hybrid dispersers. This strategy may represent a convenient tool to reduce the success of largely-non-native ponds without a costly eradication program, while maintaining the natural function of the vernal pool ecosystem. Finally, research into methods that enable rapid detection and targeted removal of hybrid individuals from key population may still be required.

Introduction:

Species conservation requires a thorough understanding of the underlying demographic processes that govern threatened populations. Adaptation of quantitative models, such as demographic models, can significantly improve conservation actions (García-Díaz et al. 2019). Such models can be used to compare alternative management strategies for taxa that would otherwise be impossible to compare (Chapron et al. 2003; Wiens et al. 2017; Brooks 2020). Though the accuracy of these models have sometimes been questioned (Beissinger & Westphal 1998; Coulson et al. 2001; Ellner et al. 2002), other studies have demonstrated the accuracy and usefulness of these models for predicting population extinctions and comparing the relative

extinction risk of different ecological scenarios (Brook et al. 2000). When properly parameterized and specifically applied, population models can be vital tools for making informed management decisions (Chaudhary & Oli 2020).

The recent development of Integral Projection Models (IPM) has greatly improved the accuracy and predictive power of demographic models (Easterling et al. 2000). IPMs eliminate the need to divide populations into discrete stage classes with constant demographic parameters for each group. Instead, IPMs predict demographic parameters as a function of a critical phenotypic character, like body mass, that allows one to incorporate individual variation in the model. Since their introduction, these models have gained considerable attention due to their increased explanatory power (Jongejans et al. 2011) and adaptability to accurately describe complex life histories (Ellner & Rees 2006). IPMs can be used to estimate demographic parameters similar to a standard matrix model (Easterling et al. 2000), including: 1) Population growth rate (λ) without density dependence (e.g. Canessa et al., 2018; Lown et al., 2020); 2) Sensitivity/Elasticity analyses of small changes in vital rate (reproduction and survival) functions on population growth (Canessa et al. 2018; Lown et al. 2020); and 3) Demographic simulations incorporating density dependent population growth with environmental stochasticity to project population dynamics (Rees & Ellner 2009). Demographic simulations built around high quality IPMs are extremely valuable for identifying and quantifying threats to specific populations. These simulations can model multiple ecological or management scenarios, providing insight into the dangers and benefits that each may afford. These simulations may include population viability analyses (PVA), which incorporate environmental variability into demographic models to assess the stability of populations (Beissinger & McCullough 2002; Lacy 2019). The ability to assess alternative scenarios and quantitatively evaluate their effects on focal populations allows

for clear and direct management recommendations that are necessary for effective conservation (García-Díaz et al. 2019).

In this study, I adapt a recently developed IPM for the endangered California tiger salamander (*Ambystoma californiense*; hereafter, "CTS") to evaluate specific ecological scenarios that may guide management of the species. The CTS is a federally and state-protected species that inhabits grassland ecosystems in central California, and is endemic to the state. Individuals spend the majority of their lives in underground in rodent burrows, from which they emerge during winter rain events both to feed and breed in temporary rain-filled vernal pools and ponds (Trenham & Shaffer 2005a). CTS lay eggs that hatch into fully aquatic larvae that require at least 90 days to grow and complete metamorphosis (Johnson et al. 2013). Ponds that fail to hold water for approximately 90 days often result in mass larval mortality. A number of threats both contribute to the decline and impede the recovery of this salamander, including invasive species (Fisher and Shaffer 1996), habitat loss (particularly agricultural conversion) and climate change (Davidson et al. 2002b). However one of the most complex issues impeding the recovery of this species is hybridization with an introduced congener, the barred tiger salamander (*Ambystoma mavortium*; hereafter "BTS"; Riley et al. 2003b; Fitzpatrick et al. 2010). After its intentional introduction approximately 50 years ago into the Salinas Valley (Monterey County, CA), non-native BTS have established and expanded their range through hybridization. Hybrids are fertile, and appear to enjoy greater fitness than either CTS or BTS parental genotypes (Fitzpatrick & Shaffer 2007d; Johnson & Johnson 2010; Cooper & Shaffer 2021). This hybrid advantage threatens to erase the unique diversity inherent in native CTS populations through genomic extinction. Furthermore, hybrid salamanders have been shown to alter California central valley vernal pool communities (Ryan et al. 2009b; Searcy et al. 2016), threatening the

persistence of these unique ecosystems and the many endangered species that they contain . A critical conservation need is to develop and evaluate management solutions that reduce the success of non-native hybrids in the field.

Hybrid success appears to be related to pond hydrology. Frequently, ponds that are used for agriculture or livestock have been modified to extend the amount of time that ponds hold water ("hydroperiod") beyond their natural ephemeral state. Previous work has demonstrated that non-native allele frequencies increase in ponds with artificially long hydroperiods (Fitzpatrick & Shaffer 2007a). Subsequently, a controlled mesocosm study investigated the effect of hydroperiod on larval survival and mass at metamorphosis (Johnson et al. 2013), two demographic parameters essential to CTS ecology (Searcy et al. 2014b, 2014c). This study found that longer hydroperiods strongly favor non-native genotypes, while shorted hydroperiods favored native genotypes, although to a much more limited degree. While promising, Johnson et al. (2013) used a controlled experimental design at low density with unlimited food resources. Before hydroperiod modification can be considered as a viable strategy for reducing relative hybrid fitness, it must be tested under natural ecological conditions.

My recent work employed 14 large (30-foot diameter) constructed ponds, situated at the edge of the CTS hybrid zone, to evaluate this apparent pattern in the most natural setting possible (Cooper et al. Ch2). The results of this multi-year experiment yielded mixed results: while long hydroperiods disproportionately favor non-native hybrids, short hydroperiods do not appear to favor native CTS. The short hydroperiod treatments reduced, but did not eliminate, the relative advantage of hybrids, and resulted in lower survival and mass at metamorphosis for all genotypes.

The present study seeks to answer three vital questions to definitively evaluate hydroperiod management as a conservation tool in the hybrid CTS/BTS system: 1) Can short hydroperiods (e.g., 80 to 95 days) support stable CTS populations? 2) Can short hydroperiods sufficiently reduce hybrid success to reduce non-native allele frequencies, or at least slow their spread? And 3) Can short hydroperiod increase population-level resistance to hybrid invasions? Adapting the most extensive and accurate IPM (Searcy et al. *in press*) for CTS enables unparalleled accuracy in evaluating these critical conservation questions. I take this approach one step further by modifying the IPM to incorporate individual-level estimates of larval survival and mass at metamorphosis derived from a previous large-scale hydroperiod experiment (Cooper et al. Chapter 2). This previous study quantified the success of hybrid and native salamanders in experimental ponds under natural conditions, across a range of experimental hydroperiods using an array of 30-foot diameter ponds. From this experiment, I parameterized functions that include breeding pond hydroperiod and an individual's non-native ancestry (Hybrid Index Score; hereafter "HIS"; Johnson et al. 2010) to estimate larval survival and mass at metamorphosis in a new, forward-in-time demographic model. From this modified IPM model, I estimate the intrinsic population growth rate, density-dependent carrying capacity, and 100-year population viability for 54 demographic scenarios spanning a wide range of hydroperiods and hybrid frequencies. I use these results to assess the potential to use hydroperiod management to reduce the success of hybrid tiger salamanders in the field.

Methods:

Integral Projection Model (IPM) Adaptation:

I adapted the CTS demographic model constructed by Searcy et al. (*in press*). This model combined multiple long term ecological studies on CTS (Trenham et al. 2000a; Searcy et al. 2014c) to construct an IPM for CTS. The authors evaluated the accuracy of the IPM by comparing model estimates with empirical demographic data. They then use PVAs to quantify the amount of upland habitat that is required to sustain a CTS population in the wild. This model has two distinct stage classes: metamorph and juvenile/adult. Metamorphs begin the season as an aquatic egg, which hatches into an aquatic larva, grows, and then undergoes metamorphosis and transitions to terrestrial life all in the first year. After this first year, all individuals transition to the juvenile/adult class, where they eventually mature into reproductive adults. All of the demographic functions in this model are fit based on an individual's mass, the importance of which has been well documented in CTS (Searcy et al. 2014b, 2014c). Specifically, the IPM uses mass from the previous year to predict the new mass of the present year using a growth function. It then uses this estimated mass to project annual survival, maturity and fecundity for each size-class. These functions are fit using long term mark-recapture data collected from two 10-year drift fence/pitfall trap studies in Solano and Monterey Counties, California.

The original IPM bins each individual by mass into 122 discrete groups. The model then uses two distinct kernels to construct the transition matrix. The first kernel is the product of survival and growth, representing the change in size of an individual if it survived to the next year. A second fecundity kernel, calculated as the sum of the size-based products of growth, survival, and fecundity, estimates the number of offspring an individual would produce given their change in size if they survived to the next year. These two kernels are applied to each stage-class of the model, using separate equations for metamorphs and juvenile/adults. This model uses

probability densities for each of the traits and vital rates, and individual variation is not identified in the model. For example, there may be 50 individuals in the 20g mass bin, so rather than predicting a new mass for each of the 50 individuals using the growth function, the model uses a probability density of new masses for the entire group simultaneously. A new distribution of masses is created, with no connection between yearly values at the individual level. While this model is incredibly useful and efficient for population level analyses, it does not allow individual traits to transition across years and therefore affect the demographic simulation.

I modified this approach, constructing an individual-based model that incorporates individual variation and projects shifts in these traits over time. Specifically, I am interested in the effects of hydroperiod and HIS on CTS demography. I include these parameters by augmenting the functions that predict mass and larval survival. From my previous hydroperiod experiment (Cooper et al. Chapter 2), I fit log-linear models that use hydroperiod and HIS to predict metamorph mass and larval survival-to-metamorphosis. These functions are applied to both density-independent and density-dependent versions of the demographic model. This individual-based implementation of the IPM includes all demographic functions taken directly from Searcy et al. (*in press*) unless otherwise stated. I accomplish this by modifying the demographic functions to accept single values for mass and return a single prediction, rather than a probability density. Each new value is assigned to that specific individual, replacing the previous value. At a basic level, this meant replacing the r-function "dnorm" (which generates a probability density from an input distribution) with "rnorm" (which draws a random number from the probability distribution). I also restructured the survival, maturity, and breeding probability functions. In the original model these represented probabilities of an event occurring (i.e., 0.2 probability of individuals in this size-bin dying). In my model, I used these probabilities

to draw a binary response for each individual using the "rbinom" function (i.e., 0 = the individual died or 1 = the individual lived). I expect that these changes to the IPM will produce more stochasticity in small populations, but that this individual-based stochasticity more accurately represents natural conditions and population variation.

Demographic Scenarios:

Each simulation included a wide range of demographic scenarios with specific conservation applications. I evaluated nine levels of the "hydroperiod" treatment, from 80 to 120 days in 5-day intervals (e.g., 80, 85, 90, etc.). These levels were chosen to replicate the previous hydroperiod study which is used to parameterize key model functions (Cooper et al. Ch2). The "proportion of hybrids" treatment consisted of populations with a specific ratio of hybrid/native individuals. I included 6 levels of hybrid proportions spanning 0 to 1 by 0.2 (0, 0.2, 0.4, …). For example, a population of 100 individuals with 0.2 proportion of hybrids initially contains 20 hybrids and 80 natives which mate at random. A hybrid proportion of 0 or 1 indicates all native or all hybrid populations, respectively. Each hybrid individual was simulated with an HIS of 0.75 and each native individual with a HIS of 0.05. This non-zero value for native HIS reflects the average native HIS recorded in the previous hydroperiod study, due to uncertainty in the HIS calculation (Cooper et al. Ch2). This resulted in a total of 54 simulations per demographic model (9 hydroperiod levels x 6 hybrid-prevalence levels).

Density-Independent Model and Population Growth Rate (λ):

Density-independent models are useful for comparing intrinsic growth rates across demographic scenarios. The intrinsic growth rate, or lambda (λ), describes population growth

under “ideal” or low-density scenarios, when density dependent factors are not operating. If lambda is less than 1, the population decreases, if lambda is greater than 1 the population increases; populations with greater lambda grow more rapidly and are thus able to recover more rapidly from events that reduce population size.

The general model framework is outlined in Table 3.1. This simulation was run for 15 years, allowing enough time for the population to experience exponential growth, without requiring excessive computational time which likewise grows exponentially. Each year the number of juveniles/adults (Nt) is recorded. Lambda is estimated as the slope of the log-transformed Nt with respect to time from years 5 to 15. Years 1-5 include transient dynamics that reflect the starting conditions and not true population growth and are therefore not considered. The slope is determined using the linear model function “lm” in the R statistical language (R. Core Team 2013). This model is iterated 100 times to fully sample the variation inherent in the stochastic functions.

Density-Dependent Model and Carrying Capacity:

The density-dependent model is the same as the density-independent model, but includes several population-limiting modifications. The original CTS IPM by Searcy et al. (*in press*) incorporates the effect of egg density on two vital rates: larval survival and metamorph mass, both of which were estimated from field and mesocosm studies. Here egg density, which was inferred from field data (Trenham et al. 2000a), serves as a proxy for larval density. The first model included log-transformed egg density and log-transformed larval survival which exhibited a negative linear relationship: as egg density increases, larval survival decreases. I adapt this model to this present simulation by centering the function on the larval survival probability that

is predicted using my hydroperiod and HIS-based model. Therefore, larval survival predicted from the hydroperiod and HIS functions represents survival at average egg density. I then use the slope determined from Searcy et al. (*in press*) to account for the increase in larval survival at low densities and decrease in survival at high densities.

The second density-dependent model from Searcy et al. (*in press*) was defined as the negative linear relationship between log-transformed egg density and log-transformed mass at metamorphosis. This relationship was adapted for the present study by similarly re-centering the predicted metamorph mass on the value determined from the HIS and hydroperiod dependent model. Thus, average egg density observed in the field would produce metamorph mass equal to the model predictions and increases or decreases in density would result in smaller or larger individuals, respectively.

These density-dependent simulations yield the estimated carrying capacity on which the population converges. The carrying capacity (K) can be estimated as the average population size (Nt) once births and deaths reach an equilibrium, which was visually confirmed in pilot simulations. I ran 100 iterations of the density-dependent model to sample the variation in estimates of K that result from the stochastic demographic functions, and report K as the median adult population size from years 50 – 100 across the 100 model iterations. The effects of hydroperiod and the initial proportion of hybrids on K were estimated using log-linear regression.

Population Viability Analysis (PVA):

Environmental stochasticity was incorporated into the model to assess the long-term viability of each demographic scenario in the model. It is well established that the amount of

rainfall in a given year drastically affects the magnitude of CTS breeding and recruitment. Low rainfall years result in fewer adults emerging from aestivation to breed (Trenham et al. 2000a), and reduces offspring survival through metamorphosis. Therefore, extended droughts may lead to significant population reduction and possibly extinction.

I adapted two functions from Searcy et al. (*in press*) to account for this environmental effect on population vital rates. First, I used the cumulative precipitation from December through January to scale the number of females that emerged to breed. I used the generalized linear model from Searcy et al. (*in press*) that estimates the number of breeding females from the December – January precipitation, the period when most females emerge to breed (Searcy & Shaffer 2011). Second, I used the cumulative precipitation from October through June to scale larval survival probability. Searcy et al. (*in press*) used empirical data to fit a three-component, piecewise linear model that predicts the proportion of successful metamorphs given the October – June precipitation. This model includes two inflection points, the lower point defines a level of rainfall (404.5mm) below which there is complete reproductive failure. This point likely reflects the minimum rainfall required for the breeding pond to support the necessary hydrology for CTS to complete their larval development. The upper inflection point corresponds to the amount of rainfall (674.5mm) above which all larvae are predicted to survive, after density-dependent larval survival is taken into account. Between these two points, the model predicts a linear increase in larval survival from the lower inflection point (survival = 0) to the upper inflection point (survival = 1). It is important to note that these values were calculated for native CTS populations, and may differ slightly for hybrids. I discuss this limitation in the discussion section.

Historical climate data were incorporated into the model to evaluate relative population viability given environmental stochasticity. I implemented the same 96-year precipitation records from the Vacaville and Nut-Tree Airport Weather Stations as Searcy et al. (*in press*) to simulate annual rainfall conditions. I randomly sampled these rainfall data, with replacement, for each year of the simulation. It is important to note that future climate may have a negative impact on population persistence, and should be considered when evaluating absolute persistence probability. However, in this study I am comparing different demographic scenarios and their relative effects on population persistence. For this reason, I chose to use historical data since it has a finer resolution and encompasses true annual variability. Each model iteration ran for 100 years, at which time the population size (N100) was recorded. Populations that dropped below the 3-individual quasi-extinction threshold used in Searcy et al (*in press*) were considered extinct. The population HIS was also recorded to track changes in the frequency of non-native alleles. Each demographic scenario was iterated 100 times (100 iterations x 100 years per iteration) to explore the variation produced by the historical environmental stochasticity.

Several statistical models were used to assess the relative effect of hydroperiod and the initial proportion of hybrids on population viability. A log-normalized linear model was constructed to explain the population size at the end of the PVA simulation, which was estimated for each iteration as the median population size from year 80 to 100. The probability of population persistence was modeled using a generalized linear model with a binomial error distribution. The change in stable population size in the PVA framework was modeled as the proportion of the carrying capacity estimated for each demographic scenario. This fraction was modeled using a generalized linear model with a quasibinomial error distribution. The number of years that elapsed before populations went extinct was recorded for each population that did not

persist. The number of years to extinction was included as a response variable in a linear model with hydroperiod and the initial proportion of hybrids as independent variables

Single-Hybrid Invasion:

Another critical aspect of hybrid zone dynamics concerns the initial invasion of hybrids into a native population at the expanding edge of the hybrid zone. I simulated this scenario as a single hybrid adult migrating into a population of all native CTS. Each single-hybrid invasion scenario was initiated with either: 1) the population at the scenario-specific carrying capacity (K) determined from the density-dependent simulations; or 2) at a standardized population size of 2000 individuals (1000 adults and 1000 metamorphs). I ran these simulations in the same manner as the other PVA analyses and recorded the number of populations that went extinct, became hybrid or remained pure native. Each simulation was iterated 600 times to capture the variability between simulations. At the end of each 100-year simulation, I considered any population with a final HIS above the starting native HIS (>0.05) to be a "hybrid" population. I used generalized linear models with a binomial error distribution to test whether pond hydroperiod affects the vulnerability of a population to hybrid invasion. The final proportion of hybrid/native adults at year 100 was calculated for each simulation, and linear models were used to assess whether longer hydroperiods decreased the likelihood of retaining native adults in the population. The final HIS of each hybrid population was then estimated as the median HIS from years 80 to 100, after it had reached a stable equilibrium. I used linear models to assess whether hydroperiod significantly affected the equilibrium HIS value. The number of years required for the population to reach the HIS equilibrium was also calculated. I fit a linear model to test if hydroperiod was

correlated with this time to equilibrium. Together, these metrics enable the comparison of population susceptibility to non-native immigration.

Statistical Methods:

All statistical analyses were conducted in the statistical language R (R. Core Team 2013). For all statistical models I report the slope (β), 95% confidence interval, and p-value for each predictor variable. All log-linear dependent variables (y) in this study were transformed by adding the smallest value observed for y to each value of y before taking the natural log, to avoid undefined values produced by zeros. Model slopes for log-linear models are reported without exponentiating. I report the percent increase in y with each unit increase in x using the equation:$(e^{x} - 1) \times 100\%$. I then scale dependent variables using the "arm" package in R (Gelman et al. 2016), to compare their relative effect on y using the equation: $\frac{\beta 1}{\beta 2}$.

Results:

All results and model statistics are summarized in Table 3.2.

Population Growth Rate (λ):

The basic density independent model enabled estimates of the intrinsic, per-capita population growth rate (λ). Across 100 model iterations of 54 different demographic scenarios λ ranged widely, from 0.886 to 1.936. Lambda was significantly correlated with both hydroperiod (log-lm: $\beta = 7.62 \times 10^{-3}$, Confidence Interval = (7.56×10^{-3}, 7.67×10^{-3}), $p < 2 \times 10^{-16}$; Figure 3.1A) and the proportion of hybrids (log-lm: $\beta = 0.111$, CI = (0.109, 0.113), $p < 2 \times 10^{-16}$; Figure 3.1B).

Exponentiating these independent variables reveals that a 1-day increase in pond hydroperiod duration results in a 0.76% increase in λ, and a one unit increase in hybrid proportion (0 to 1, or all native to all hybrid) increases λ by 117%. Scaling the input variables before exponentiating reveals that hydroperiod ($\beta = 1.22$) has a 1.13x greater effect on λ than the proportion of hybrids ($\beta = 1.08$). The scenarios that produced a λ of less than one, signifying an unsustainable population, were mostly distributed in the short hydroperiods: 484/600 (81%) in the 80-day, 144/600 (24%) in the 85-day and only 5/600 (0.8%) in the 90-day scenarios. These unstable populations were most frequent in populations with a lower proportion of hybrids, ranging from 205/900 (23%) in the full native populations to only 17/900 (1.9%) in the full hybrid populations. The greatest value for λ across all iterations was 1.936 from a completely hybrid population with a 120-day hydroperiod pond, this demographic scenario had an average λ of $1.72 \pm 3.4\text{x}10^{-3}$ (median $\pm$ standard error). The lowest value for λ (0.886) was in a full native population with an 80-day hydroperiod pond, which had an average λ of $0.91 \pm 9.0\text{x}10^{-4}$ (med $\pm$ SE).

Carrying Capacity (K):

The density dependent model yielded different adult carrying capacity (K) estimates for each combination of hydroperiod and proportion hybrid initial conditions. Both hydroperiod (log-lm: $\beta = 0.131$, confidence interval = (0.130, 0.133), $p < 2\text{x}10^{-16}$; Figure 3.2A and B) and the proportion of hybrids (log-lm: $\beta = 1.65$, CI = (1.59, 1.71), $p < 2\text{x}10^{-16}$; Figure 3.2C and D) significantly affected the estimate for K. Exponentiating the slope estimates from this model shows that a 1-day increase in hydroperiod results in a 14% increase in K. A one unit increase in hybrid proportion results in a 422% increase in K. When the predictors are scaled and then

exponentiated, hydroperiod ($\beta = 29.6$) had a 9.6x greater effect on K than the proportion of hybrids ($\beta = 3.1$). Estimates of K ranged from 0 to 6,953 adults (Figure 3.2). There were 198 simulations that resulted in population collapse (i.e., K=0). The majority of these simulations were in the 80-day hydroperiod (182/600 or 30%), with a few in the 85-day scenario (16/600 or 2.7%; Figure 3.2). The distribution of failed populations was more evenly spread across the proportion of hybrids with 114/900 (13%) in full native populations to 2/900 (0.7%) in the 80% hybrid scenarios; none of the full hybrid population simulations went to 0 (Figure 3.2). The demographic scenario that resulted in the greatest K (6,953 adults) was the 120-day hydroperiod, all-hybrid simulation which had an average K of 6709 $\pm$ 7.3 adults (median $\pm$ SE). Conversely, the all-native population with an 80-day hydroperiod had the lowest estimate, with an average K of 0 $\pm$ 0.1 adults.

Population Viability Analysis:

The population size at the end of the 100-year PVA was positively correlated with hydroperiod (log-lm: $\beta = 0.178$, CI = (0.176, 0.180), $p < 2x10^{-16}$; Figure 3.3A and B) and the initial proportion of hybrids (log-lm: $\beta = 1.70$, CI = (1.61, 1.80), $p < 2x10^{-16}$; Figure 3.3C and D). Exponentiating the model predictors shows that the population size increases by 19.5% with each additional day of pond duration. A one unit increase in the starting proportion of hybrids resulted in a 450% increase in stable population size. When the model is re-evaluated using exponentiated and scaled predictors, hydroperiod ($\beta = 99.0$) is estimated to have a 31x greater effect on population size than the starting proportion of hybrids ($\beta = 3.2$).

The addition of environmental stochasticity into the demographic model negatively impacted population size and persistence. Across all simulations, the population size at the end of the PVA

was 36.2% ± 2% (median ± SE) of the carrying capacity estimated for each specific scenario (Figure 3.4). Longer hydroperiods significantly increased the population's percent of K (GLM-quasibinomial: $\beta = 0.044$, CI = (0.032, 0.058), $p = 2.37 \times 10^{-8}$; Figure 3.4A), however the proportion of hybrids in the population did not (glm-quasibinomial: $\beta = 0.41$, CI = (-0.07, 0.89), $p = 0.10$; Figure 3.4B).

The probability of a population persisting to the end of the 100-year simulation is positively associated with hydroperiod (GLM-logit: $\beta = 0.73$, CI = (0.67, 0.80), $p < 2 \times 10^{-16}$; Figure 3.5A and B) and the initial proportion of hybrids (GLM-logit: $\beta = 6.20$, CI = (5.53, 6.91), $p < 2 \times 10^{-16}$; Figure 3.5C and D). When the predictors are scaled, a unit increase in hydroperiod ($\beta = 18.87$) has a 4.4x greater effect on the probability of persistence than the proportion of hybrids ($\beta = 4.23$). The number of years a population persists before dropping below the quasi-extinction threshold (3 adults) is also positively correlated with hydroperiod (lm: $\beta = 4.51$, CI = (4.23, 4.79), $p < 2 \times 10^{-16}$; Figure 3.6A) and the starting proportion of hybrids (lm: $\beta = 39.78$, CI = (36.69, 42.88), $p < 2 \times 10^{-16}$; Figure 3.6B). When the independent variables are scaled, hydroperiod ($\beta = 116.5$) has a 4.3x greater effect on the time to extinction than the proportion of hybrids ($\beta = 27.2$).

Single-Hybrid Invasion:

The simulations of a single hybrid adult migrating into native populations yielded information about the resiliency of populations to hybrid invasion. In the simulations that began at carrying capacity, the majority of the short hydroperiod populations went extinct: 600/600 (100%) of the 80-day, 598/600 (99%) of the 85-day, 423/600 (71%) of the 90-day went extinct over 100 years (Figure 3.7A). There were similar rates of extinction when all populations began

at 2,000 individuals (1,000 adults and 1,000 metamorphs): 600/600 at 80-days, 597/600 at 85-days, 423/600 at 90-days resulted in population extinction (Figure 3.7B). Across the simulations that persisted, there was no detectable effect of hydroperiod on the number of populations that retained non-native genes when populations started at K (GLM-logit: $\beta = -0.009$, CI = (-0.019, 0.002), $p = 0.098$). However, when populations began at 2000 individuals there was a strong effect of hydroperiod (GLM-logit: $\beta = 0.043$, CI = (0.034, 0.053), $p < 2x10^{-16}$), where longer pond duration increased the probability of successful hybrid establishment. In most populations where hybrids persisted (median HIS > native HIS), every surviving adult was a hybrid to some degree. However, a few populations retained a small number of native adults: for populations at K there were 34 (0.6%) populations that retained native adults at the end of the 100-year simulation (1 in 100, 3 in 105, 6 in 110, 14 in 115, and 10 in 120-day hydroperiods), which marginally increased with longer hydroperiods (lm: $\beta = 0.58$, CI = (-0.02, 1.2), $p = 0.05$). Similarly, for populations starting at 2000 individuals there were 27 (0.5%) simulations that retained some native adults (1 in 95, 1 in 105, 6 in 110, 8 in 115, and 11 in 120-day hydroperiods), which increased with longer pond duration (lm: $\beta = 0.42$, CI = (0.11, 0.74), $p = 0.02$). The equilibrium HIS was correlated with hydroperiod in both the simulations initiated at K (lm: $\beta = -0.0014$, CI = (-0.0017, -0.0013), $p < 2x10^{-16}$; Figure 3.7E) and at 2000 individuals (lm: $\beta = -5.78x10^{-4}$, CI = ($-6.9x10^{-4}$, $-4.6x10^{-4}$), $p < 2x10^{-16}$; Figure 3.7F). In populations at K, there was no effect of hydroperiod on the time to HIS equilibrium (lm: $\beta = -0.07$, CI = (-0.23, 0.08), $p = 0.36$; Figure 3.8A). However, simulations starting at 2000 individuals revealed a significant correlation between hydroperiod and time to HIS equilibrium (lm: $\beta = -0.36$, CI = (-0.50, -0.22), $p = 4.76x10^{-7}$; Figure 3.8B), where increased pond duration resulted in a smaller time to equilibrium.

Discussion:

In this study, I adapt the most comprehensive demographic model (Searcy et al. *in press*) constructed for the endangered California tiger salamander, to evaluate the potential for hydroperiod management to reduce the success of non-native hybrids. I restructured the model to include individual-level traits, primarily genotype and mass, to track allele frequencies, persistence, and vulnerability to hybrid invasion at the population level. I adapted statistical models from previous studies to incorporate the effects of genotype and pond duration into population vital rate estimates. These new models were used to evaluate 54 different demographic scenarios spanning a range of hydroperiod and hybrid abundance combinations. Here I discuss the results from each level of model implementation, and evaluate the effects of hydroperiod manipulation as a management strategy.

Population Growth Rate:

The density-independent model confirmed my prediction that populations in longer duration ponds have greater intrinsic growth rates. Based on these results (Figure 3.2A) there appears to be diminishing returns from hydroperiods in excess of 110 days, a pattern that is more pronounced in ponds with a greater proportion of hybrid individuals. In contrast, ponds with 85 - 90-day hydroperiods have exceptionally low λ estimates, a pattern that is most pronounced in native populations. Based on these results, it would be unadvisable to reduce breeding pond duration to less than 90 days, especially in native populations, since it is likely that those populations will have intrinsic growth rates of 1 or less. By definition, populations with a lambda of less than 1 have a much greater chance of going extinct (Lande 1993), and have less ability to

recover from negative stochastic events which reduce the population size (Lennartsson & Oostermeijer 2001; Kissel et al. 2019).

The range of λ determined in this study generally agrees with previous model implementations. Searcy et al. (*in press*) reported a density independent λ of 1.42 (CI = 0.74, 2.35), which is only slightly greater than my estimate of 1.39 for native CTS in the 120-day hydroperiod. This minor difference may reflect the marginally longer hydroperiod experienced by the CTS population at Olcott lake (Solano County, USA), which was used to construct Searcy's model. If these CTS experienced a longer hydroperiod, it may result in greater growth rates since larval survival and mass at metamorphosis are both positively influenced by hydroperiod in my adaptation of the model.

Hydroperiod and the proportion of hybrids in a population are both positively correlated with λ. All demographic parameters investigated here were more sensitive to changes in hydroperiod compared to the proportion hybrids. However, for λ the proportion of starting hybrids had the greatest *relative* effect (0.88x), though still less than the effect of hydroperiod. This likely reflects the importance of individual biology and reproduction on intrinsic growth rates. Because hybrid individuals achieve a greater mass at metamorphosis and have higher larval survival rates (Cooper et al. Ch2), each individual achieves greater fecundity and lifetime reproduction (Tucker 1999; Trenham et al. 2000a). It is therefore likely that populations with a greater proportion of hybrid individuals are able to grow more rapidly, regardless of pond hydroperiod. This explains why the proportion of hybrids parameter has a larger relative effect on λ compared to other population metrics such as K.

Carrying Capacity:

Hydroperiod had a large effect on the number of individuals that the environment can support. For every additional day of pond inundation, the carrying capacity of the population was predicted to increase by about 14%. While hydroperiod has always been highlighted as a critical feature of CTS ecology (Fitzpatrick & Shaffer 2007a; Johnson et al. 2013), this result quantifies the benefit of longer pond duration on theoretical estimates of K. Hydroperiod had a much larger impact (9.6x) on population K than the starting proportion of hybrids. This agrees with previous studies on *Ambystoma* that demonstrate the strong influence of hydroperiod on K (Baldwin et al. 2006; McMenamin & Hadly 2010). Hydroperiod is an environmental feature that impacts every individual in the population. Longer duration ponds yield higher larval survival rates resulting in a greater number of metamorphs that partially counteract density-dependent reduction in larval survival.

Management actions that increase K (e.g., increasing hydroperiod) are important goals for conservation efforts and are therefore essential factors for managing wild CTS populations. However, these modifications will likely benefit hybrid individuals more than natives based on the results presented here and in previous hydroperiod studies (Cooper et al., Ch 2; B. Fitzpatrick & Shaffer, 2007; J. R. Johnson et al., 2013). This suggests that while increased hydroperiod may be essential for supporting large populations of native CTS, they also make these populations more suitable for hybrid infiltration.

Population Viability Analysis:

The addition of environmental stochasticity negatively affected all simulated populations. Short pond hydroperiods experienced the greatest reduction in population size, however the average across each demographic scenario was never greater than 40% of K. This suggests that

variability in rainfall has a dramatic effect on realized population size. Years with below-average precipitation reduce the number of females that emerge from aestivation to breed, reducing total reproduction in ponds (Trenham et al. 2000a; Trenham & Shaffer 2005a). Additionally, low rainfall reduces the survival of the offspring that are produced (Searcy et al. 2014b), sometimes resulting in complete reproductive failure if ponds dry before larvae are able to complete metamorphosis (Loredo & van Vuren 1996; Trenham & Shaffer 2005a).

The viability of simulated CTS populations was largely driven by pond hydroperiod. Most populations in the 80 and 85-day hydroperiods were predicted to go extinct over the 100-year interval (Figure 3.5A), with population extinction occurring on average in 24 and 38 years, respectively (Figure 3.6A). The poor success of populations centered around short duration ponds is likely the culmination of two vital rates. First, low population growth rates prevent the population from rebounding after periods of low recruitment (Turkalo et al. 2017). This increased time below carrying capacities can increase sensitivity to environmental stochasticity (Lande 1993). Second, lower carrying capacities limit the number of adults in the population, rendering them more susceptible to stochastic events (Foley 1994). Together, these values result in populations that are unsustainable in the long-term at 80 and 85-day hydroperiods, corroborating previous studies that suggest a minimum 90-day hydroperiod to facilitate CTS larval development (Petranka 1998; Stebbins 2003; Johnson et al. 2013). Though the naturalistic hydroperiod experiment that parameterized this model did identify some individuals that successfully emerged from the 80 and 85-day hydroperiod treatments, they represented a small fraction of surviving metamorphs (Cooper et al. Ch2). It appears that, while some individuals are capable of emerging from ponds that do not last for 90 days, this hydrology will not support a

stable population. This unfortunately is at odds with the conservation goal of exploiting short hydroperiod ponds to reduce hybrid advantage (Cooper et al. Ch 2).

The 90-day hydroperiod treatment appears to be on the threshold of supporting a viable population, with approximately 30% of simulated populations going extinct over the 100-year interval. Although a 70% survival probability is unlikely to be an attractive conservation option for managers, it does provide a critical threshold below which almost all populations are predicted to go extinct. At 95 days, 98% of all simulated populations are predicted to persist, a drastic increase that may prove to be a desirable management goal.

In summary, very short hydroperiods do reduce or potentially eliminate hybrid advantage, but only at a point so extreme that populations are not viable in the long run.

Single Hybrid Migrant:

The effect of a single hybrid individual dispersing into a native population was modeled using the general PVA with a very low frequency (1 individual) of hybrid individuals. This scenario mimics the most probable scenario of hybrid dispersal and range expansion in nature at the leading edge of the hybrid invasion. Quantifying the relative impact of the single hybrid individual on population persistence and HIS offers insight into the resilience of native populations to hybrid immigration. Given the similarity to the general PVA, it follows that the rates of extinction in this simulation are equivalent to that of the PVA. Few populations survive below the 90-day hydroperiod mark, at which 71% of the populations persist. A large proportion of simulations successfully repel the non-native invasion given the extremely low initial frequency of hybrids, consisting of a single adult migrant. In populations that begin the simulation at K, I was unable to detect an effect of hydroperiod on the number of successful

invasions (Figure 3.7A and C). However, populations that begin at a constant 2,000 individuals demonstrate a significant increase in the number of successful hybrid immigrations (Figure 3.7B and D). This is likely due to the relative proportion of the carrying capacity at which each population begins. At 2,000 individuals in the short hydroperiod treatments, the population is far above K, resulting in greater mortality and a greater probability of the single hybrid to be eliminated before successfully reproducing. In longer hydroperiod simulations, the population is below K and each individual contributes more offspring to the growing population, increasing the likelihood that a hybrid migrant can successfully reproduce in its lifetime. For these reasons, the simulations that begin at K are likely more appropriate when comparing the vulnerability of the different hydroperiod treatments. However, it is important to note that populations which are currently below their realized K are more susceptible to non-native immigration events. This is a relevant issue on the landscape since many populations are imperiled by variable precipitation (Holland et al. 1990) and transient anthropogenic disturbances (Barry & Shaffer 1994), which likely translate to populations existing below their true K.

Most populations that retained non-native alleles were composed of only hybrid individuals by the end of the 100-year simulation. This is largely a reflection of the mating system dynamics in this model. All individuals selected mates randomly from the adults that entered the pond to breed in a given year, resulting in a fairly rapid population-level admixture. However, a few simulations retained some completely native adults at year 100. While these numbers were quite small (0.5-0.6%) they appeared to increase with longer hydroperiods. This is likely an effect of the larger population size reducing the probability of a native CTS mating with a hybrid. Although this result is intuitive, it is an important factor to consider: larger, healthier CTS populations may have a greater chance of retaining some pure native genotypes even within

the hybrid zone, albeit at a very low frequency. This pattern is also evident in the equilibrium HIS, where longer hydroperiod ponds show a lower average HIS than shorter duration ponds. Again, this is likely a reflection of the larger population size which has a greater abundance of native alleles which further diminishes the effect of a single hybrid migrant. This may be significant on the landscape since the number of hybrid migrants is likely independent of the focal population's size. In sum, these results suggest that larger native populations that are supported by longer pond hydroperiods, have a greater chance of retaining at least some completely native genotypes and experience a smaller overall shift in HIS per unit of time. Although the fraction of the population that is expected to remain pure CTS is small, retaining any full native genotypes on the landscape may be a critical management goal, because it at least allows one to entertain the possibility of removing hybrid individuals and rebuilding pure native populations. If additional practices are implemented, such as targeted removal of hybrid adults, these native survivors may be vital in recovering a greater portion of the native genome.

Caveats and Future Work:

While this study utilizes the best available science to address CTS management concerns, it is also limited by some key factors. First, and probably most importantly, some aspects of this model have been parameterized using data collected for pure native CTS (Trenham et al. 2000a; Searcy et al. 2014c). It is likely that hybrids differ in demographic factors that are not strictly explained by size and genotype. However, several important components of this model were parameterized using empirical data collected on native and hybrid CTS, including the effects of hydroperiod and genotype on larval survival and mass at metamorphosis. While I believe that these two components are the predominant factors in salamander demography (Searcy et al.

2014b, 2014c), there are several demographic functions that would benefit from additional empirical data on hybrids. Critically, future studies should determine the effect of HIS on juvenile/adult growth and maturation rates, which were shown to have the greatest effect on population growth rate (Searcy et al. *in press*). This model would also benefit from empirical studies that incorporate the effect of HIS on density-dependent larval mortality. While this study does include models that predict larval survival based on HIS, I do not have data on how this relationship changes with different levels of larval density, which has a large effect on the estimates of carrying capacity in native CTS (Searcy et al. *in press*).

In this study, I include hydroperiod as a fixed environmental characteristic, however variability in rainfall will significantly change pond hydrology. Given expectations of climate change, future work should combine climate projections with rainfall-dependent hydroperiod to simulate real-world pond scenarios and evaluate their expected viability. Additionally, the current model assumes that mating occurs randomly between any mature adult that enters the breeding pond each year. This results in rapid homogenization of native/non-native allele frequencies in the population. However, in the field, a significant degree of variability in HIS among individuals within ponds and between years has been observed (McCartney-Melstad et al. unpublished data; Shaffer et al. 2020). This pattern may be the result of assortative mating, potentially through behavioral or temporal isolating mechanisms. In addition, I model all diagnostic loci as neutral, when in the wild there is likely selection on specific CTS/BTS alleles (Cooper et al. Ch2) which may alter the spread of non-native alleles in a population. Future studies should investigate these possibilities to improve the accuracy of demographic modelling efforts. Finally, I do not evaluate the effect of paedomorphic salamanders in this study, which may significantly augment hybrid recruitment and fitness in permanent ponds. These

paedomorphs become sexually mature and remain in the ponds indefinitely, and therefore require constant water. It is likely critical for ponds to dry frequently (at minimum every few years) to preclude both paedomorph and invasive species (Fisher and Shaffer, 1996) occupancy. The demographic contribution of paedomorphic salamanders is a critical addition to this model, which future studies should investigate.

The model presented here has wide-reaching implications for the persistence of CTS populations afflicted by non-native introgression. However, these model predictions should be empirically tested in future studies. One strategy could involve tracking the frequency of non-native alleles in a single pond through time, using historical sampling, to evaluate how well the model captures the pattern of introgression and eventual equilibrium of non-native alleles. Another may involve modeling the spread of non-native alleles after the initial introduction to compare model simulations with historical sampling of non-native allele distributions through time. These studies may identify aspects of the model that do not align with empirical data, and suggest modifications to improve model performance.

Conclusion:

Simply managing all ponds to reduce hydroperiod does not appear to be a reliable method for reducing the success of non-native alleles on the California landscape. Both native and hybrid individuals are negatively affected by shortened pond duration through reductions in intrinsic growth rate and carrying capacity of focal populations. However, this uniform degradation does not appear to confer an advantage for native genotypes or individuals in any of the scenarios examined in this study. Instead, my results highlight the need for large robust native populations which are maintained with long hydroperiod ponds. These large populations near their K have

the greatest chance of resisting non-native immigration and experience the smallest shift in non-native allele frequencies following successful hybrid establishment. Populations that are significantly below their realized K are the most susceptible to hybrid infiltration. However, it is also vital to reduce the degree of hybrid immigration into these native ponds, since there is no clear way to select against hybrid genotypes.

From these results, I suggest the following strategy for reducing non-native tiger salamander success on the landscape.

- Native populations around the periphery of the hybrid zone should be managed intensively to improve their resiliency against hybrid migrants. This may include managing ponds to ensure an adequately long hydroperiod in most years, to support the healthiest population possible.
- Ponds within the hybrid zone should be managed to limit population sizes and discourage hybrid dispersal. Hydroperiod may offer a convenient management tool to reduce the success of largely-non-native ponds without a costly eradication program. Modifying hybrid ponds to reduce hydroperiod is straightforward (e.g., cutting the berm, adding overflow pipes, etc.) and may sufficiently hinder the hybrid populations, reducing their numbers and dispersers. This would also avoid the need to completely drain ponds for 10-15 years in order to effectively eradicate hybrid populations, given the lifespan of CTS (Searcy et al. 2014c). Reduced hydroperiod would retain the natural function of the vernal pool ecosystem, without promoting additional hybrid success.
- Remove large hybrid individuals from populations. This would require rapid detection molecular techniques that could quantify an individual's HIS within hours. Removing

large hybrid adults could significantly reduce hybrid reproductive success, since these individuals enjoy much greater fecundity and offspring success.

Future studies will need to evaluate how effective these strategies are at maintaining native genotypes in hybrid ponds. Although hydroperiod management does not offer the "silver bullet" for reversing the pattern of non-native introgression, it may be a useful tool in a multi-faceted approach to CTS management.

Chapter 3 Figures

Step	Model Function	Description
1	Survival	Survival probability is estimated, and a binary state (alive or dead) is randomly chosen. Individuals that are greater than 15 years old are automatically coded as dead.
2	Growth	Growth is randomly selected from the predicted probability density distribution and used as the new mass.
3	Maturity	Maturity probability is estimated, and a binary state (mature or immature) is randomly chosen. Individuals that have reached maturity remain mature in all subsequent years (until death).
4	Fertility	Fertility is estimated from the new mass and fertility that is less than zero is truncated at zero
5	Breeding	Breeding probability is estimated, and a binary state (breeding or not breeding) is chosen for the year.
6	Death	All individuals that did not survive are removed from the data frame.
7	Select Breeders	A separate data frame is created for males and females that include metamorphs and adults that are mature and breeding that year. If there are 0 male or female breeders, then no offspring are created.
8	Pair Breeders	Each female is randomly assigned one male as a mate. Each male may mate multiple times. All females breed, but not all males necessarily breed.
9	Larval Survival	The offspring HIS and pond hydroperiod are used as parameters in the larval survival function determined from the hydroperiod experiment (Cooper et al. Ch2).
10	Fecundity	Female fecundity, estimated as the product of fertility and larval survival.
11	Metamorphs	A new data frame is created with each row representing a new metamorph derived from female fecundity.
12	Metamorph HIS	The mid-parent value (mean) of HIS is assigned to each offspring.
13	Metamorph Mass	The mass at metamorphosis for each offspring is predicted using the HIS and hydroperiod as inputs into the metamorph mass model derived from the present hydroperiod study (Cooper et al. Ch2).

Table 3.1: The major steps in the density-independent model. All steps are repeated for each year the model is run. This is used as the basic model framework that is further augmented for additional model implementation (i.e., density-dependent and PVA)

Test	Parameter	Raw β	Scaled β	Upper CI	Lower CI	p-value	Model Type
Population Growth Rate (λ)							
	Hydroperiod	7.62×10^{-3}	1.22	7.56×10^{-3}	7.67×10^{-3}	$p < 2 \times 10^{-16}$	log-lm
	Proportion Hybrid	0.111	1.08	0.109	0.113	$p < 2 \times 10^{-16}$	log-lm
Carrying Capacity (K)							
	Hydroperiod	0.131	29.6	0.130	0.133	$p < 2 \times 10^{-16}$	log-lm
	Proportion Hybrid	1.65	3.1	1.59	1.71	$p < 2 \times 10^{-16}$	log-lm
PVA - Population Size							
	Hydroperiod	0.178	99.0	0.176	0.180	$p < 2 \times 10^{-16}$	log-lm
	Proportion Hybrid	1.70	3.2	1.61	1.80	$p < 2 \times 10^{-16}$	log-lm
PVA - Percent of K							
	Hydroperiod	0.044	NA	0.032	0.058	$p = 2.37 \times 10^{-8}$	glm-binom
	Proportion Hybrid	0.41	NA	-0.07	0.89	$p = 0.10$	glm-binom
PVA - Prob. of Persistence							
	Hydroperiod	0.73	18.87	0.67	0.80	$p < 2 \times 10^{-16}$	glm-logit
	Proportion Hybrid	6.20	4.23	5.53	6.91	$p < 2 \times 10^{-16}$	glm-logit
PVA - Time to Extinction							
	Hydroperiod	4.51	116.5	4.23	4.79	$p < 2 \times 10^{-16}$	lm
	Proportion Hybrid	39.78	27.2	36.69	42.88	$p < 2 \times 10^{-16}$	lm
Single hybrid - Prob. of Hybrid Persistence							
	Pop. Size = K	0.009	NA	-0.019	0.002	$p = 0.098$	glm-logit
	Pop. Size = 2000	0.043	NA	0.034	0.053	$p < 2 \times 10^{-16}$	glm-logit
Single hybrid - Number of Pure Native							
	Pop. Size = K	0.58	NA	-0.02	1.2	$p = 0.05$	lm
	Pop. Size = 2000	0.42	NA	0.11	0.74	$p = 0.02$	lm
Single hybrid - Equilibrium HIS							
	Pop. Size = K	-0.0014	NA	-0.0017	-0.0013	$p < 2 \times 10^{-16}$	lm
	Pop. Size = 2000	-5.78×10^{-4}	NA	-6.9×10^{-4}	-4.6×10^{-4}	$p < 2 \times 10^{-16}$	lm
Single hybrid - Time to HIS Equilibrium							
	Pop. Size = K	-0.07	NA	-0.23	0.08	$p = 0.36$	lm
	Pop. Size = 2000	-0.36	NA	-0.50	-0.22	$p = 4.76 \times 10^{-7}$	lm

Table 3.2: Statistical model output for each test performed. Raw β is the unscaled model estimate for each parameter. Scaled β is the model estimate for parameters after standardization (mean = 0 and SD = 0.5).

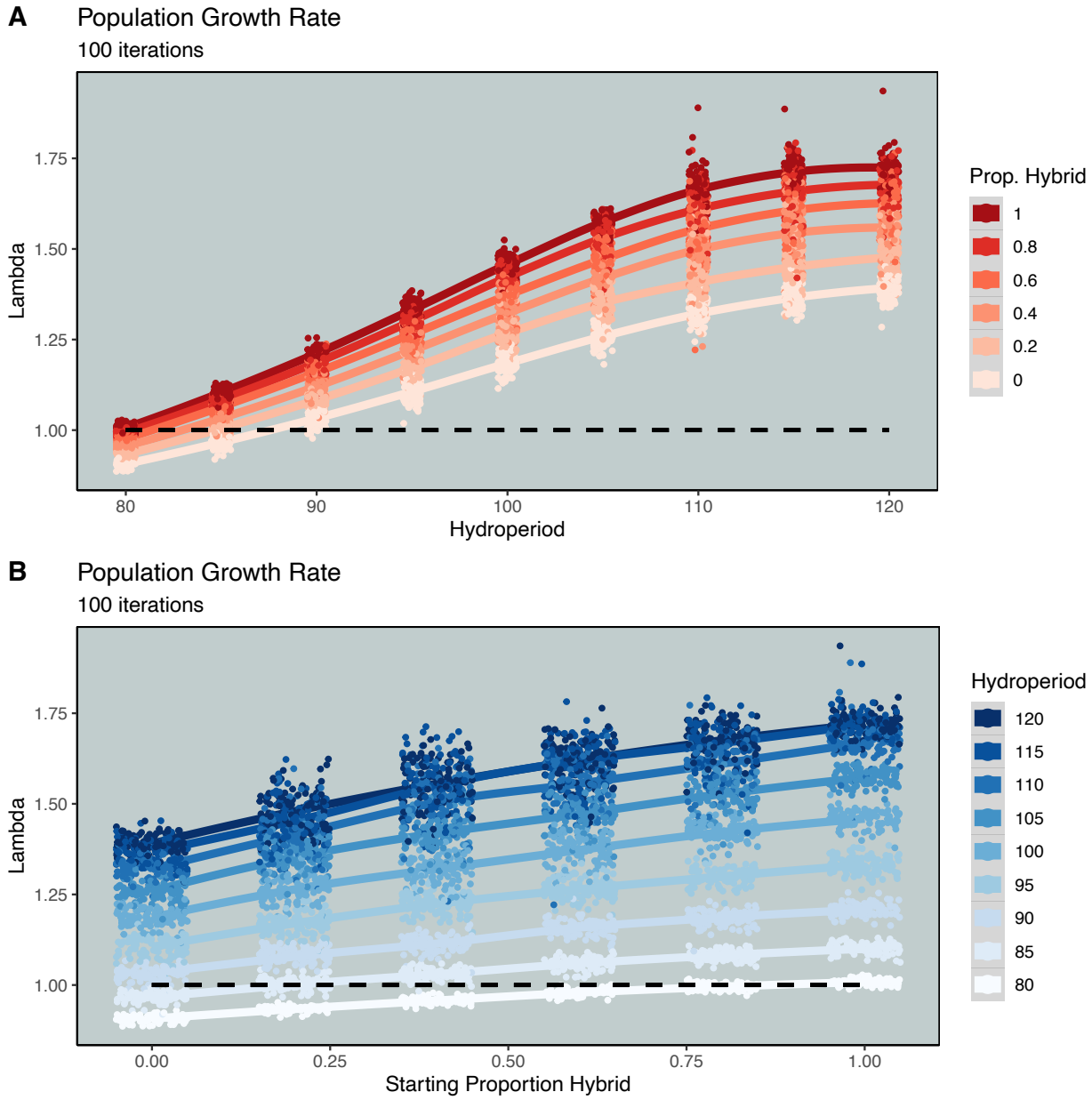

Figure 3.1: Density-independent model estimates of population growth rate ("Lambda" or λ) across demographic scenarios. Lambda is estimated as the slope of the log-normalized adult population size and time (years), after an initial burn-in of 5 years. The simulation was initiated with different combinations of pond hydroperiod (A) and the starting proportion of hybrid individuals (B) in the population. Longer hydroperiods and higher hybrid frequencies yield greater λ estimates. Short hydroperiods and more native populations result in lower values for λ, some of which are less than 1 (dashed black lines), indicating a declining population.

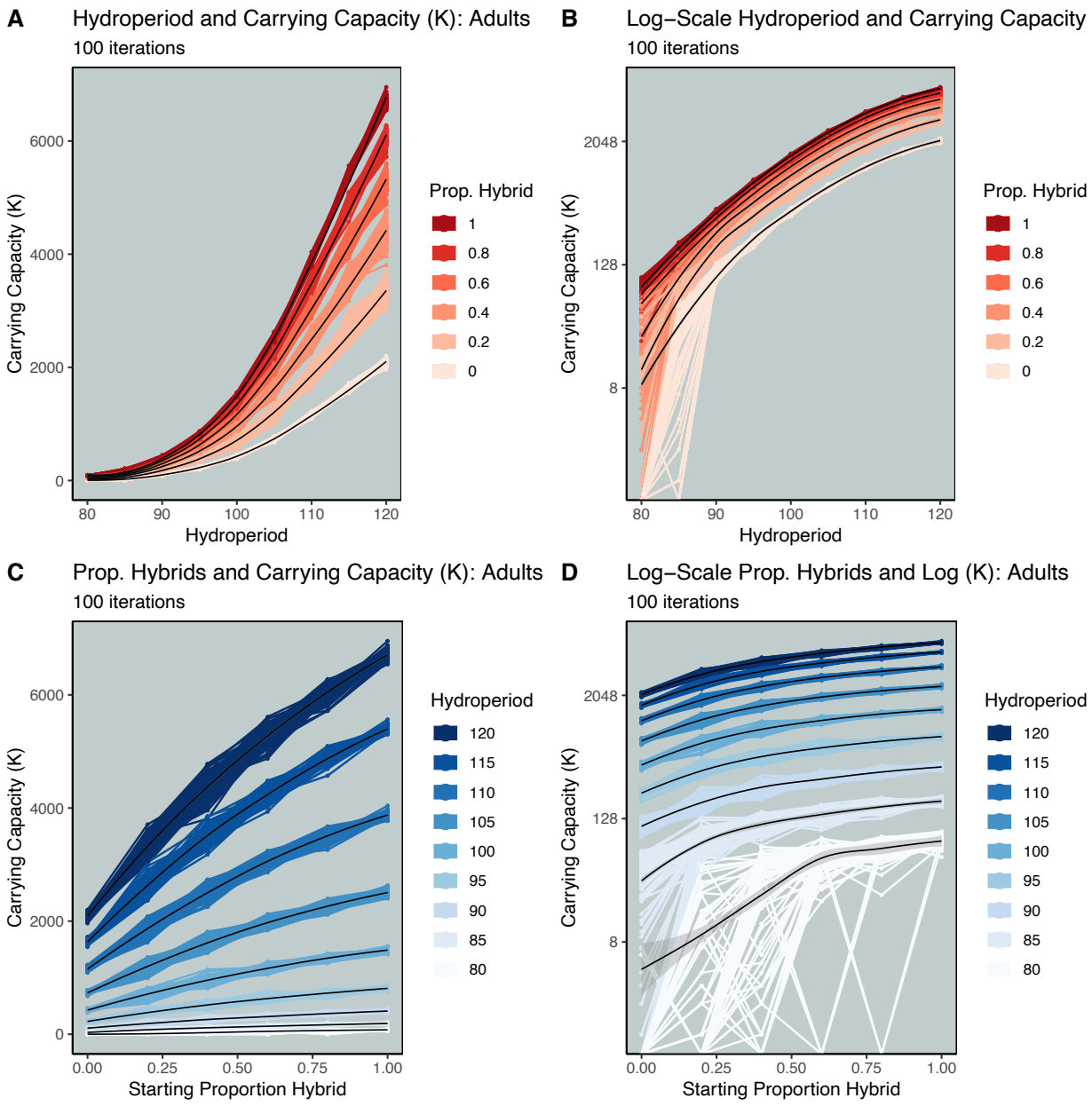

Figure 3.2: Density-Dependent model estimates for carrying capacity (K) across a range of pond hydroperiods (panels A, B) and starting proportion of hybrids (panels C, D). Panels show the median population size from years 50 to 100 of the 100-year simulations. The first 50 years are removed to allow the populations sufficient time to reach their stable equilibrium. Colored lines represent each of the 100 iterations, black lines depict the median values with standard error. Panels B and D show the population size at K on a logarithmic scale to improve resolution at low values for K.

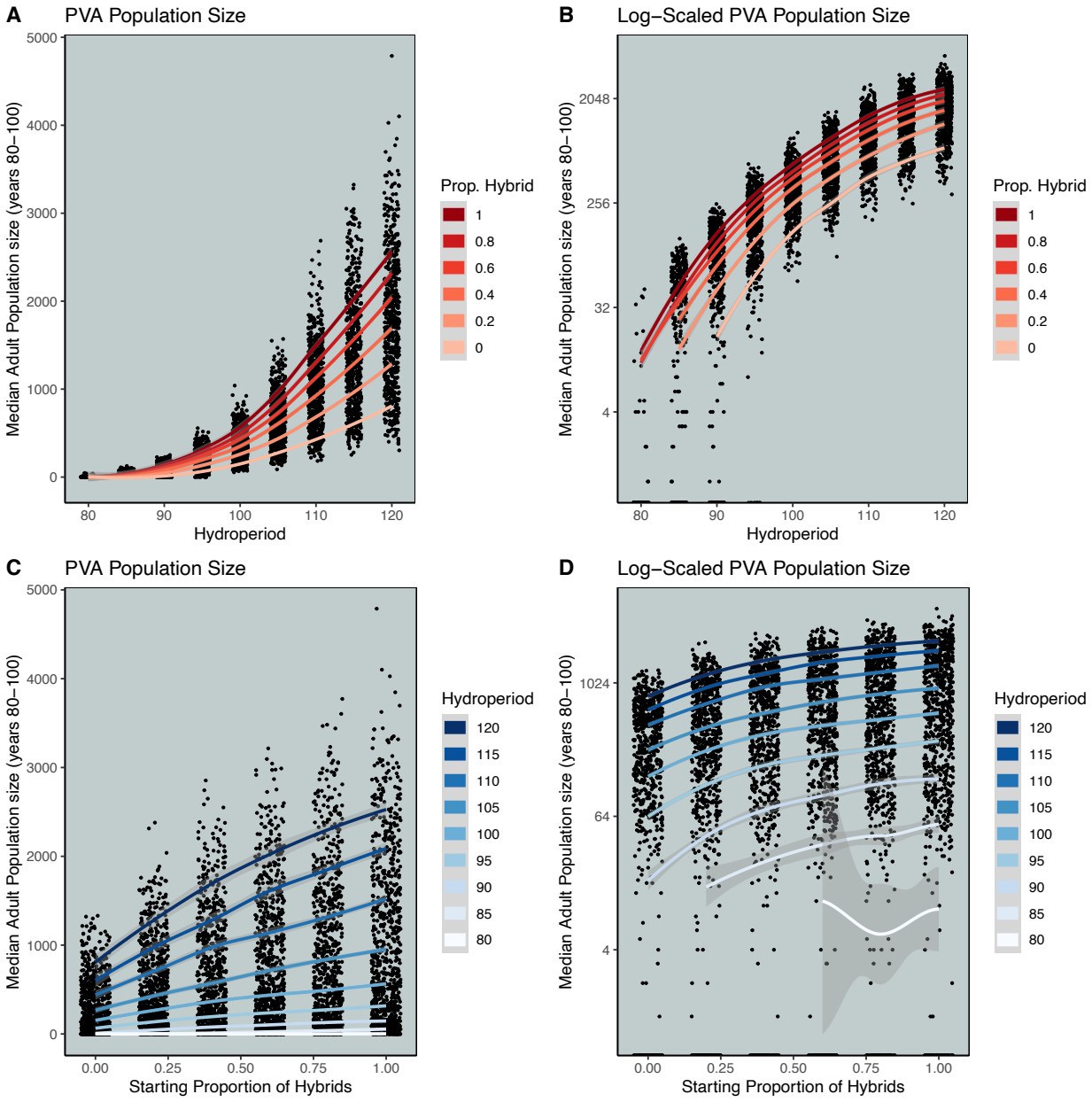

Figure 3.3: Population Viability Analysis (PVA) model estimates of stable population size. Population size is calculated as the median number of adults in the population in years 80 - 100. The PVA model includes environmental stochasticity which yields new estimates for the estimated population size equilibrium. Shown are estimates for a range of hydroperiod (panels A, B) and initial hybrid proportion (panels C, D), each iterated 100 times. Figures B and D show population sizes on a logarithmic scale to increase the resolution of low population scenarios.

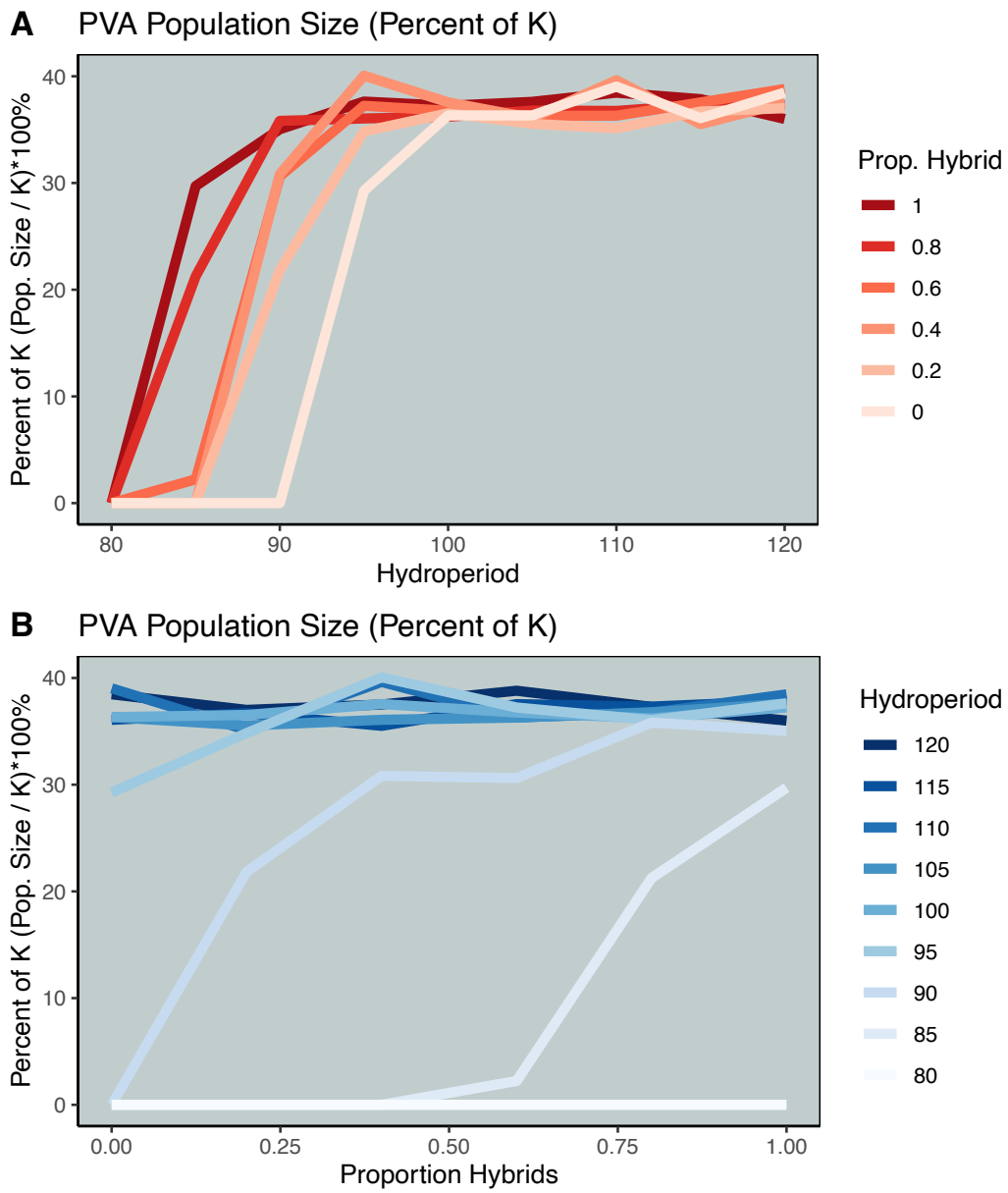

Figure 3.4: Population Viability Analysis (PVA) estimates of population size reported as the percent of carrying capacity (K). The PVA incorporates environmental stochasticity, which reduces the stable population size below the value for K determined in the density-dependent model. All populations begin the simulation at their estimated value for K, then the PVA population estimate is taken as the median number of individuals in the population from years 80-100. This figure shows the median percent of K across 100 model iterations for a range of hydroperiod (A), and initial hybrid proportion (B) scenarios. Several scenarios with short hydroperiod and low hybrid proportions are consistently predicted to go extinct within the 100 simulated years.

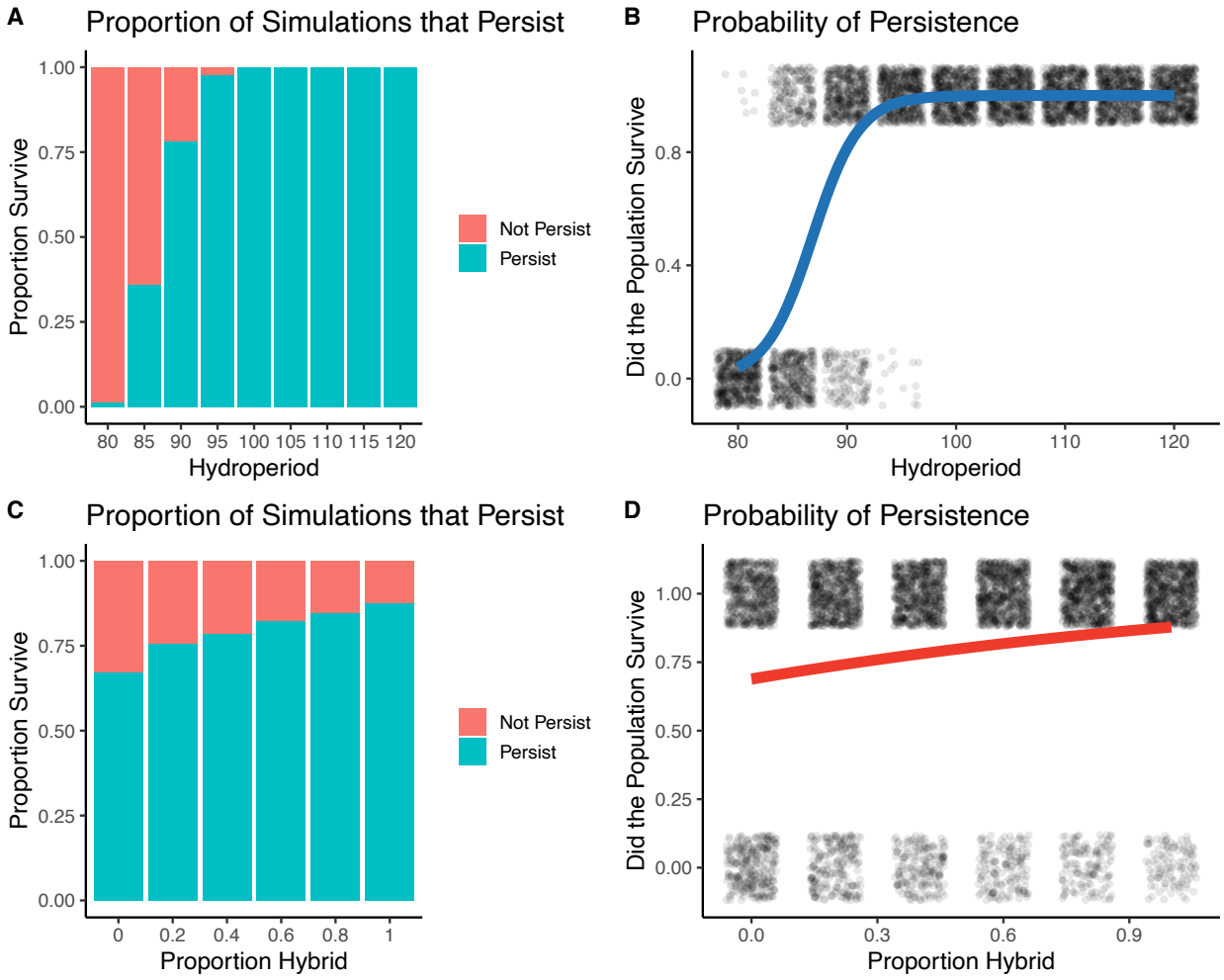

Figure 3.5: Population Viability Analysis model estimates of population persistence across a range of hydroperiod (panels A, B) and initial hybrid proportion (panels C, D) scenarios. Populations that drop below the quasi-extinction threshold of 3 adults at any point in the 100-year simulation are considered to have gone extinct. All simulations that consistently maintain more than 3 adults are considered to have persisted. Panels A and C show the relative frequency of extinct vs. persistent populations across 600 model iterations per hybrid proportion scenario. Panels B and D show the logistic regression model that predicts population persistence given pond hydroperiod (B) or initial proportions of hybrids (D). In these figures, extinction is displayed as y = 0 and persistence as y = 1, points are jittered to show relative density.

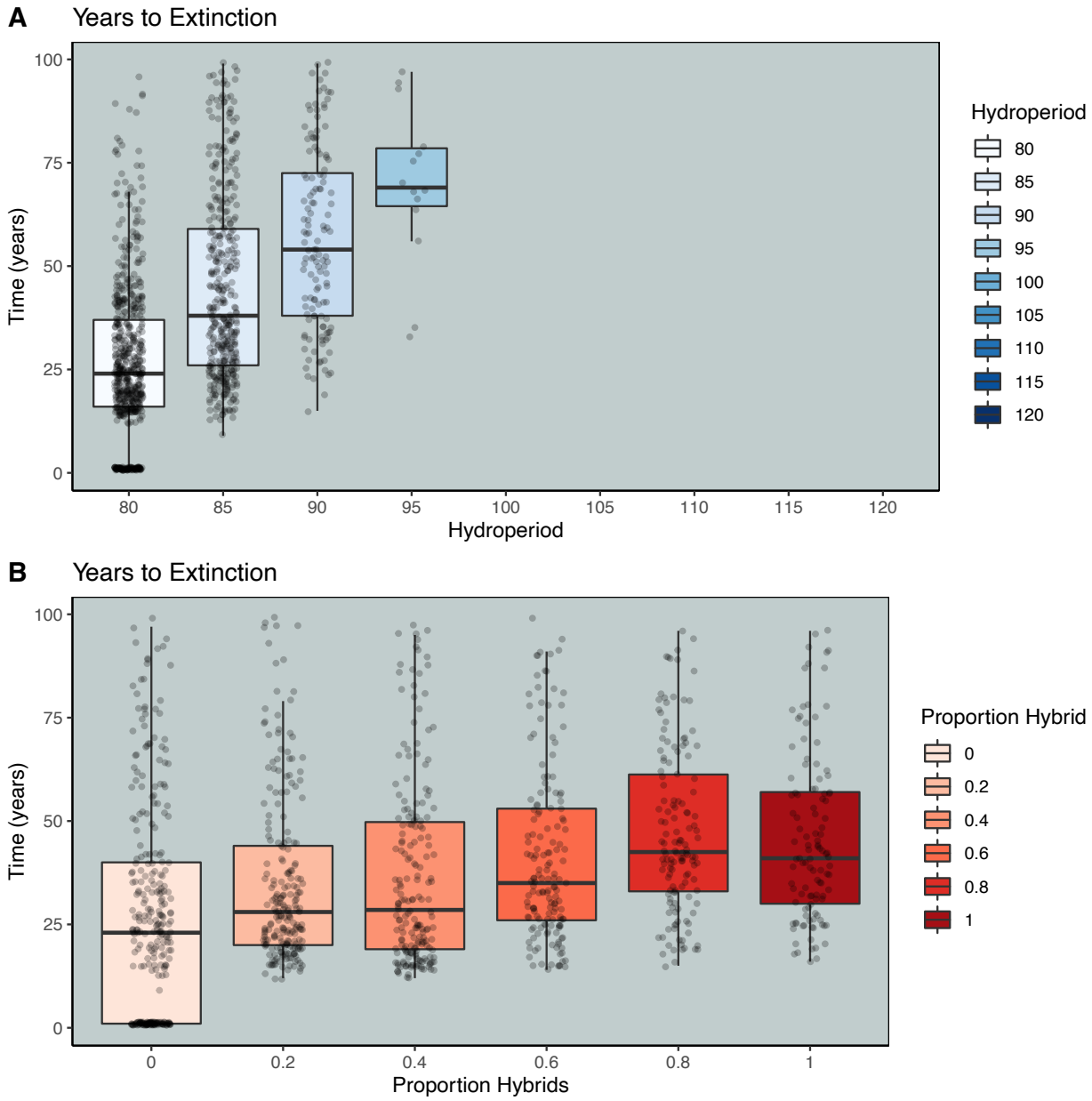

Figure 3.6: Population Viability Analysis model estimates of the time to extinction across hydroperiod (panel A) and initial hybrid proportion (panel B) scenarios. This figure only plots populations that dropped below the quasi-extinction threshold of 3 adults at any point in the 100-year simulation. Populations drawn from longer hydroperiod duration ponds tend to persist longer up to 95-day hydroperiods (panel A). For hydroperiods longer than 100 days, no populations went extinct. A similar, but far less pronounced trend is apparent with the initial proportion of hybrids (panel B).

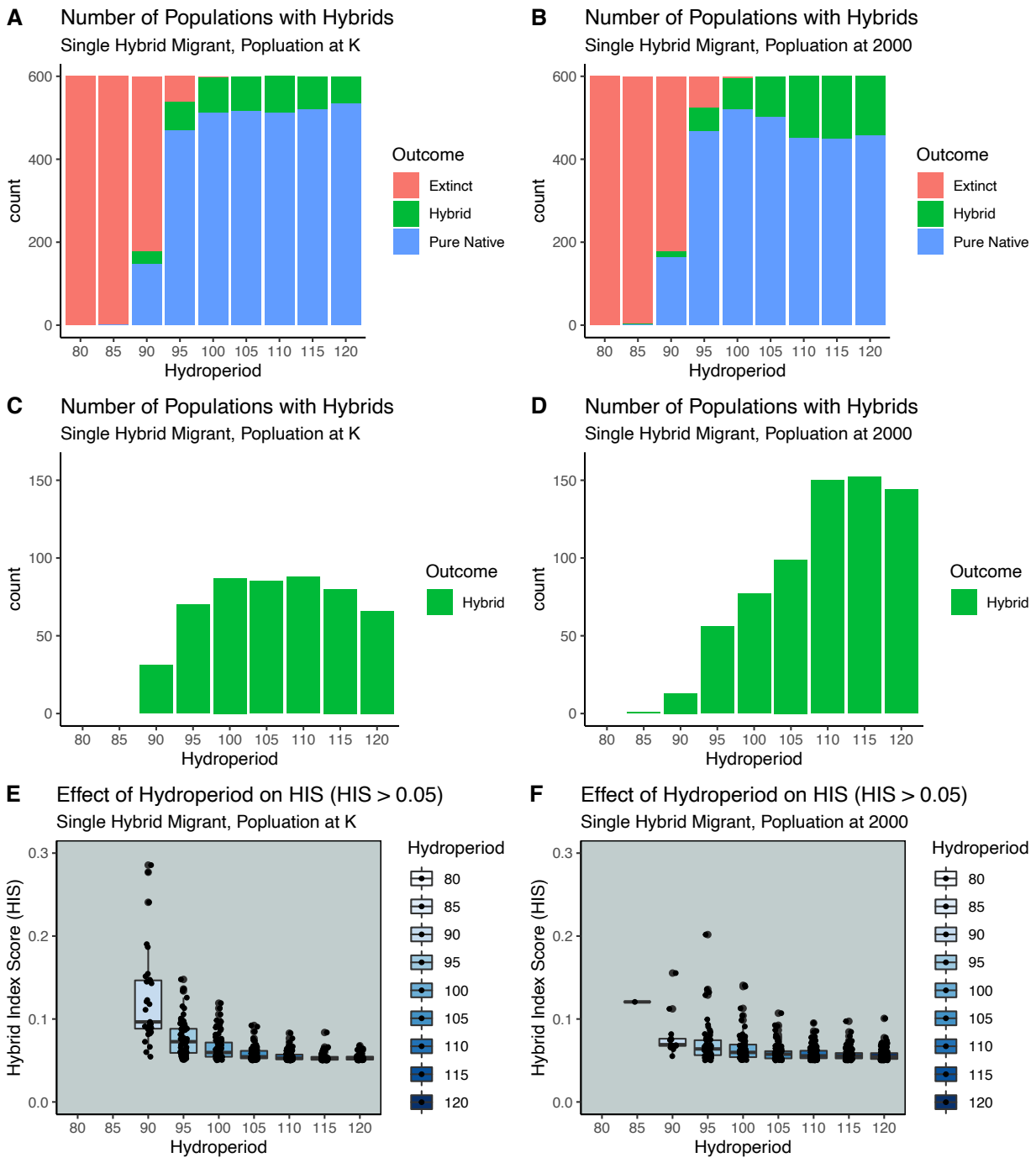

Figure 3.7: The effect of a single hybrid migrant into native populations on pond Hybrid Index Score (HIS). This model shows a special case of the Population Viability Analysis model where populations were completely native except for a single hybrid adult. Panels A, C, and E show the effect of a single hybrid on native populations at their carrying capacity, while B, D, and F show the effect of populations at a standard size of 2000 individuals (1000 adults and 1000 metamorphs). Panels A and B show the counts of populations that went extinct, survived but retain hybrid alleles, and survived without supporting hybrid alleles (full native). Panels B and D show only the counts of populations that support hybrid alleles (that is, the green bars from

panels A and B). Panels E and F show the final HIS at year 100 across hydroperiod treatments. Full native populations have a HIS of 0.05, and the relative increase in HIS demonstrates the relative success of the single hybrid migrant. In both scenarios it appears that increased pond duration leads to an increase in the number of ponds that retain hybrid alleles, but less relative increase in overall pond HIS.

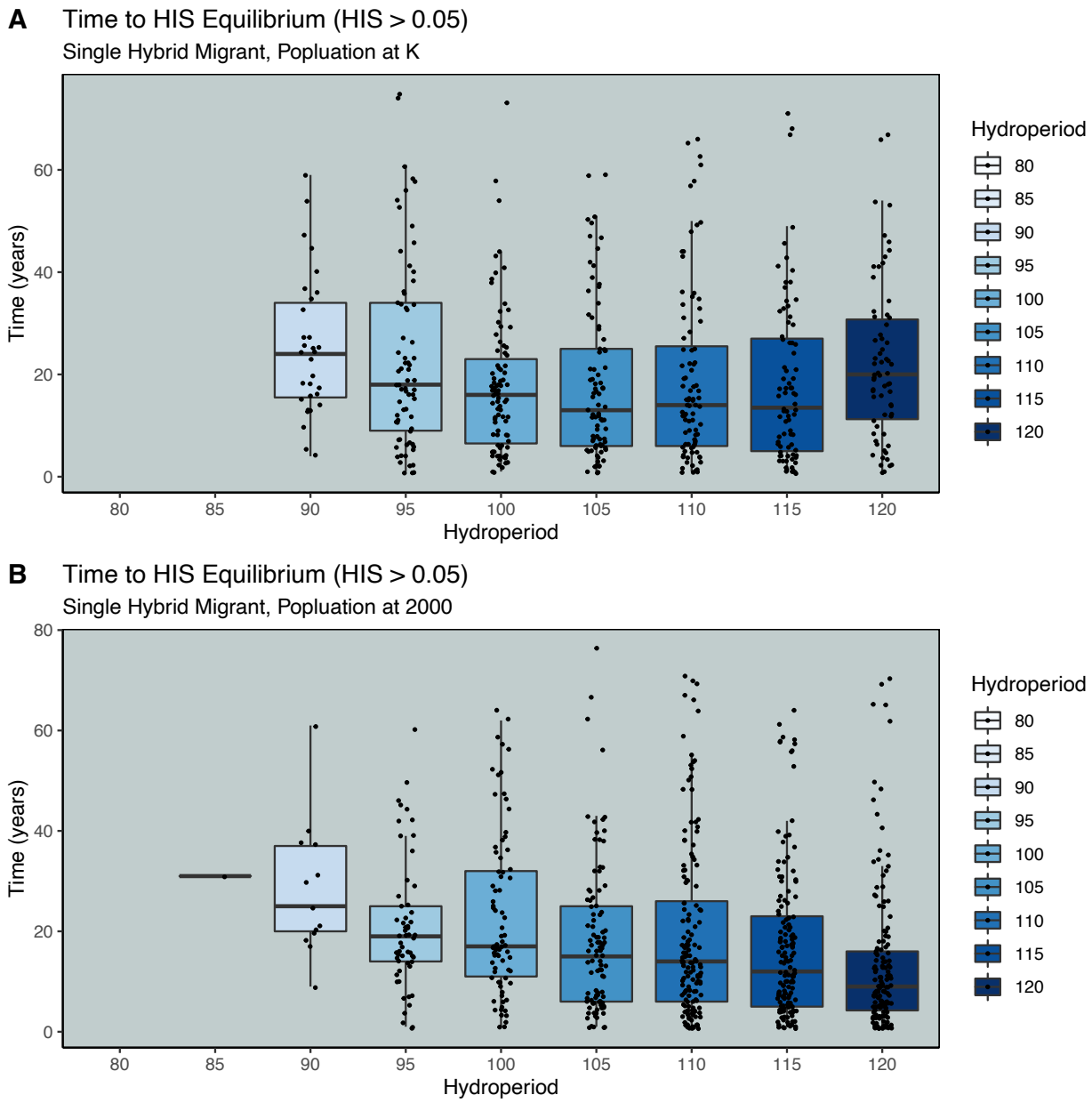

Figure 3.8: Single hybrid migrant model estimates for the time to HIS equilibrium across hydroperiod treatments. This model is a special case of the Population Viability Analysis model where ponds begin at either their specific carrying capacity (panel A), or at 2000 individuals (1000 adults and 1000 metamorphs; panel B). Equilibrium HIS was estimated as the median HIS from years 80 to 100 of the 100 year simulation. The number of years before the population reached this median HIS was recorded as the time to HIS. There is no significant trend in populations that start at K, but populations at 2000 individuals show a decrease in time to HIS equilibrium as hydroperiod increases.

Appendix A

Supplemental Information for Chapter 3

Estimating Larval Survival from HIS:

In this study, I use data collected in a previous experiment (Cooper et al. Chapter 2) to parameterize demographic functions in this present study. These updated methods are required to model the effect of an individual's Hybrid Index Score (HIS) on larval survival and mass at metamorphosis.

I used a bootstrap resampling technique to test the effects of hydroperiod and HIS on individual larval survival probability. Although I was unable to collect genetic tissue from every larva at the start of each years' experiment, I did collect genetic and morphological data from a representative sample of larvae from each source pond. I used random resampling to reconstruct the full dataset of input larvae, using the observed distribution of individuals across each source pond and family group. Briefly, source pond represents the original wild pond from which larvae were collected and family group represents the probable full-sibling groups estimated from genomic data (Cooper et al. Chapter 2). For example, exactly 40 larvae from source pond JCLH were added into experimental "pond D" in 2019. I additionally derived the relative distribution of larvae across family groups in JCLH from genomic analyses. I can therefore randomly sample the 60 representative larvae 40 times to simulate the individual larvae that were added into that experimental pond. I repeat this process for each source pond across all experimental ponds to simulate a complete dataset of larvae that entered the various pond treatments. I then use the observed metamorph survival data to assign "survivors". I use the family group assignment to accurately select which simulated input larvae "survived". For example, if 6 metamorphs from family group JCLH_1 emerged from "pond D" in 2019, then I would remove 6 larvae from

family group JCLH_1, and replace them with the known metamorphs. I match the simulated larval dataset to the observed metamorph dataset using family group to maximize accuracy in genotype assignment, since siblings are more likely to exhibit the same genotype than other family groups. I then use this complete, partially simulated dataset to construct a Generalized Linear Mixed Model (GLMM) with a binomial error distribution and using larval treatment as a random effect. This model uses Hydroperiod and HIS to predict the binomial Survival probability (0,1) of each larva.

Since this dataset is simulated using the representative larvae, I acknowledge that there will be variability in the assemblage of input larvae. I quantify this variation using a resampling bootstrap technique, where I iterate the described process 10,000 times, sampling the representative larvae with replacement each iteration. This bootstrap instance represents the "true" data. In order to assess the probability that patterns in the "true" data could have arisen by chance, I also repeat this resampling technique using "random" survivorship data. Here I randomly assign "survival" based on the number of surviving metamorphs that year, without regard to the experimental pond, source pond, family group or hydroperiod. I compare the distribution of "true" GLMM model estimates to those of the "random" analysis. Similar to a bootstrap technique, I consider any overlap between the two distributions as evidence of a false discovery, I therefore report p-values as the degree of overlap between the "random" and "true" model estimates. Once significance is established, I explore the effect of Hydroperiod and HIS on survival by estimating predicted marginal effect sizes using the function "ggpredict" in the eponymous R package GGPREDICT. I assessed the interaction between Hydroperiod and HIS by generating model predictions for mostly native (HIS=0.10) and mostly non-native (HIS=0.90), and comparing the marginal effect on survival. Specifically, for each level of hydroperiod, I

subtracted the native effect from the hybrid effect to quantify how much more hybrids benefit from each treatment.

Larval survival is strongly correlated with hydroperiod (bootstrap: estimate = 0.079, iterations = 10,000, $p < 1x10^{-4}$) and HIS (bootstrap: estimate = 0.66, iterations = 10,000, p = 0.005). The difference in predicted survival between hybrid and native genotypes was positive and increasing throughout the range of Hydroperiod. This suggests that hybrids have a survival advantage in all hydroperiod levels tested, and this advantage increases concurrently with pond duration.

Supplemental Figures for Chapter 3:

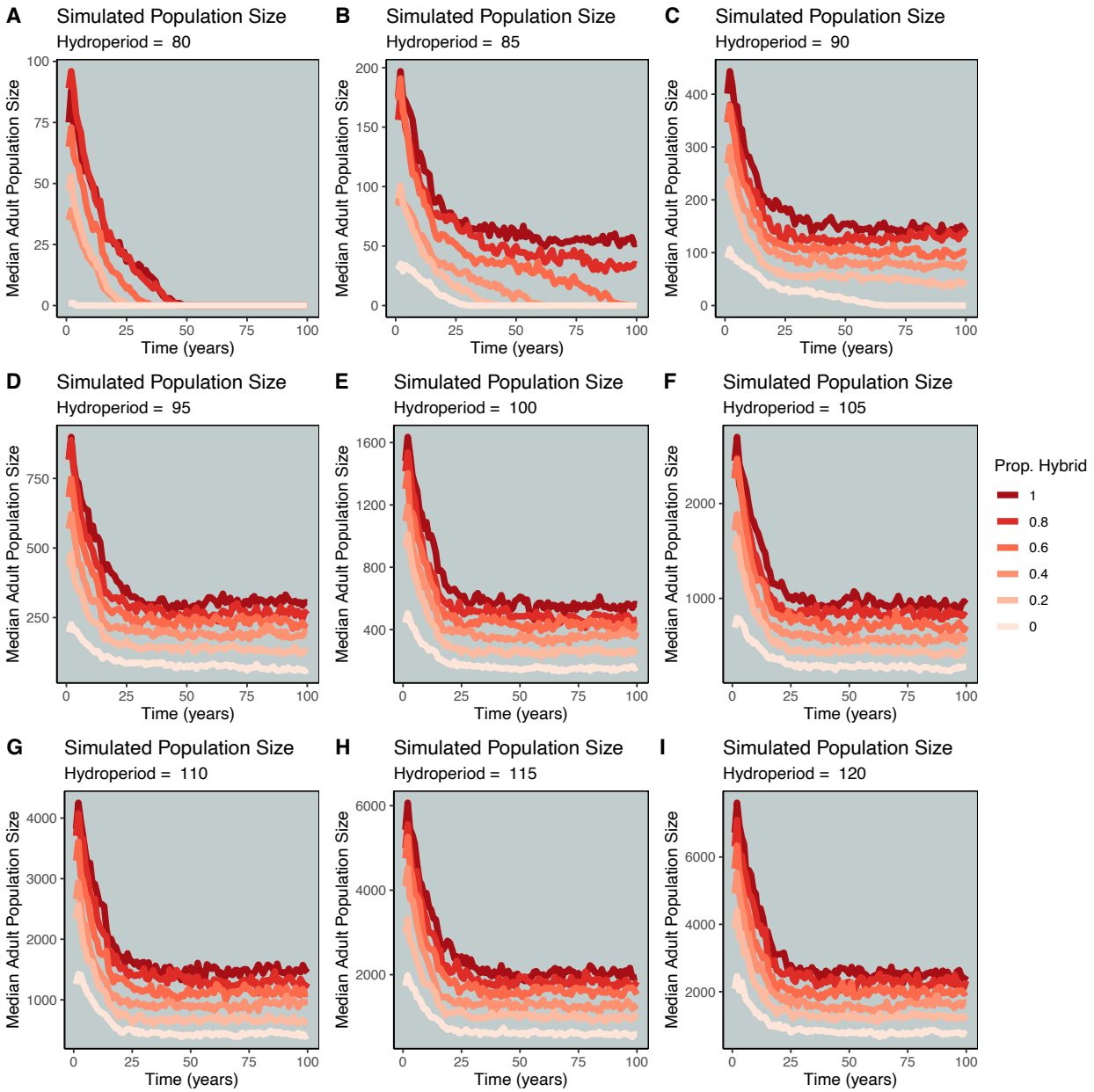

Figure 3.S1: Simulated population size across 100-years for multiple demographic scenarios using the population viability model. Each panel shows the difference in population size between starting hybrid proportions for a specific hydroperiod treatment.

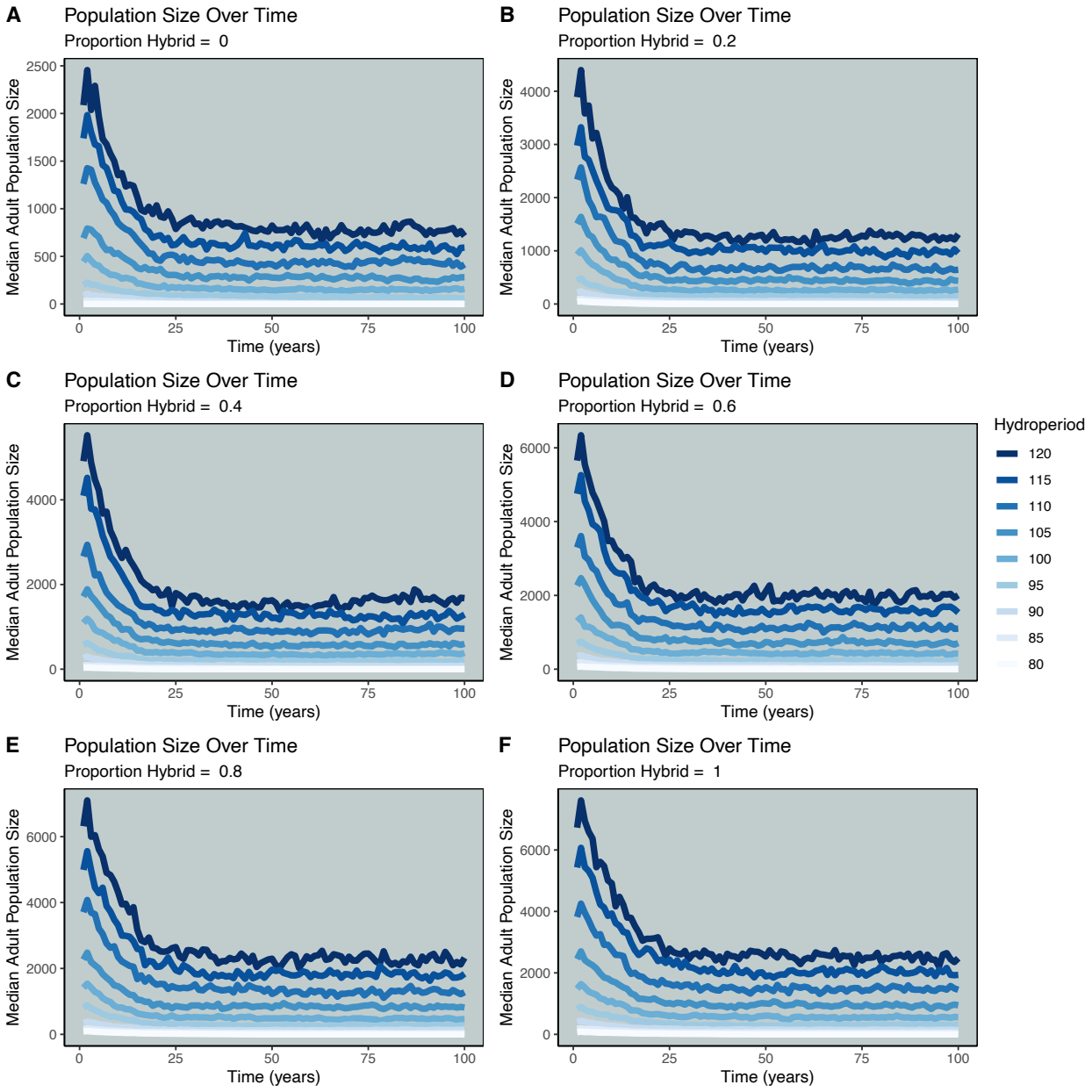

Figure 3.S2: Simulated population size across 100-years for multiple demographic scenarios using the population viability model. Each panel shows the difference in population size across hydroperiods for each initial hybrid proportion.

Literature Cited

Alexander ME, Dick JTA, Weyl OLF, Robinson TB, Richardson DM. 2014. Existing and emerging high impact invasive species are characterized by higher functional responses than natives. Biology Letters **10**:20130946. Royal Society.

Allendorf F, Hohenlohe P, Luikart G. 2010. Genomics and the future of conservation genetics.

Allendorf FW, Leary RF, Spruell P, Wenburg JK. 2001. The problems with hybrids: setting conservation guidelines. Trends in Ecology & Evolution **16**:613–622.

Anderson TL, Mott CL, Levine TD, Whiteman HH. 2013. Life Cycle Complexity Influences Intraguild Predation and Cannibalism in Pond Communities. Copeia **2013**:284–291.

Auwera GAV der et al. 2013. From FastQ Data to High-Confidence Variant Calls: The Genome Analysis Toolkit Best Practices Pipeline. Current Protocols in Bioinformatics **43**:11.10.1-11.10.33.

Baldwin RF, Calhoun AJK, deMaynadier PG. 2006. The significance of hydroperiod and stand maturity for pool-breeding amphibians in forested landscapes. Canadian Journal of Zoology.

Barnard R, Futo V, Pecheniuk N, Slattery M, Walsh T. 1998. PCR Bias Toward the Wild-Type k-ras and p53 Sequences: Implications for PCR Detection of Mutations and Cancer Diagnosis. BioTechniques **25**:684–691. Future Science.

Barry SJ, Shaffer HB. 1994. The Status of the California Tiger Salamander (Ambystoma californiense) at Lagunita: A 50-Year Update. Journal of Herpetology **28**:159–164. Society for the Study of Amphibians and Reptiles.

Beavis WD. 1998. QTL Analyses: Power, Precision, and Accuracy. Page Molecular Dissection of Complex Traits. CRC Press.

Beissinger SR, McCullough DR. 2002. Population viability analysis. University of Chicago Press.

Beissinger SR, Westphal MI. 1998. On the use of demographic models of population viability in endangered species management. The Journal of wildlife management:821–841. JSTOR.

Berger J, Pericchi L. 2015. Bayes Factors. Pages 1–14 Wiley StatsRef: Statistics Reference Online. American Cancer Society.

Biebighauser TR. 2011. Wetland restoration and construction: a technical guide. Upper Susquehanna Coalition Horseheads, New York, USA.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics:btu170.

Bronner IF, Quail MA, Turner DJ, Swerdlow H. 2013. Improved Protocols for Illumina Sequencing. Current Protocols in Human Genetics **79**:18.2.1-18.2.42.

Brook BW, O'Grady JJ, Chapman AP, Burgman MA, Akçakaya HR, Frankham R. 2000. Predictive accuracy of population viability analysis in conservation biology. Nature **404**:385–387. Nature Publishing Group.

Brooks GC. 2020. On the use of demographic models to inform amphibian conservation and management: A case study of the reticulated flatwoods salamander.

California Department of Fish Game. 2010. A status review of the California tiger salamander (Ambystoma californiense). Report to the Fish and Game Commission, State of California. Nongame Wildlife Program Report **4**.

Canessa S et al. 2018. Decision-making for mitigating wildlife diseases: From theory to practice for an emerging fungal pathogen of amphibians. Journal of Applied Ecology **55**:1987–1996.
Chapron G, Legendre S, Ferrière R, Clobert J, Haight RG. 2003. Conservation and control strategies for the wolf (Canis lupus) in western Europe based on demographic models. Comptes Rendus Biologies **326**:575–587.
Chaudhary V, Oli MK. 2020. A critical appraisal of population viability analysis. Conservation Biology **34**:26–40.
Coop G, Witonsky D, Rienzo AD, Pritchard JK. 2010. Using Environmental Correlations to Identify Loci Underlying Local Adaptation. Genetics **185**:1411–1423. Genetics.
Cooper RD, Shaffer HB. 2021. Allele-specific expression and gene regulation help explain transgressive thermal tolerance in non-native hybrids of the endangered California tiger salamander (Ambystoma californiense). Molecular Ecology **30**:987–1004.
Coulson T, Mace GM, Hudson E, Possingham H. 2001. The use and abuse of population viability analysis. Trends in Ecology & Evolution **16**:219–221.
Cummins D, Kennington WJ, Rudin-Bitterli T, Mitchell NJ. 2019. A genome-wide search for local adaptation in a terrestrial-breeding frog reveals vulnerability to climate change. Global Change Biology **25**:3151–3162.
Danecek P et al. 2011. The variant call format and VCFtools. Bioinformatics **27**:2156–2158.
Danecek P et al. 2021. Twelve years of SAMtools and BCFtools. GigaScience **10**.
David P, Thébault E, Anneville O, Duyck P-F, Chapuis E, Loeuille N. 2017. Chapter One - Impacts of Invasive Species on Food Webs: A Review of Empirical Data. Pages 1–60 in D. A. Bohan, A. J. Dumbrell, and F. Massol, editors. Advances in Ecological Research. Academic Press.
Davidson C, Shaffer HB, Jennings MR. 2002a. Spatial Tests of the Pesticide Drift, Habitat Destruction, UV-B, and Climate-Change Hypotheses for California Amphibian Declines. Conservation Biology **16**:1588–1601.
Davidson C, Shaffer HB, Jennings MR. 2002b. Spatial Tests of the Pesticide Drift, Habitat Destruction, UV-B, and Climate-Change Hypotheses for California Amphibian Declines. Conservation Biology **16**:1588–1601.
Easterling MR, Ellner SP, Dixon PM. 2000. Size-Specific Sensitivity: Applying a New Structured Population Model. Ecology **81**:694–708.
Ellner SP, Fieberg J, Ludwig D, Wilcox C. 2002. Precision of Population Viability Analysis. Conservation Biology **16**:258–261.
Ellner SP, Rees M. 2006. Integral Projection Models for Species with Complex Demography. The American Naturalist **167**:410–428. The University of Chicago Press.
Everson KM et al. 2021. Geography is more important than life history in the recent diversification of the tiger salamander complex. Proceedings of the National Academy of Sciences **118**. National Academy of Sciences.
Feit B, Dempster T, Jessop TS, Webb JK, Letnic M. 2020. A trophic cascade initiated by an invasive vertebrate alters the structure of native reptile communities. Global Change Biology **26**:2829–2840.
Fitzpatrick B, Shaffer H. 2007a. Introduction history and habitat variation explain the landscape genetics of hybrid tiger salamanders.

Fitzpatrick BM, Johnson JR, Kump DK, Shaffer HB, Smith JJ, Voss SR. 2009. Rapid fixation of non-native alleles revealed by genome-wide SNP analysis of hybrid tiger salamanders. BMC Evolutionary Biology **9**:176.
Fitzpatrick BM, Johnson JR, Kump DK, Smith JJ, Voss SR, Shaffer HB. 2010. Rapid spread of invasive genes into a threatened native species. Proceedings of the National Academy of Sciences **107**:3606–3610.
Fitzpatrick BM, Ryan ME, Johnson JR, Corush J, Carter ET. 2015. Hybridization and the species problem in conservation. Current Zoology **61**:206–216.
Fitzpatrick BM, Shaffer HB. 2007b. Introduction History and Habitat Variation Explain the Landscape Genetics of Hybrid Tiger Salamanders. Ecological Applications **17**:598–608.
Fitzpatrick BM, Shaffer HB. 2007c. Hybrid vigor between native and introduced salamanders raises new challenges for conservation. Proceedings of the National Academy of Sciences **104**:15793–15798.
Fitzpatrick BM, Shaffer HB. 2007d. Hybrid vigor between native and introduced salamanders raises new challenges for conservation. Proceedings of the National Academy of Sciences **104**:15793–15798.
Foley P. 1994. Predicting Extinction Times from Environmental Stochasticity and Carrying Capacity. Conservation Biology **8**:124–137.
García-Díaz P, Prowse TAA, Anderson DP, Lurgi M, Binny RN, Cassey P. 2019. A concise guide to developing and using quantitative models in conservation management. Conservation Science and Practice **1**:e11.
Gelman A, Su Y-S, Yajima M, Hill J, Pittau MG, Kerman J, Zheng T, Dorie V, Su MY-S. 2016. Package 'arm.' data analysis using regression and multilevel/hierarchical models. R package, version **9**.
Günther T, Coop G. 2013. Robust Identification of Local Adaptation from Allele Frequencies. Genetics **195**:205–220.
Gurevitch J, Padilla DK. 2004. Are invasive species a major cause of extinctions? Trends in Ecology & Evolution **19**:470–474.
Hassel D, Cheng P, White MP, Ivey KN, Kroll J, Augustin HG, Katus HA, Stainier DYR, Srivastava D. 2012. MicroRNA-10 regulates the angiogenic behavior of zebrafish and human endothelial cells by promoting vascular endothelial growth factor signaling. Circulation Research **111**:1421–1433.
Holland D, Hayes M, E M. 1990. Late summer movement and mass mortality in the California tiger salamander (Ambystoma californiense).
Holomuzki JR, Collins JP, Brunkow PE. 1994. Trophic Control of Fishless Ponds by Tiger Salamander Larvae. Oikos **71**:55–64. [Nordic Society Oikos, Wiley].
Hulme PE. 2020. Plant invasions in New Zealand: global lessons in prevention, eradication and control. Biological Invasions **22**:1539–1562.
Johnson J, Johnson B. 2010. Genotype and temperature affect locomotor performance in a tiger salamander hybrid swarm.
Johnson JR, Fitzpatrick BM, Shaffer HB. 2010a. Retention of low-fitness genotypes over six decades of admixture between native and introduced tiger salamanders. BMC Evolutionary Biology **10**.
Johnson JR, Fitzpatrick BM, Shaffer HB. 2010b. Retention of low-fitness genotypes over six decades of admixture between native and introduced tiger salamanders. BMC Evolutionary Biology **10**:147.

Johnson JR, Johnson BB, Bradley Shaffer H. 2010c. Genotype and temperature affect locomotor performance in a tiger salamander hybrid swarm. Functional Ecology **24**:1073–1080.
Johnson JR, Ryan ME, Micheletti SJ, Shaffer HB. 2013. Short pond hydroperiod decreases fitness of nonnative hybrid salamanders in California. Animal Conservation **16**:556–565.
Johnson JR, Thomson RC, Micheletti SJ, Shaffer HB. 2011. The origin of tiger salamander (Ambystoma tigrinum) populations in California, Oregon, and Nevada: introductions or relicts? Conservation Genetics **12**:355–370.
Jones OR, Wang J. 2010. COLONY: a program for parentage and sibship inference from multilocus genotype data. Molecular Ecology Resources **10**:551–555.
Jongejans E, Shea K, Skarpaas O, Kelly D, Ellner SP. 2011. Importance of individual and environmental variation for invasive species spread: a spatial integral projection model. Ecology **92**:86–97.
Kass RE, Raftery AE. 1995. Bayes Factors. Journal of the American Statistical Association **90**:773–795. Taylor & Francis.
King JL. 1998. Loss of diversity as a consequence of habitat destruction in California vernal pools. Pages 119–123 Ecology, Conservation, and Management of Vernal Pool Ecosystems—Proceedings from a 1996 Conference. California Native Plant Society, Sacramento, CA, USA.
Kissel AM, Palen WJ, Ryan ME, Adams MJ. 2019. Compounding effects of climate change reduce population viability of a montane amphibian. Ecological Applications **29**:e01832.
Lacy RC. 2019. Lessons from 30 years of population viability analysis of wildlife populations. Zoo Biology **38**:67–77.
Lampert A, Hastings A, Grosholz ED, Jardine SL, Sanchirico JN. 2014. Optimal approaches for balancing invasive species eradication and endangered species management. Science **344**:1028–1031. American Association for the Advancement of Science.
Lampert A, Liebhold AM. 2021. Combining multiple tactics over time for cost-effective eradication of invading insect populations. Ecology Letters **24**:279–287.
Lande R. 1993. Risks of Population Extinction from Demographic and Environmental Stochasticity and Random Catastrophes. The American Naturalist **142**:911–927. The University of Chicago Press.
Leary SL, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre C, Gwaltney-Brant S, McCrackin MA, Meyer R. 2013. AVMA guidelines for the euthanasia of animals: 2013 edition. American Veterinary Medical Association Schaumburg, IL.
Lennartsson T, Oostermeijer JGB. 2001. Demographic variation and population viability in Gentianella campestris: effects of grassland management and environmental stochasticity. Journal of Ecology **89**:451–463.
Liebhold AM, Kean JM. 2019. Eradication and containment of non-native forest insects: successes and failures. Journal of Pest Science **92**:83–91.
Loredo I, van Vuren D. 1996. Reproductive Ecology of a Population of the California Tiger Salamander. Copeia **1996**:895–901. [American Society of Ichthyologists and Herpetologists (ASIH), Allen Press].
Lown AE, Hepburn LJ, Dyer R, Cameron TC. 2020. From individual vital rates to population dynamics: An integral projection model for European native oysters in a marine protected area. Aquatic Conservation: Marine and Freshwater Ecosystems **30**:2191–2206.
Mallet J. 2005. Hybridization as an invasion of the genome. Trends in ecology & evolution **20**:229–237.

McCartney-Melstad E, Mount GG, Shaffer HB. 2016. Exon capture optimization in amphibians with large genomes. Molecular Ecology Resources **16**:1084–1094.
McMenamin SK, Hadly EA. 2010. Developmental dynamics of Ambystoma tigrinum in a changing landscape. BMC Ecology **10**:10.
McMenamin SK, Hadly EA, Wright CK. 2008. Climatic change and wetland desiccation cause amphibian decline in Yellowstone National Park. Proceedings of the National Academy of Sciences **105**:16988–16993. National Academy of Sciences.
Messerman AF, Clause AG, Catania SVL, Shaffer HB, Searcy CA. 2021. Coexistence within an endangered predator–prey community in California vernal pools. Freshwater Biology **66**:1296–1310.
Morais P, Reichard M. 2018. Cryptic invasions: A review. Science of The Total Environment **613–614**:1438–1448.
Mott CL, Dzaferbegovic H, Timm SR, Whiteman HH. 2019. Influences of facultative paedomorphosis on kin selection in a larval salamander, Ambystoma talpoideum. Behaviour **156**:287–306. Brill.
Nowoshilow S et al. 2018. The axolotl genome and the evolution of key tissue formation regulators. Nature **554**:50–55.
Palacios-Martinez J, Caballero-Perez J, Espinal-Centeno A, Marquez-Chavoya G, Lomeli H, Salas-Vidal E, Schnabel D, Chimal-Monroy J, Cruz-Ramirez A. 2020. Multi-organ transcriptomic landscape of Ambystoma velasci metamorphosis. Developmental Biology **466**:22–35.
Panetta FD. 2015. Weed eradication feasibility: lessons of the 21st century. Weed Research **55**:226–238.
Petranka JW. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press.
Pfennig DW, Collins JP, Ziemba RE. 1999. A test of alternative hypotheses for kin recognition in cannibalistic tiger salamanders. Behavioral Ecology **10**:436–443.
Pfennig DW, Sherman PW, Collins JP. 1994. Kin recognition and cannibalism in polyphenic salamanders. Behavioral Ecology **5**:225–232.
R. Core Team. 2013. R: A language and environment for statistical computing. Vienna, Austria.
Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, Vilo J. 2019. g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Research **47**:W191–W198.
Rees M, Ellner SP. 2009. Integral projection models for populations in temporally varying environments. Ecological Monographs **79**:575–594.
Reilly SM, Lauder GV, Collins JP. 1992. Performance Consequences of a Trophic Polymorphism: Feeding Behavior in Typical and Cannibal Phenotypes of Ambystoma tigrinum. Copeia **1992**:672–679. [American Society of Ichthyologists and Herpetologists (ASIH), Allen Press].
Riley SPD, Bradley Shaffer H, Randal Voss S, Fitzpatrick BM. 2003a. Hybridization Between a Rare, Native Tiger Salamander (ambystoma Californiense) and Its Introduced Congener. Ecological Applications **13**:1263–1275.
Riley SPD, Bradley Shaffer H, Randal Voss S, Fitzpatrick BM. 2003b. Hybridization Between a Rare, Native Tiger Salamander (ambystoma Californiense) and Its Introduced Congener. Ecological Applications **13**:1263–1275.
Ritchie EG, Johnson CN. 2009. Predator interactions, mesopredator release and biodiversity conservation. Ecology Letters **12**:982–998.

Rödin-Mörch P, Palejowski H, Cortazar-Chinarro M, Kärvemo S, Richter-Boix A, Höglund J, Laurila A. 2021. Small-scale population divergence is driven by local larval environment in a temperate amphibian. Heredity **126**:279–292.

Rogers HS, Buhle ER, HilleRisLambers J, Fricke EC, Miller RH, Tewksbury JJ. 2017. Effects of an invasive predator cascade to plants via mutualism disruption. Nature Communications **8**:14557. Nature Publishing Group.

Ryan ME, Johnson JR, Fitzpatrick BM. 2009a. Invasive hybrid tiger salamander genotypes impact native amphibians. Proceedings of the National Academy of Sciences **106**:11166–11171.

Ryan ME, Johnson JR, Fitzpatrick BM. 2009b. Invasive hybrid tiger salamander genotypes impact native amphibians. Proceedings of the National Academy of Sciences **106**:11166–11171.

Ryan ME, Johnson JR, Fitzpatrick BM, Lowenstine LJ, Picco AM, Shaffer HB. 2013. Lethal Effects of Water Quality on Threatened California Salamanders but Not on Co-Occurring Hybrid Salamanders. Conservation Biology **27**:95–102.

Sacerdote AB, King RB. 2009. Dissolved oxygen requirements for hatching success of two ambystomatid salamanders in restored ephemeral ponds. Wetlands **29**:1202–1213.

Sambrook J, Russel DW. 2001. Molecular cloning: a laboratory manual, volume 1, 2, 3 Cold Spring Harbour Laboratory Press. Cold Spring Harbour, New York.

Scott DE, Casey ED, Donovan MF, Lynch TK. 2007. Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. Oecologia **153**:521–532.

Searcy C, Gray LN, Trenham PC, Shaffer HB. 2014a. Delayed life history effects, multilevel selection, and evolutionary trade-offs in the California tiger salamander. Ecology **95**:68–77.

Searcy C, Snaas H, Shaffer H. 2014b. Determinants of size at metamorphosis in an endangered amphibian and their projected effects on population stability. Available from http://dx.doi.org/10.1111/oik.01775.

Searcy CA, Clause AG, Gray LN, Krkosek M, Rollins HB, Trenham PC, Shaffer HB. In Press. Building accurate integral projection models from sparse data: insights from the endangered California tiger salamander. Ecological Applications **In Press**.

Searcy CA, Gabbai-Saldate E, Bradley Shaffer H. 2013. Microhabitat use and migration distance of an endangered grassland amphibian. Biological Conservation **158**:80–87.

Searcy CA, Gray LN, Trenham PC, Shaffer HB. 2014c. Delayed life history effects, multilevel selection, and evolutionary trade-offs in the California tiger salamander. Ecology **95**:68–77.

Searcy CA, Rollins HB, Shaffer HB. 2016. Ecological equivalency as a tool for endangered species management. Ecological Applications **26**:94–103.

Searcy CA, Shaffer HB. 2011. Determining the migration distance of a vagile vernal pool specialist: how much land is required for conservation of California tiger salamanders? Studies From the Herbarium:73–87.

Searcy CA, Snaas H, Shaffer HB. 2015. Determinants of size at metamorphosis in an endangered amphibian and their projected effects on population stability. Oikos **124**:724–731.

Semlitsch R, Scott D, Pechmann J. 1988. Time and size at metamorphosis related to adult fitness in Ambystoma talpoideum.

Semlitsch R, Wilbur H. 1988. Effects of pond drying time on metamorphosis and survival in the salamander Ambystoma talpoideum.

Semlitsch RD. 1987. Relationship of Pond Drying to the Reproductive Success of the Salamander Ambystoma talpoideum. Copeia **1987**:61–69. [American Society of Ichthyologists and Herpetologists (ASIH), Allen Press].
Shaffer HB, McCartney-Melstad E, Toffelmier E. 2020. Landscape Genomics Study on California Tiger Salamander. State of California, Department of Transportation, Division of Research , Innovation & System Information:29.
Shaffer HB, McKnight ML. 1996. The Polytypic Species Revisited: Genetic Differentiation and Molecular Phylogenetics of the Tiger Salamander Ambystoma Tigrinum (amphibia: Caudata) Complex. Evolution **50**:417–433.
Sheridan MarkA, Kao Y-H. 1998. Regulation of Metamorphosis-Associated Changes in the Lipid Metabolism of Selected Vertebrates1. American Zoologist **38**:350–368.
Smith JJ, Timoshevskaya N, Timoshevskiy VA, Keinath MC, Hardy D, Voss SR. 2019. A chromosome-scale assembly of the axolotl genome. Genome Research **29**:317–324.
Stebbins RC. 2003. A field guide to western reptiles and amphibians. Houghton Mifflin Harcourt.
Storfer A, Eastman JM, Spear SF. 2009. Modern Molecular Methods for Amphibian Conservation. BioScience **59**:559–571.
Takatsu K, Kishida O. 2020. Enhanced recruitment of larger predators in the presence of large prey. Journal of Animal Ecology **89**:1615–1627.
Todesco M et al. 2016. Hybridization and extinction. Evolutionary Applications.
Treangen TJ, Salzberg SL. 2012. Repetitive DNA and next-generation sequencing: computational challenges and solutions. Nature Reviews Genetics **13**:36–46.
Trenham P, Shaffer BH, Koenig W. 2000a. Life history and demographic variation in the California tiger salamander (Ambystoma californiense).
Trenham PC. 2001. Terrestrial Habitat Use by Adult California Tiger Salamanders. Journal of Herpetology **35**:343–346.
Trenham PC, Bradley Shaffer H, Koenig WD, Stromberg MR. 2000b. Life history and demographic variation in the California tiger salamander (Ambystoma californiense). Copeia **2000**:365–377.
Trenham PC, Shaffer HB. 2005a. Amphibian Upland Habitat Use and Its Consequences for Population Viability. Ecological Applications **15**:1158–1168.
Trenham PC, Shaffer HB. 2005b. Amphibian Upland Habitat Use and Its Consequences for Population Viability. Ecological Applications **15**:1158–1168.
Tucker JK. 1999. Fecundity in the tiger salamander (Ambystoma tigrinum) from west-central Illinois. Amphibia-Reptilia **20**:436–438. Brill.
Turkalo AK, Wrege PH, Wittemyer G. 2017. Slow intrinsic growth rate in forest elephants indicates recovery from poaching will require decades. Journal of Applied Ecology **54**:153–159.
U.S. Fish and Wildlife Service. 2004. Endangered and threatened wildlife and plants; determination of threatened status for the California tiger salamander; and special rule exemption for existing routine ranching activities; final rule. Federal Register **69**:47212–47248.
Villemereuil P de, Frichot É, Bazin É, François O, Gaggiotti OE. 2014. Genome scan methods against more complex models: when and how much should we trust them? Molecular Ecology **23**:2006–2019.

Wayne RK, Shaffer HB. 2016. Hybridization and endangered species protection in the molecular era. Molecular Ecology **25**:2680–2689.
Whiteman HH, Wissinger SA, Brown WS. 1996. Growth and foraging consequences of facultative paedomorphosis in the tiger salamander, Ambystoma tigrinum nebulosum. Evolutionary Ecology **10**:433–446. Springer.
Wiens JD, Schumaker NH, Inman RD, Esque TC, Longshore KM, Nussear KE. 2017. Spatial Demographic Models to Inform Conservation Planning of Golden Eagles in Renewable Energy Landscapes. Journal of Raptor Research **51**:234–257.
Wilbur HM, Collins JP. 1973. Ecological Aspects of Amphibian Metamorphosis: Nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. Science **182**:1305–1314. American Association for the Advancement of Science.
Wu RS, Lam II, Clay H, Duong DN, Deo RC, Coughlin SR. 2018. A Rapid Method for Directed Gene Knockout for Screening in G0 Zebrafish. Developmental Cell **46**:112-125.e4.
Zacharias I, Zamparas M. 2010. Mediterranean temporary ponds. A disappearing ecosystem. Biodiversity and Conservation **19**:3827–3834.
Zhang X, Azhar G, Zhong Y, Wei JY. 2004. Identification of a novel serum response factor cofactor in cardiac gene regulation. The Journal of Biological Chemistry **279**:55626–55632.
Zhang X, Williams ED, Azhar G, Rogers SC, Wei JY. 2016. Does p49/STRAP, a SRF-binding protein (SRFBP1), modulate cardiac mitochondrial function in aging? Experimental Gerontology **82**:150–159.
Zheng L, Lv G, Sheng J, Yang Y. 2010. Effect of miRNA-10b in regulating cellular steatosis level by targeting PPAR-α expression, a novel mechanism for the pathogenesis of NAFLD. Journal of Gastroenterology and Hepatology **25**:156–163.