Abstract

ADAPTATION IN A CHANGING WORLD:

EVOLUTIONARY MECHANISMS OF SALT TOLERANCE IN A COASTAL AMPHIBIAN

By

Molly Ann Albecker

July 2018

Director of Dissertation: Michael W. McCoy

Major Department: Biology

In order to predict evolutionary outcomes of environmental change on populations in nature, we need an improved understanding of the biological mechanisms that affect whether organisms will adapt to a changed environment. This dissertation capitalizes on the unlikely discovery of a freshwater treefrog (*Hyla cinerea*) inhabiting brackish marshes along the coast of North Carolina to better understand adaptive evolution to a changed environment. The goals of this research are to (1.) examine the extent that salt-exposed, coastal frog populations are diverging from salt-naïve, inland populations in response to saltwater exposure across life stages, (2.) determine the molecular and life history mechanisms that permit this species to persist in brackish habitats, and (3.) explore factors that influence likelihoods of evolution (e.g., density dependence, phenotypic plasticity).

Chapter 1 used field surveys, meta-analysis, and common garden experiments to show that *Hyla cinerea* are unique among frog species in their ability to inhabit saline wetlands. Coastal *H. cinerea* laid more eggs in saltwater compared to inland *H. cinerea*, more coastal eggs

hatched in saltwater compared to inland eggs, and in the highest experimental treatment (12ppt), early-stage coastal tadpoles had higher survival rates than inland tadpoles. Chapter 2 investigated the role of plasticity in generating divergent phenotypes across larval development. Regardless of salinity, coastal tadpoles grew faster and initiated metamorphosis sooner but at a smaller size compared to inland tadpoles, and more coastal tadpoles survived to metamorphosis. Chapter 3 used individual-based modeling to explore how density dependence and selection interact to affect evolution in complex life cycle organisms. Density dependence increased genetic variation across populations by reducing population size, and evolutionary rescue was most likely to occur when selection precedes density dependence. Chapter 4 used transcriptomics to explore the mechanisms that produce differences across inland and coastal populations. We identified 1,924 differentially expressed genes between coastal and inland frog populations. We found that differentially expressed genes encode diverse molecular functions including ionic and osmotic transporters and stress response pathways.

This dissertation shows that coastal *H. cinerea* can become locally adapted to inhabit brackish habitats and explores several mechanisms that affect adaptive evolution to environmental change.

ADAPTATION IN A CHANGING WORLD:

EVOLUTIONARY MECHANISMS OF SALT TOLERANCE IN A COASTAL AMPHIBIAN

A Dissertation

Presented To

The Faculty of the Interdisciplinary Program in Biological Sciences

Department of Biology, Harriot College of Arts and Sciences

East Carolina University

Submitted In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Interdisciplinary Biological Sciences

By

Molly Ann Albecker

July 2018

ADAPTATION IN A CHANGING WORLD:
EVOLUTIONARY MECHANISMS OF SALT TOLERANCE IN A COASTAL AMPHIBIAN

By

Molly Ann Albecker

APPROVED BY:

DIRECTOR OF DISSERTATION: _____
MICHAEL MCCOY, PhD

COMMITTEE MEMBER: _____
BURRELL MONTZ, PhD

COMMITTEE MEMBER: _____
MATTHIAS LEU, PhD

COMMITTEE MEMBER: _____
KRISTA MCCOY, PhD

COMMITTEE MEMBER: _____
MARCELO ARDON SAYAO, PhD

COMMITTEE MEMBER: _____
DAVID KIMMEL, PhD

CHAIR OF DEPARTMENT OF BIOLOGY: _____
JEFF McKINNON, PhD

DEAN OF THE GRADUATE SCHOOL: _____
PAUL J. GEMPERLINE, PhD

ACKNOWLEDGEMENTS

The past several years have been some of the most rewarding and fun years of my life, and I am ever grateful to have found such an interesting and productive research question and study system. I am grateful for research funding provided by North Carolina Sea Grant (Project No. 2014-R/14/HCE-3I), National Science Foundation Doctoral Dissertation Improvement Grant (#1701690), Graduate Women in Science Nell Mondy Fellowship, Garden Club of America Fellowship, North Carolina Herpetological Society, Explorer's Club research grant, and Pitt County Wildlife Club. I want to thank the members of my committee (Dr. Ardon, Dr. Kimmel, Dr. Leu, Dr. K. McCoy, and Dr. Montz) for their helpful feedback and advice through the years. I am particularly indebted to Krista McCoy, who has graciously shared her time, expertise, lab space, and equipment with me. I am also very thankful for the sustained support from the Department of Biology and East Carolina University. Friends and family have given me generous and continual love and support throughout this endeavor, and they have added much color to the past several years. I am especially thankful for my husband, Adam, who has given me three things I needed throughout this process: love, companionship, and laughter. Adam has also helped with this research in multiple ways from assisting with frog-catching in the field to patiently teaching me bioinformatics pipelines. Finally, I will always be thankful for Mike and his advising. We have been through the "trials of miles" together – both literally and figuratively. He is truly exceptional as an advisor and friend, and I look forward to many more races, collaborations, and memories.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

IUCN = International Union for the Conservation of Nature

Ca. = circa (around)

Cm = centimeter

NC = North Carolina

Mm = millimeter

Yr = year

$MgCl_2$ = magnesium chloride

KCl = potassium chloride

$CaCl_2$ = Calcium chloride

NaCl = Sodium chloride

N = Number or North

Ppt = parts per thousand

GPS = Global positioning system

ECU = East Carolina University

IACUC = Institutional Animal Care and Use Committee

Oz. = ounce

H = hours

Hr = hours

FW = freshwater

L = Liter

mL = milliliter

C = Celsius

F = Fahrenheit

G = grams

Std. dev. = standard deviation

MCMC = Markov chain monte carlo

LC_{50} = Lethal Concentration that induces 50% mortality

BCI = Bayesian credible interval

Km = kilometers

NWR = national wildlife refuge

LCI = Lower Credible interval

UCI = Upper Credible interval

AICc = Akaike information criterion, corrected

dAICc = delta Akaike information criterion, corrected

df = degrees of freedom

C.I. = confidence interval

SE = standard error

Fig. = Figure

Psu = partial salinity units

MS-222 = Tricaine methanosulfate

RNA = ribonucleic acid

RNA-seq = Sequencing ribonucleic acid

mRNA = messenger ribonucleic acid

qPCR = quantitative polymerase chain reaction

ORP = oyster river protocol

Kmer = length of sequenced RNA

mOsm/L = milliosmoles per liter

GO = gene ontology

DNA = deoxyribonucleic acid

I. ADAPTIVE RESPONSES TO SALINITY STRESS ACROSS MULTIPLE LIFE STAGES IN ANURAN AMPHIBIANS

Abstract

In many regions, freshwater wetlands are increasing in salinity at rates exceeding historic levels. Some freshwater organisms, like amphibians, may be able to adapt and persist in salt-contaminated wetlands by developing salt tolerance. Yet adaptive responses may be more challenging for organisms with complex life histories because the same environmental stressor can require responses across different ontogenetic stages. Here we investigated responses to salinity in anuran amphibians, a common, freshwater taxon with a complex life cycle. We conducted a meta-analysis to define how the lethality of saltwater exposure changes across multiple life stages, surveyed wetlands in a coastal region experiencing progressive salinization for the presence of anurans and used common garden experiments to investigate whether chronic salt exposure alters responses in three sequential life stages (reproductive, egg, and tadpole life stages) in *Hyla cinerea*, a species repeatedly observed in saline wetlands. Meta-analysis revealed differential vulnerability to salt stress across life stages with the egg stage as the most salt-sensitive. Field surveys revealed that 25% of the species known to occur in the focal region were detected in brackish habitats. Remarkably, *Hyla cinerea* was found in large abundances in multiple wetlands with salinity concentrations 450% higher than the tadpole-stage LC_{50}. Common garden experiments showed that coastal (chronically salt exposed) populations of *H. cinerea* lay more eggs, have higher hatching success, and greater tadpole survival in higher salinities compared to inland (salt naïve) populations. Collectively, our data suggest that some species of anuran amphibians have divergent and adaptive responses to salt exposure across

populations and across different life stages. We propose that anuran amphibians may be a novel and amenable natural model system for empirical explorations of adaptive responses to environmental change.

Introduction

Accumulating greenhouse gas concentrations are increasing the heat retained in the atmosphere, which is in turn causing global mean sea levels to rise through intensified ice sheet and glacier melting and thermal expansion of ocean water (Meehl et al. 2005, Nicholls and Tol 2006, Domingues et al. 2008, Church et al. 2013). Sea levels have already risen 17-21cm over the past 110 years, and current models forecast that sea levels could rise an additional 40-63 cm over the next century with additions expected if ice sheets on Greenland and West Antarctica collapse (Scavia et al. 2002, Senior et al. 2002, Domingues et al. 2008, Kemp et al. 2011, Rahmstorf et al. 2012, Church et al. 2013). Ancillary impacts of climate change on coastal wetlands include alterations in the frequency and intensity of storm surges and coastal flooding, which may compound the effects of coastal erosion and saltwater inundation (Peng et al. 2004, Mulligan et al. 2012). The magnitude of sea level rise and impact on coastal ecosystems will vary depending on glacial isostatic adjustment, tectonic processes, oceanic circulation patterns, sediment compaction and accretion, wind patterns, and gravitational changes (DaLaune and Pezeshki 1994, Abrams 1995, Michener et al. 1997, Loaiciga 2003, Day et al. 2008, Meyssignac and Cazenave 2012, Church et al. 2013, Williams 2013), yet many areas are already being affected by sea level rise (Knighton et al. 1991, Baldwin and Mendelssohn 1998, Williams et al. 1999, Geddes and Mopper 2006, Kopp et al. 2015). Rising salinities are broadly anticipated to negatively impact freshwater organisms inhabiting coastal regions by reducing both the quality and quantity of suitable habitat, lowering individual fitness (e.g., increased physiological stress,

morphological deformities, reduced fecundity, and modifications to growth, development, and mortality), reducing population carrying capacity, and by altering biological interactions, disease risk, species movement, and community structure (Morris et al. 2002, Hamer and McDonnell 2008, Foden et al. 2009, Reed et al. 2011).

To survive in higher salinity habitats, osmoregulators require a wide variety of physiological, morphological, life historical, and behavioral traits to conserve water and expel enough excess ions. Although examples of adaptive responses across strong abiotic clines are multiplying quickly (Lee 1999, Reznick and Ghalambor 2001, Fraser et al. 2011, Brady 2012, Lamichhaney et al. 2012a, Mopper and Strauss 2013, Anderson et al. 2015), adaptive responses might be slowed by an organism's life history strategy, amount of standing genetic variation, demographic constraints (e.g., competition), or decoupling of environmental cue from response (Pfennig et al. 2010, Reed et al. 2010, Wund 2012, Hendry 2015, Nonaka et al. 2015). For example, organisms with complex life cycles, such as amphibians, have different ontogenetic life stages that are typically marked by abrupt shifts in morphology, physiology, behavior, and often distinct changes in habitat use. Therefore, the same stressor may differently impact each life stage, and require multiple adaptive responses across life stages to successfully adapt to an emerging environmental stressor.

Amphibians are a classic model for exploring responses to environmental stressors such as salinity (Morgan and Stockard 1907, Schmidt-Nielsen and Forster 1954, Thorson 1955, Thorson 1956, Christensen et al. 1961, McClanahan 1964, Gordon 1965, Gordon and Tucker 1965). Amphibians are widely regarded as important indicator species of wetland quality due to a life history tied to freshwater coupled with unique characteristics such as permeable skin, an inability

to concentrate and excrete excess salts, and poor dispersal capabilities (Neill 1958, Vitt et al. 1990, Carignan and Villard 2002, Hopkins and Brodie 2015). Additionally, amphibians comprise a significant proportion of the vertebrate biomass in wetland ecosystems (Gibbons et al. 2006, McCoy et al. 2009) and have been classified by the IUCN as "climate change susceptible" (Foden et al. 2009). Most amphibians have a complex life cycle in which they are obligatorily aquatic throughout the egg and larval period and become semi-terrestrial upon metamorphosis. Depending on the species, amphibians typically return to water as adults to breed or rehydrate.

A recent review identified ca. 140 anuran amphibian species that have been observed in saline habitats (ranging from tidal mangrove swamps to inland freshwater habitats contaminated with road deicing salts). Yet these species represent only 2% of all known species (Neill 1958, Hopkins and Brodie 2015), supporting the widely held belief that anurans are a generally salt-sensitive, freshwater order. A few notable species of amphibians such as *Fejervarya cancrivora*, *Bufo viridis,* and *Rhinella marina* are known to tolerate brackish conditions (Neill 1958, Christman 1974, Gibbons and Coker 1978, Balinsky 1981, Gomez-Mestre and Tejado 2003, Wu and Kam 2009a, Hopkins and Brodie 2015, Wijethunga et al. 2016), but these species still require freshwater habitats to complete their life cycles suggesting differential vulnerability to salt exposure across life stages even in specialist salt-tolerant species (Gordon et al. 1961, Gordon 1962, 1965, Gordon and Tucker 1965).

In addition to field observations, there are many published studies that experimentally explore egg, tadpole, or adult responses to salt stress (Hopkins and Brodie 2015). These studies typically evaluate how saltwater impacts anuran survivorship and behavior in a single life stage, and in

doing so, provide informative data on expected responses across a range of salinities. Hopkins and Brodie published an extensive review of saltwater tolerance in amphibians (Hopkins and Brodie 2015), which provides a useful framework to better understand and predict how salinization affects anuran populations. Yet the information about how salt impacts frog survivorship contained in these studies has not yet been coalesced to precisely quantify how salt tolerance changes across different life stages. Moreover, to best predict how anurans will respond to progressively increasing salinities, we not only need to define how salinity affects each life stage, but also how labile salt-tolerant responses are across populations.

In this study, we use multiple, complementary strategies to evaluate salt sensitivity in anurans generally, and substitute space for time to explore whether populations that inhabit coastal wetlands with a history of increasing salt exposure demonstrate adaptive responses across multiple life stages. First, we conducted a meta-analysis to establish an empirically derived quantitative framework of expected survivorship following exposure to saltwater in anuran amphibians for different life stages. Second, we performed a field survey of brackish and freshwater wetlands to describe and characterize amphibian distributions along a salt gradient in a coastal location predicted to be among the most impacted by sea level rise. Third, we substitute space for time in common garden experiments to investigate how exposure to saltwater across life stages differs among chronically salt-exposed (coastal) and salt-naïve (inland) anuran populations. If coastal frogs are responding adaptively, we expect to find differences in oviposition site choice behaviors, higher hatching rates, and higher tadpole survival in coastal populations compared to inland populations.

We focus on reproductive behaviors, egg hatching patterns, and post-hatching tadpole survival for our common garden experiments. During breeding events, male frogs amplex females and then the females will transport the males to assess potential egg laying sites. Females of some species are highly discriminatory and choose among oviposition sites to avoid a variety of biotic and abiotic stressors (Hsu et al. 2012, Wilder and Welch 2014). Oviposition site choice behaviors are under strong selection because her choice can strongly affect fertilization success, mortality risk to offspring, as well as resource availability to offspring, thus impacting offspring survival and performance (Sanzo and Hecnar 2006, Wu and Kam 2009b, Hsu et al. 2012, Wu et al. 2012, Wilder and Welch 2014, Nakkrasae et al. 2015). After eggs have been deposited, developing clutches are vulnerable to aquatic contaminants because frog eggs are enclosed by a permeable, jelly coat and lack a hard, protective shell (Touchon 2006, Haramura 2007). Upon hatching, many species frog larvae (tadpoles) are obligatorily aquatic and cannot survive on land until the completion of metamorphosis. During this period, tadpoles respire and osmoregulate via gills that function similar to freshwater teleosts such that ions and salts are conserved, and excess water is expelled (Dietz and Alvarado 1974, Uchiyama and Yoshizawa 1992, Wu et al. 2014). Saltwater is known to impact each of these stages (Christy and Dickman 2002, Dougherty and Smith 2006, Smith et al. 2006, Wu and Kam 2009a, Haramura 2011, Bernabò et al. 2013, Wilder and Welch 2014), and so we chose reproductive choices, embryo hatching success, and tadpole survival because these stages are key periods in the anuran life cycle that are highly vulnerable to external stressors, and strongly influence individual fitness and population persistence.

Methods

Study Location: We conducted these studies in eastern North Carolina, USA. North Carolina's coastline, barrier islands, and coastal habitats are predicted to be among the most significantly impacted by sea level rise due to the geomorphology of the Northern coastal zone (Albemarle embayment), coastal subsidence rates (-1mm±0.15mm/yr.), and gently sloped coastal plains (Titus and Richman 2001, Kemp et al. 2009, Williams 2013, Kopp et al. 2015). Indeed, the North Carolina coast has already seen intensified coastal flooding, and increased saltwater intrusion into coastal lowlands and freshwater aquifers making it an important location for investigating the impacts of sea level rise and increasing salinities on coastal organisms (Parkinson 1994, Michener et al. 1997, Kopp et al. 2015).

Meta-Analysis

Literature Search: We searched Google Scholar and Scopus databases for experimental studies evaluating the survivorship of anuran amphibians after experimental exposure to saltwater. We conducted the primary, exhaustive searches on December 16-20, 2014. Literature was checked again on July 14, 2015, September 23, 2015, February 25, 2016, and February 2, 2017 to ensure recently published work was included. We used the search terms (and all combinations of): "frog" OR "anuran" OR "amphibian" AND "saltwater" OR "salt" OR "salinity" OR "ocean" OR "NaCl" AND "mortality" OR "survivorship". Initial searches returned ~24,500 hits in total. These studies were further refined by scanning titles and abstracts. We excluded studies that did not mention survivorship or mortality of anurans (e.g., excluding *Caudata* and *Gymnophonia* amphibian orders) and exposure to saltwater in the abstract. We also cross checked against the

list of studies in Hopkins and Brodie's review of amphibian salt tolerance to ensure all appropriate studies were included (Hopkins and Brodie 2015).

Data Extraction: After refining our database to 129 studies, each study was read in detail and data were extracted from the text or from the figures. We extracted data only on studies that experimentally and directly manipulated salt concentrations against known sample sizes (e.g., field observations and studies with incidental, non-targeted salt exposure were excluded). We used studies that exposed frogs to saltwater solutions comprised of sodium chloride (NaCl), (e.g. InstantOcean® or natural seawater) and excluded studies that exposed frogs to mixed salt solutions (e.g., mixed road salt solutions) (Hintz and Relyea 2017). In studies where multiple saltwater compositions (e.g., $MgCl_2$, KCl, $CaCl_2$) were tested, we only used data from the trials that utilized NaCl. See supplementary material for detailed list of studies.

We used GraphClick® software version 3.0.3 (Arizona Software) to extract estimates from published figures and graphs. We report the mean survivorship (with error) for studies containing multiple replicates across salinities. For studies that compare survivorship across replicate populations, we present global averages across all populations tested. Although three studies report intra-specific differences in saltwater tolerance across different populations (e.g., (Gomez-Mestre et al. 2003, Crother and Fontenot 2006, Wu et al. 2014)), there were too few studies available to permit a meaningful formal analysis on population level differences in saltwater tolerance across studies or species. We recorded species identity, family, life stage (tadpole, egg, or adult), experimental salinity concentrations, sample size (N), survivorship (as proportion), the standard deviation of survivorship (converted from standard error when necessary), location of

the study, and length of exposure (in hours) for each study. Because different studies reported salinity using different units, we used standard conversions to transform all salinity measurements to parts per thousand (ppt).

Field Survey

Study Sites: We monitored wetlands regularly to make sure species that breed at different times could be detected. We surveyed 55 salt and freshwater wetlands in eastern North Carolina between February and September of 2014 for the presence of anuran amphibians. We included bogs, retention areas, marshes, ponds, ditches, and swamps, but excluded estuaries, sea grass beds, and other large, open water habitats. The most southern and eastern location was Cape Hatteras National Seashore and the survey extended northward to the town of Nags Head. Along this transect, we surveyed wetlands along Rodanthe, New Inlet, Bodie Island, Oregon Inlet, and Pea Island National Wildlife Refuge. We also sampled wetlands along an east to west transect spanning from the outer banks of NC, across Roanoke Island, which lies between the inner and outer banks and throughout Alligator River National Wildlife Refuge located on the Albemarle peninsula. The geographic bounds of the study area are 35º55'7"N to 35º14'7"N, and between 75º48'43"W to 75º27'27"W, excluding the Atlantic Ocean and the Pamlico, Croatan, and Roanoke sounds.

Survey Techniques: We used standard sampling methods to characterize anuran presence and relative abundance including auditory call surveys, standardized dip netting for larvae, and active searching for adults (Rader et al. 2001, Heyer et al. 2014). Our primary approach used auditory surveys to identify and locate frog populations, as well as to determine species identities and

relative abundances of the anurans present. When frogs were detected via call, the site was geo-referenced using a Garmin® GPSMAP 60CSx GPS navigator (Garmin, Ltd., Olathe, KS) and salinity (in ppt) and the temperatures of the air and water were measured using YSI Professional Plus multiparameter meter (Xylem, Inc., Yellow Springs, OH). We returned the following day (auditory surveys occurred at night) to the geo-referenced sites to determine egg mass/larvae presence using fixed-effort dip netting, and visual transect surveys (Rader et al. 2001, Heyer et al. 2014). To ensure that we thoroughly surveyed all wetlands for the presence of amphibians (and not just wetlands with detectable choruses), we used Google Maps® and visual surveys to identify additional wetlands that were not identified using call surveys, and sampled these wetlands using visual transect surveys and dip-netting for the presence of adult and/or larval anuran species. Tuberville et al. (Tuberville et al. 2005) conducted a thorough amphibian field survey along the North Carolina coast that included Cape Hatteras and Cape Lookout National Seashore and documented the current or historic presence of 17 anuran species, and we use the results of this study as a comparison for our own observations. Notably, the Tuberville study did not record salinity of locations in which anurans were observed.

Common Garden Experiments

We used *Hyla cinerea*, the American green tree frog (average size: 3.2-5.7 cm), for each of our common garden experiments, as this species is common across the Southeastern United States and has been repeatedly documented in brackish environments (Crother and Fontenot , Neill 1958, Wells 2007, Brown and Walls 2013, Wilder and Welch 2014). These experiments were conducted between May and August 2015. To characterize and identify how responses to saltwater differ among populations, we compared individuals from chronically salt-exposed *Hyla*

cinerea populations (hereafter referred to as "coastal" populations) against individuals from freshwater, salt-naive *Hyla cinerea* populations (hereafter referred to as "inland" populations). We located coastal and inland populations via the field survey. All coastal individuals were collected from sites in which salinities remained at or above 3ppt over the course of the breeding season, and all inland individuals were collected from populations with salinities below 1ppt. Coastal populations and inland populations were geographically separated from one another by at least 190 kilometers, so we assume that pairs collected from populations within these locations are sufficiently distant both geographically and environmentally to provide an accurate assessment of population-level differences produced by the different salinity of their habitats. The protocols for these experiments were approved by East Carolina's Animal Care and Use committee (D328 and D314) and collected under North Carolina Wildlife Collection License (#16-SC00840).

Oviposition Site Choice and Egg Hatching: We tested oviposition site choice by collecting four amplexed pairs of *Hyla cinerea* from either coastal or inland populations. Each pair was placed into an 18-Liter clear bin, the bottom of which was lined with six pint cups. Three of the six cups contained 400ml tap water (0ppt) treated with API® Tap Water Conditioner (Chalfont, PA), and the remaining cups contained 400ml saltwater prepared by mixing treated tap water with InstantOcean Sea Salt® (Blacksburg, VA). Each bin contained a single saltwater concentration that was either 4ppt, 6ppt, 8ppt, or 10ppt. In doing so, we presented each pair with a binary choice between laying eggs in freshwater or saltwater. The four salt concentration treatments collectively comprised a single replicate (i.e., four bins = one replicate). On nights when multiple replicates were conducted, each replicate was arranged in a spatial block at the site of collection.

Bins were left *in situ* overnight to allow pairs to complete breeding. The following morning, adult frogs were released, lids fastened to each cup, and bins were transported to the laboratory. Each cup was individually photographed, the salinity measured, and then monitored for hatching. Eggs hatched after 72-96 hours, defined as the point in which individuals were no longer retained in egg matrix and have functional gills (Gosner stage 20 (Gosner 1960)). Hatchlings were counted and recorded.

Tadpole Survivorship: To determine the effects of salinity on tadpole survival, we used the individuals hatched from eggs laid in freshwater during the previous oviposition experiments. Hatchlings were held in the laboratory that was maintained at 26.67ºC (~80ºF) and allowed to develop until reaching Gosner stage 25 (approximately 5 days) (Gosner 1960). Several studies have indicated that acclimatizing anurans to elevated salinities reduces mortality (Gordon 1962, Gordon and Tucker 1968, Hsu et al. 2012), and natural salinity fluctuations typically do not exceed +/- 2ppt per day, excluding an extreme event such as storm surge or flooding event. Therefore, to best mimic natural conditions and quantify survival, tadpoles were gradually acclimatized to a specified target salinity over 6 days. We chose five target salinities, 0.5ppt, 4ppt, 6ppt, 8ppt, and 12ppt, which are representative of natural salinities observed in coastal wetlands. Freshwater treatments (0.5ppt) were maintained at 0.5ppt throughout the six-day acclimatization period. The 4ppt treatments were raised by 0.67ppt per day, 6ppt treatments were raised by 1ppt per day, 8ppt treatments raised by 1.33ppt per day, and 12ppt treatments were raised by 2ppt per day, with final target salinities reached on day 6.

We divided each clutch into five groups of fifty tadpoles, which were then randomly assigned to one of the five salinity treatments, replicated 8 times for each location. Each clutch divided into five groups comprised a single replicate block to account for potential parental effects. Groups of tadpoles were placed into 350mL glass containers containing 300 mL of treated tap water (treated with API® Tap Water Conditioner (Chalfont, PA)) within a laboratory with 12-hour light/dark cycle. After acclimatizing overnight, salinity was increased incrementally each day according to treatment. Prior to water changes each day, tadpole mortality in each cup was assessed and recorded, and deceased individuals were removed. Tadpoles were fed 0.01g of Spirulina fish food flakes (Ocean Star International, Coral Springs, FL) each day following the water change. To perform water changes, tadpoles were carefully poured into a small holding container. 300mL of new, treated water with experimentally raised saltwater concentrations (InstantOcean Sea Salt® (Blacksburg, VA)) was poured into glass containers.

Statistical Analyses

We use a Bayesian approach to analyze data. For all statistical analyses we used JAGS interfaced with the R statistical programming environment, version 3.2.3 (2017) via "R2jags" (Su and Yajima 2015), "rjags"(Plummer 2015), and "coda"(Plummer et al. 2006) packages. For each analysis, we ran 5,000 iterations of three separate Markov Chain Monte Carlo (MCMC) chains with starting values that varied by an order of magnitude, each with a burn in of 2500 unless otherwise specified (Gelman et al. 2004). We used Gelman-Rubin diagnostics to assess model convergence in each analysis (Gelman et al. 2004).

Meta-Analysis: To estimate the probability of survival in saltwater for each life stage across anuran taxa and across salinities, we tested how increasing salinity affects anuran survivorship across clades for each life stage (e.g., egg, larvae, adult). We did not use phylogenetically corrected data because a recent review of all instances of amphibians in saline environments revealed no phylogenetic signal (Hopkins and Brodie 2015) and we detected no signal of phylogeny in the unexplained deviance from our analysis. We performed a Bayesian beta regression with an uninformative (relatively flat; mean = 0, std. dev. = 0.001) Gaussian prior. We chose the beta distribution because the data extracted for the meta-analysis were often only reported as "proportion survived" or "proportion killed" and lacked the necessary information (e.g., sample sizes and replicate numbers) required to back-calculate starting densities. In this analysis, survivorship and salinity were considered fixed effects, with individual studies treated as random effects.

Field Survey: We used the posterior distribution from the meta-analysis of all anuran species to predict the probability of anuran survivorship across several salinities including the salinities where we observed coastal *Hyla cinerea* during field surveys. Specifically, we generated a survival curve (with uncertainty) across salinities ranging from 1ppt (freshwater) up to 40ppt, and estimated the expected probability and credible intervals for finding frogs in sites with salinity concentrations we found in our field observations. Although 40ppt exceeds the salinity of natural seawater (35ppt), Gordon and colleagues observed *Fejervarya cancrivora* tadpoles in 39 ppt water in 1961 (Gordon et al. 1961). While this particular observation was not included in our meta-analysis due to its non-experimental nature, we wanted to ensure that all possible salinities were considered in our meta-analysis.

Common Garden Experiments: We used ImageJ® software to quantify the number of eggs that were laid in each cup. Briefly, files for each container were imported and changed to 8-bit images. The image background was subtracted, images were made binary, and files were converted to a mask. To separate groups of eggs that were clumped together, we used the watershed feature to demarcate individual egg boundaries. Outputs were visually inspected to ensure that all eggs were included and correctly counted.

We ran two-stage tests for both oviposition site-choice and hatching data. In the first step, we analyzed the data in binary form to ask if the probability of egg deposition or hatching changed as a function of the interaction between source population (e.g., coastal vs. inland) and salinity. In the second step, given that egg deposition or hatching occurred (i.e., excluding all cups in which zero eggs were laid or hatched), we analyzed the proportion of eggs deposited into freshwater and the proportion of offspring hatched as a function of the interaction between source population and salinity. These dual approaches answer distinct but complementary questions. Regarding oviposition, the first test asks if the probability of depositing eggs into saltwater or freshwater reflects a choice between salinities, while the second test reveals how parental investment differs according to salinity. Regarding hatching, the first test uncovers differences in the probability of complete loss due to salinity, while the second test reveals thresholds of sensitivity to salt.

To test the probability of oviposition, we ran Bernoulli regression to test for a relationship between egg presence or absence according to salinity and location (step one above). To test

whether there were differences in investment (step two above), we ran a binomial regression to examine whether salinity and location affected the proportion of eggs deposited by a female into saltier water. For both of these analyses, we used uninformative Gaussian priors (mean = zero and precision as a decaying power function with exponent = -2). To test the probability of hatching and proportion that hatched, we use informed priors based on the posterior distribution produced by the egg stage meta-analysis. Similar to the oviposition analyses, we ran Bernoulli regression to determine the relationship between egg hatching and salinity and location. We then used a binomial regression to analyze differences in the proportion of eggs that hatched in each salinity and location. Each of these four models considers salinity and location (e.g., coastal or inland) as fixed effects with "bin" nested in location as a random effect to account for parental effects (Bennett et al. 1996).

Tadpole Survivorship: To quantify how salinity, location, and time (e.g., day) affect tadpole survivorship, we used a binomial regression with informed priors based on the posterior distribution produced by the tadpole stage meta-analysis. This model considers salinity and location (e.g., coastal or inland) as fixed effects with "clutch" included as a random effect to account for sibship (Bennett et al. 1996). For this analysis we ran four separate MCMC chains with 50,000 iterations, each with a burn in of 25,000 (Gelman et al. 2004).

Results

Meta-Analysis

Effects of Salt on Amphibian Survivorship: We utilized data from 39 papers published between 1961 to early 2017 (see supplementary materials for detailed information). Overall, the literature

uniformly demonstrates that increasing saltwater concentrations lowers anuran survivorship across all three life-stages (Fig. 1-1). We found that across all studies included in this analysis, the lethal concentration of saltwater required to impose 50% mortality (LC_{50}) to anuran amphibian eggs is 4.15ppt (95% Bayesian credible interval [BCI] = 2.25 to 6.25ppt). The LC_{50} for larval anurans is 5.5ppt (4.24-6.65ppt BCI), while the LC_{50} for adults is 9.0ppt (0-19.9ppt BCI).

Field Surveys

Species Presence: We surveyed 55 wetlands along North Carolina's coastal plain (Fig. 1-2). In coastal freshwater habitats (<3ppt) with no connection to saltwater influence (e.g., municipal retention ponds), we documented the regular presence of 16 of the 17 anuran species found in the Tuberville study including *Hyla cinerea, Hyla chrysoscelis, Hyla squirella, Hyla femoralis, Anaxyrus fowleri, Anaxyrus quercicus, Anaxyrus terrestris, Lithobates sphenocephalus, Lithobates clamitans, Lithobates virgatipes, Lithobates catesbeianus, Gastrophryne carolinensis, Pseudacris ocularis, Pseudacris crucifer,* and *Acris gryllus*. We did not detect *Scaphiopus holbrookii* (Tuberville et al. 2005). In brackish wetlands (>3ppt), we documented the presence of 4 of those 16 species (*Hyla cinerea, Gastrophryne carolinensis, Lithobates catesbeianus,* and *Lithobates sphenocephalus*) (Table1).

Relative Abundance: In general, we noted that relative abundances of all species (except *Hyla cinerea*) declined as wetlands grew more saline. *Hyla cinerea* demonstrated unique distribution patterns along North Carolina's coast as the most abundant species found within brackish habitats along both the estuarine shoreline and outer banks. Notably, in some locations we

observed that the relative abundance of *Hyla cinerea* actually increased with increasing salinity, a pattern not shared with any of the other species found in brackish wetlands. We observed early and late stage *Hyla cinerea* tadpoles, metamorphs (between Gosner stages 31-39 (Gosner 1960)), and adults from multiple locations including from ponds and marshes with 3.9ppt, 8.3ppt, 11ppt, 16.8ppt, and 23.4ppt water (Table 1-1).

Probability of Field Findings: Using the posterior probability distributions from our meta-analysis we examined the relative probability of survival for frogs in the observed salinities: 3.9ppt, 8.3ppt, 11ppt, 16.8ppt, and 23.4ppt saltwater. The expected probability of survival for an individual anuran following exposure to a 3.9ppt saltwater solution during the egg stage is 0.52 (0.39-0.66 95% BCI), 0.60 (0.51-0.68 BCI) for larval anurans, and 0.62 (0.39-0.84 BCI) for adults. The probability of survival in 8.3ppt water for eggs is 0.25 (0.09-0.45 BCI), 0.32 (0.23-0.39 BCI) for larvae, and 0.53 (0.32 – 0.74 BCI) for adult frogs. At 11ppt, the survivorship for eggs is 0.15 (0.03-0.35 BCI), larval survivorship is 0.18 (0.12-0.25 BCI), with adult survivorship predicted at 0.46 (0.25 – 0.70 BCI). Wetlands at 16.8ppt have 0.04 (0.002-0.19 BCI) expected egg survivorship, 0.04 (0.02-0.07 BCI) expected larval survivorship, and 0.35 (0.13-0.61 BCI) expected adult survivorship. In 23.4ppt wetlands, 0.01 (0.00-0.008 BCI) eggs are expected to survive, larval survivorship is 0.01 (0.002-0.02 BCI), and expected adult survivorship is 0.25 (0.05-0.57 BCI) (Table 1-1).

Common Garden Experiments

The oviposition site choice experiment used *Hyla cinerea* pairs collected from three geographically discrete populations from inland and coastal locations in eastern North Carolina.

The subsequent egg hatching and tadpole survivorship experiments utilized the offspring of the collected pairs. For the coastal locations, we sampled three discrete populations along the inner and outer banks of North Carolina. We collected 1 replicate from a population near New Inlet bridge (35º41'11.5" N, 75º29'03.92"W), 1 replicate from Coastal Studies Institute on Roanoke Island (35º52'26.14" N, 75º39'38.54" W), and 2 replicates from Point Peter Road, Alligator River National Wildlife Refuge (35º46'13.1" N, 75º44'30.1" W). These populations are separated by the Croatan and/or Roanoke Sounds. For the inland locations, we sampled three discrete populations around Greenville, North Carolina. Specifically, we collected 1 replicate from a population near MacGregor Downs Road (35º37'15.8" N, 77º26'45.29" W), 1 replicate along Pactolus Highway (35º37'18.9" N, 77º20'43.8" W), and 2 replicates from a retention pond on 10th Street (35º35'26.49" N, 77º19'09.89" W). Each inland population is at least 5km apart from other populations with the Tar River and multiple highways between populations.

Oviposition Site Choice: We conducted four replicates in coastal and inland locations. Pairs successfully bred in every bin except one that contained a coastal pair. On average, females laid 1,363 eggs (minimum = 713 eggs, maximum = 3,039 eggs) per bin. We found that location (e.g., coastal vs. inland) and salinity both affected the probability that a female will lay her eggs in a particular pool (Fig. 1-3). As salinity increased, pairs from inland populations were less likely to deposit eggs in salinized water, while coastal females maintained a high probability of laying eggs in the higher salinity treatments (Fig. 1-3). For example, in the lower salinity treatments (4ppt), coastal females showed no divergence with inland females having 0.87 (0.85-0.91 BCI) probability of laying any eggs in the 4ppt water, and coastal females having 0.84 (0.81-0.88 BCI) probability of laying eggs. Yet in the higher salinity treatments in which females chose between

fresh or 12ppt water, inland females had a 0.51 (0.41- 0.61 BCI) probability of laying any eggs into 12ppt water, while coastal females exhibited 0.91 (0.88-0.96 BCI) probability of laying eggs. Source population and salinity both affected the proportion of eggs laid in freshwater (Fig. 1-4). Pairs from both locations tended to lay the majority of their eggs into freshwater as salinity increased, but at 12ppt, pairs from inland populations laid only 6% (0.04-0.07 BCI) into the saline water, while coastal pairs laid 16% (0.14-0.18 BCI) of their eggs in the saline water (Fig. 1-4).

Egg Hatching: Salinity and source population affect the probability that any eggs would hatch out of a particular treatment (Fig. 1-5). At 4ppt, the probability that an egg sourced from inland parents would hatch is 0.31 (0.24-0.38 BCI), while the probability that an egg laid by coastal parents would hatch is 0.54 (0.47-0.61 BCI). At higher salinities (10ppt), eggs from both populations had an exceedingly low probability of hatching (inland probability: 0.02 (0.007-0.03 BCI); coastal probability: 0.04 (0.02-0.06 BCI)) (Fig. 1-5). We also observed that although the proportion of eggs that hatched in 3ppt was similar across locations (inland proportion hatched: 0.33 (0.27-0.38 BCI); coastal proportion hatched: 0.36 (0.31-0.42 BCI)), 10% (0.07-0.11 BCI) of the coastal-sourced eggs hatched at 6ppt compared to 3% (0.02-0.04 BCI) of the eggs sourced from inland populations (Fig. 1-6).

Tadpole Survivorship: Following the 6-day acclimation, the predicted survival probability for coastal and inland *Hyla cinerea* tadpoles in freshwater for coastal-sourced tadpoles is 0.98 (0.96-0.99 BCI) and 0.98 (0.96-0.99 BCI) for inland-sourced tadpoles (Fig. 1-7). At 4ppt, predicted survivorship for coastal offspring is 0.96 (0.92-0.98 BCI) while inland offspring survivorship is

0.97 (0.95-0.99 BCI). Survivorship in 6ppt treatments is 0.94 (0.90-0.97 BCI) for coastal tadpoles and 0.95 (0.89-0.98 BCI) from inland tadpoles. In the 8ppt treatments, coastal tadpoles had higher survivorship at 0.97 (0.94-0.99 BCI) than inland tadpoles at 0.84 (0.73-0.92 BCI). At 12ppt, we again observed higher survivorship among coastal tadpoles with 0.24 (0.14-0.39 BCI) survivorship compared to inland tadpoles with 0.09 (0.04-0.16 BCI) survivorship. The random effect standard deviation representing parental influence is 0.17. Fixed effect slope and intercept estimates are listed in Table 1-3 in the supplementary material.

Discussion

We are at the precipice of dramatic environmental transformation as a result of global climate change, which provides the ideal canvas for exploring organismal responses to environmental change. Wetlands in coastal zones around the globe are among those anticipated to be most severely impacted from climate change due to increased frequency and intensity of coastal storms as well as increased flooding and secondary salinization from sea level rise (Titus and Richman 2001, Meehl et al. 2005, Domingues et al. 2008, Craft et al. 2009, Nicholls and Cazenave 2010, Kemp et al. 2011, Kopp et al. 2015). Yet despite the amount of cultural and research attention that climate change garners, a distressing deficiency exists in our empirical understanding of how rising salinities will impact coastal freshwater habitats and the animal communities sustained therein.

Ecological niche models aimed at understanding how environmental changes will impact affected populations typically predict that species that cannot emigrate to more suitable habitats are at risk of being locally extirpated as environmental quality degrades (Walther et al. 2002,

Thomas et al. 2004, Davis et al. 2005, Bradshaw and Holzapfel 2006, Nicholls and Tol 2006, Parmesan 2006, Chen et al. 2011, Dawson et al. 2011, Harley 2011, Moritz and Agudo 2013). This forecast is rational for freshwater organisms (like amphibians) that inhabit coastal wetlands given the lethal nature of osmotic stress (Stuart et al. 2004, Thomas et al. 2004, Lewis 2006, Schwartz et al. 2006, Traill et al. 2010, Maclean and Wilson 2011, Chown 2012, Moritz and Agudo 2013). However, an important assumption inherent in most model predictions is that species either completely lack or have limited capacity to respond to environmental change -- an assumption that can lead to overestimates of extinction rates or expected range contraction (Davis and Shaw 2001, Holt and Gomulkiewicz 2004, Thomas et al. 2004, Lewis 2006, Schwartz et al. 2006, Lawler et al. 2010, Reed et al. 2011, Moritz and Agudo 2013). Although adaptive evolution is increasingly well appreciated as a potential source of rescue for some, it is unclear whether organisms with complex life history strategies will be able to adapt to environmental change. In amphibians, we currently lack the ability to make more informed predictions that include adaptation for two main reasons. First, we do not know how sensitivity to salt stress varies across different life stages, and second, we know little about whether salt-tolerant responses are evolutionarily labile across life stages. In this paper, we address these gaps using a variety of tools (e.g., meta-analysis, field surveys, and common-garden experiments).

Meta-Analysis and Field Surveys: Studies on amphibian responses to saltwater often begin with some variant of the statement, *it is well accepted that frogs do not belong in saline habitats* (Neill 1958*)*. These statements stem from long standing dogma that amphibians are not physiologically equipped to osmoregulate in non-freshwater environments. Nonetheless, we observed *Lithobates catesbeieanus*, *Lithobates sphenocephalus*, *Gastrophryne carolinensis*, and

Hyla cinerea in brackish marshes in coastal North Carolina. These four species have been reported in brackish habitats previously (Hardy 1953, Hardy 1972, Christman 1974, Gunzburger 2006, Brown and Walls 2013) and the recurrence of these observations draws attention to the paucity of information explaining why some species are repeatedly observed inhabiting brackish wetlands while other closely related species are absent (Hopkins and Brodie 2015). A particularly interesting contribution on this subject stems from our repeated field observations of abundant and thriving *Hyla cinerea* populations in salt marshes with salinities 450% higher than the expected larval LC_{50} concentration (as revealed by the meta-analysis). Indeed, these findings were deemed inconceivable by the authors at the outset of the survey. While previous studies reported *Hyla cinerea* from saltmarshes along the Chesapeake Bay in Maryland in salinities up to 15ppt (Hardy 1953), we found populations in salinities as high as 23ppt, which is also the highest salinity that any North American frog species has been found to date (though Puerto Rican populations of *Rhinella marina, Eleutherodactylus coqui,* and *Lithobates grylio* come close at 20.5ppt (Rios-López 2008)).

Hopkins and Brodie recently updated (2015) Neill's 1958 review and provide a valuable and thorough review of all published observations of amphibians in saltwater (Neill 1958, Hopkins and Brodie 2015). In their review, Hopkins and Brodie present a range of salinity tolerances revealed by experimental and field studies and suggest that the median maximum experimental salinity that can be tolerated by anuran amphibians falls between 9ppt-12ppt (Ruibal 1959, Gordon et al. 1961, Munsey 1972, Hopkins and Brodie 2015). Our meta-analysis refines and builds upon these estimates by providing an empirically derived range of survival probability estimates for each salinity and life stage. For example, at 9ppt we may expect around 21% of

eggs to survive, 27% of larvae to survive, and 50% of adult to survive – fundamental information for managing anuran populations across landscapes affected by salinization.

The meta-analysis underlines the fact that amphibians have different abilities to persist in saline environments according to life stage. Though most studies test the effects of salt on a single life stage, our meta-analysis integrates the findings of all of these studies to better understand how salt sensitivity changes through each life stage and provides a quantitative baseline and important context for our common garden experiments and field observations of anurans in salinities as high as 66% seawater. Broadly, all studies examined in our meta-analysis demonstrate declines in survivorship as salinity increased across each life stage, our analyses, which includes studies on 35 species representing 26 different genera across 10 families. We found that eggs are the most sensitive to osmotic stress across the anuran clade, followed by the larval stage, and adults are the least susceptible. The results of the meta-analysis indicate that the lethal experimental salt concentration in which 50% mortality (LC_{50}) is expected for eggs occurs at approximately 4.15ppt for anuran eggs, 5.5ppt for larvae, and 9.0ppt for adults. Although the uncertainty in LC_{50} concentrations identified in the meta-analysis stems largely from differences in sample sizes (only three studies on adult frogs met our criteria for the meta-analysis), it might also reflect greater sensitivity during particular stages among species.

Embryos, for example, are expected to be more sensitive to external stressors than other stages because important developmental pathways are initiated during the early embryonic period and so perturbations at this stage may be teratogenic or fatal (Wilbur 1980, Meteyer et al. 2000). It has been shown that pathogens (e.g., bacteria, endoparasites, or water-borne fungi), predators,

ultraviolet radiation, and toxins can all have strong effects on embryonic survival, and induce effects that carry over to affect developmental outcomes in later life stages (Grant and Licht 1995, Kiesecker and Blaustein 1997, Burkhart et al. 1998, Touchon et al. 2006, Rohr and McCoy 2010). Tadpoles are also expected to be more sensitive to water quality than are adults because they are obliged to the aquatic habitat. Larvae may be more tolerant of osmotic stress than embryos if they can increase the activity or concentration of ion pumps in the gills. However, tadpoles raised in saltwater tend to have stunted developmental rates and metamorphose at smaller sizes compared to freshwater-raised tadpoles (Uchiyama and Yoshizawa 1992, Christy and Dickman 2002, Langhans et al. 2009, Wu and Kam 2009a, Hsu et al. 2012, Bernabò et al. 2013, Wood and Welch 2015), which can affect adult survival and reproductive success (Berven 1990). Adults, on the other hand, are less confined to aquatic environments and thus can reduce contact with stressful habitats via behavioral avoidance or dispersal. Additionally, adults can likely physiologically tolerate a greater degree of osmotic stress and/or desiccation by increasing urea in the blood (Balinsky 1981, Shoemaker et al. 1992), altering cellular ion or water transport (Uchiyama and Yoshizawa 1992, Konno et al. 2006, Uchiyama and Konno 2006, Wu et al. 2014), or adjusting the permeability of the skin (McClanahan et al. 1978, Lillywhite 2006).

Common Garden Experiments: In the oviposition, hatching, and tadpole survivorship experiments, we find evidence for altered and adaptive responses to salinization across multiple life stages in *Hyla cinerea*. Specifically, we report differences in egg deposition patterns, hatching success, and tadpole survivorship between salt-exposed coastal and salt-naïve inland populations of North American green treefrogs *(Hyla cinerea)*. We focus on reproductive behaviors, egg hatching, and tadpole viability because they are stages and traits that are highly

vulnerable to environmental quality, and directly affect fitness and population viability (Gordon et al. 1961, Roberts 1970, Chinathamby et al. 2006, Brand et al. 2010, Petranka and Doyle 2010, Bernabò et al. 2013, Thirion 2014, Hopkins and Brodie 2015). Female oviposition site selection directly affects the fitness of both the parents and the offspring, so decisions about oviposition sites should reflect an adaptive response. Therefore, we expected strong patterns of saltwater avoidance among both coastal and inland populations if salt were equally lethal to eggs and offspring from both inland and coastal populations (Rieger et al. 2004, Refsnider and Janzen 2010, Haramura 2011, Wilder and Welch 2014). However, we found that coastal and inland frogs exhibited different patterns of oviposition site selection across the experimental salt gradient. Both inland and coastal pairs increasingly avoided saline water as salinity increased but inland frogs had greater response and did not deposit any eggs in salinities above ~12ppt, whereas coastal pairs laid approximately 24% of their eggs in the highest salinities (Fig. 1-3). Additionally, eggs laid by coastal parents have higher probabilities of hatching in higher salinities and more coastal tadpoles survive in higher salinities when compared to inland-sourced conspecifics. Our inferences are based on experiments on three coastal and three inland populations and so should be extrapolated more broadly with caution. However, collectively our results provide evidence that some coastal populations of *Hyla cinerea* are responding adaptively to saltwater exposure across multiple life stages, which is contrary to expected outcomes given the general reputation of anuran amphibians as a highly salt sensitive order. Gomez-Mestre and Tejado report similar findings in *Bufo calamita*, the Natterjack Toad, in which embryos and tadpoles from brackish populations demonstrate higher survival compared to tadpoles from inland, freshwater populations (Gomez-Mestre and Tejado 2003, Gomez-Mestre and Tejado 2005). Together these studies suggest that the ability to respond adaptively to saltwater exposure

may be more possible than previously appreciated, and future studies may consider using comparative, common-garden approaches to not only determine how salt-exposure affects various endpoints, but also whether other species also exhibit population-level differences in salt tolerance across species and life stages.

The physiological mechanisms that explain why coastal pairs have relaxed salt avoidance behaviors, higher hatching success, and higher tadpole survivorship are likely to be numerous and spread across the different life stages. In adults, coastal male *Hyla cinerea* may have more viable and motile sperm in saline water. A recent study examined sperm survivorship and motility in *Hyla cinerea* located in Charleston, South Carolina (a coastal location) and found that 4ppt saltwater reduced ability of sperm to survive and swim, but that study did not compare coastal and inland populations (Wilder and Welch 2014). Alternatively, adult coastal females may increase the partitioning of yolk resources into eggs, or alter the egg coat matrix to provide additional protection against osmotic stressors compared to inland eggs. In tadpoles, coastal individuals may have an increased abundance of water channels (AQPs) and ion pumps (e.g., Na^+/K^+-ATPase) in the gills that enhance the ability to maintain internal water and ion balance, thus improving survival. Several studies have demonstrated that exposure to saltwater can increase the quantity and activity of sodium-potassium pumps in tadpole gills (Bernabò et al. 2013, Havird et al. 2013, Wu et al. 2014). These hypotheses remain to be tested in coastal *Hyla cinerea*, leaving the exact mechanisms explaining the observed patterns undefined. Moreover, the adaptive processes that produce advantageous physiological responses also remain largely unknown.

There are three possible overlapping adaptive processes that may explain the divergence in responses that we observed between coastal and inland anuran populations: local adaptation, phenotypically plastic responses, and/or maternal effects. Local adaptation occurs when populations have higher fitness in their local environmental conditions compared to populations from other environments, and our results are consistent with expected outcomes if coastal populations are becoming locally adapted to tolerate elevated salt concentrations across different life stages (Savolainen et al. 2013). Adaptive evolution is a well-appreciated process that can sustain or rescue populations facing strong selection gradients (Bell 2013, Gonzalez et al. 2013, Martin et al. 2013, Bourne et al. 2014, Carlson et al. 2014). Yet several criteria must be met before local adaptation can be confirmed. "Adaptive" phenotypes must be shown to correlate positively with fitness, and the production of putatively adaptive phenotypes should be directly linked to specific environmental drivers, and studies on adaptive responses must demonstrate a genetic basis for differences observed among populations (Merilä and Hendry 2014). Our results are consistent with the expectations of the first two criteria, but we are not yet able to deduce whether there is a genetic basis for such changes.

Phenotypic plasticity (defined here as the ability to modulate phenotype in response to environmental cues) can also produce phenotypes that appear different and adaptive, yet may be genetically indistinguishable from other populations (Merilä and Hendry 2014, Urban et al. 2014, Urban et al. 2016). Because plasticity can promote adaptation, inhibit adaptation, or be the adaptive response itself, uncovering the role of phenotypic plasticity remains one of the most important challenges for understanding and predicting adaptive responses to climate change (Lande 2009, Whitman and Agrawal 2009, Chevin et al. 2010, Pfennig et al. 2010, Wund 2012,

Urban et al. 2014, Hendry 2015, Murren et al. 2015b, Nonaka et al. 2015). Indeed, some degree of phenotypic plasticity has been observed in nearly every trait that has been measured to date, which underlines the importance of examining the contribution of plasticity in studies of adaptive responses (Whitman and Agrawal 2009, Wund 2012, Urban et al. 2014, Forsman 2015, Hendry 2015, Murren et al. 2015b, Nonaka et al. 2015).

Maternal effects induced by environmental conditions experienced by the parents are also emerging as important factors that influence offspring fitness in different environments (Marshall and Uller 2007, Räsänen and Kruuk 2007). Increased prevalence of maternally affected traits is expected when the environment experienced by the mother matches the environment experienced by the offspring (Kirkpatrick and Lande 1989), and in such situations, can explain up to 96% of the variation in improved offspring fitness in stressful environments (Chirgwin et al. 2016).

The divergent responses that we present in this paper may be the production of either maternal effects, phenotypic plasticity, or local adaptation alone. However, some blend of these mechanisms is more likely. For example, exposure to saltwater during the ontogeny of coastal individuals may have initiated cascades of plastic responses that predisposed females from coastal populations toward salt tolerant responses. These responses may have transferred to offspring, which mixes plasticity with maternal effects. Alternatively, coastal individuals with increased ability to tolerate salt through enhanced plasticity may have been favored by selection. Presumably, selecting for more plastic individuals would gradually increase the overall amount of plasticity observed in coastal populations, which blends plasticity with genetic adaptation

(*sensu* Baldwin effect) (Crispo 2007). In reality, there is a multitude of possible mechanistic combinations as plasticity, local adaptation, and maternal effects can be reciprocal processes that serve as both the product and raw material for selection and adaptation. Future research should prioritize discerning how adaptive evolution, phenotypic plasticity, and maternal effects are interwoven to produce different responses to environmental stressors especially in organisms with complex life cycles. A more complete understanding of all contributing processes will help managers identify thresholds of tolerance, detect vulnerable populations, and determine which organisms are likely to successfully tolerate novel stressors and persist in their environments.

Despite the consistent differences in behavior, embryo, and larval survivorship we observed between inland and coastal populations, our results indicate that all populations and life stages of *Hyla cinerea* (coastal and inland populations) are salt-sensitive. Frog pairs laid the majority of eggs into freshwater in all populations; saltwater negatively affected hatching rates across all populations, and saltwater reduced survivorship for both coastal and inland tadpoles. While we have focused on the degree to which these responses differed among populations as indications of adaptive responses, we believe that it should be noted that anurans on the whole, remain an osmotically sensitive group of organisms even in chronically salt-exposed populations. The continued preference for, and higher performance in, freshwater, even among coastal populations, may indicate that thresholds of saltwater tolerance exist.

Conclusions: This study provides the following insights. First, our meta-analysis offers a quantitative baseline for salt tolerance in anurans and provides important context for future field observations and experimental studies exploring saltwater tolerance in anurans. The meta-

analysis also shows that generally, anurans are salt-sensitive across species and across life stages and are therefore likely to be adversely affected by progressive salinization of freshwater systems. Second, we show different sensitivities and responses to salt stress across life stages and across populations, significant information for future studies and management. Third, we provide initial evidence that despite their sensitivity, some anuran species (*Hyla cinerea*) have populations that are able to respond adaptively to salt stress across different life stages. Though these findings are an encouraging indication that some frog populations may persist through salinization, our results also illuminate that much more remains to be known. Key unknowns include the physiological mechanisms and adaptive processes that underlie salt tolerance in anurans, determining whether we can expect adaptive responses to match the pace and intensity of environmental change (i.e., define the limits of tolerance and rates of adaptation), and exploring the factors that govern amphibian distributions across brackish landscapes (i.e., why only 4 out of the 17 possible species occur in brackish wetlands).

Testing multiple mechanistic hypotheses about adaptive processes (e.g., maternal effects, genetic evolution, and phenotypic plasticity) in ecological time in wild macro-organisms has remained an empirical challenge. Yet identifying populations with complex life cycles that demonstrate divergent responses to an environmental stressor across life stages (such as coastal frog populations adapting to saline environments) may provide unique and valuable opportunities to empirically address questions about the etiology of adaptive and non-adaptive responses, how novel adaptive phenotypes emerge, and how population and demographic dynamics interact with adaptive processes.

Acknowledgements: We thank members of McCoy lab group, K. McCoy, A. Stuckert, and J. Touchon for their thoughtful insights during the development of this work. We also extend thanks to T. McFarland and C. Thaxton for field and laboratory assistance. This manuscript was improved by helpful reviews by Gareth Hopkins and Ilaria Bernabò. This work was funded by North Carolina Sea Grant (Project No. 2014-R/14-HCE-3), National Science Foundation Doctoral Dissertation Improvement Grant (#1701690) awarded to McCoy and Albecker, the Graduate Women in Science Nell Mondy Fellowship, as well as research grants from the North Carolina Herpetological Society, East Carolina University's Coastal Maritime Council, and Explorer's Club.

Literature Cited

2017. R: A language and environment for statistical computing. . R Core Development Team. Vienna, Austria, R Foundation for Statistical Computing.

Abrams, P. A. 1995. Implications of dynamically variable traits for identifying, classifying, and measuring direct and indirect effects in ecological communities. American Naturalist:112-134.

Anderson, J. T., N. Perera, B. Chowdhury, and T. Mitchell-Olds. 2015. Microgeographic patterns of genetic divergence and adaptation across environmental gradients in Boechera stricta (Brassicaceae). American Naturalist **186**:S60-S73.

Baldwin, A. H., and I. A. Mendelssohn. 1998. Effects of salinity and water level on coastal marshes: an experimental test of disturbance as a catalyst for vegetation change. Aquatic Botany **61**:255-268.

Balinsky, J. B. 1981. Adaptation of Nitrogen metabolism to hyperosmotic environment in amphibia. Journal of Experimental Zoology **215**:335-350.

Bell, G. 2013. Evolutionary rescue and the limits of adaptation. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**:20120080.

Bennett, J. E., A. Racine-Poon, and J. C. Wakefield. 1996. MCMC for nonlinear hierarchical models. London, UK: Chapman and Hall.

Bernabò, I., A. Bonacci, F. Coscarelli, M. Tripepi, and E. Brunelli. 2013. Effects of salinity stress on Bufo balearicus and Bufo bufo tadpoles: Tolerance, morphological gill alterations and Na(+)/K(+)-ATPase localization. Aquat Toxicol **132-133**:119-133.

Berven, K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology **71**:1599-1608.

Bourne, E. C., G. Bocedi, J. M. Travis, R. J. Pakeman, R. W. Brooker, and K. Schiffers. 2014. Between migration load and evolutionary rescue: dispersal, adaptation and the response of spatially structured populations to environmental change. Proceedings of the Royal Society of London B: Biological Sciences **281**:20132795.

Bradshaw, W. E., and C. M. Holzapfel. 2006. Climate change. Evolutionary response to rapid climate change. Science **312**:1477-1478.

Brady, S. P. 2012. Road to evolution? Local adaptation to road adjacency in an amphibian (Ambystoma maculatum). Scientific reports **2**.

Brand, A. B., J. W. Snodgrass, M. T. Gallagher, R. E. Casey, and R. Van Meter. 2010. Lethal and sublethal effects of embryonic and larval exposure of Hyla versicolor to stormwater pond sediments. Archives of Environmental Contamination and Toxicology **58**:325-331.

Brown, M. E., and S. C. Walls. 2013. Variation in salinity tolerance among larval anurans: Implications for community composition and the spread of an invasive, non-native species. Copeia **2013**:543-551.

Burkhart, J. G., J. C. Helgen, D. J. Fort, K. Gallagher, D. Bowers, T. L. Propst, M. Gernes, J. Magner, M. D. Shelby, and G. Lucier. 1998. Induction of mortality and malformation in *Xenopus laevis* embryos by water sources associated with field frog deformities. Environmental Health Perspectives **106**:841.

Carignan, V., and M.-A. Villard. 2002. Selecting Indicator Species to Monitor Ecological Integrity: A Review. Environmental Monitoring and Assessment **78**:45-61.

Carlson, S. M., C. J. Cunningham, and P. A. H. Westley. 2014. Evolutionary rescue in a changing world. Trends in Ecology & Evolution **29**:521-530.

Chen, I. C., J. K. Hill, R. Ohlemuller, D. B. Roy, and C. D. Thomas. 2011. Rapid range shifts of species associated with high levels of climate warming. Science **333**:1024-1026.
Chevin, L. M., R. Lande, and G. M. Mace. 2010. Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. PLoS Biol **8**:e1000357.
Chinathamby, K., R. D. Reina, P. C. Bailey, and B. K. Lees. 2006. Effects of salinity on the survival, growth and development of tadpoles of the brown tree frog, Litoria ewingii. Australian Journal of Zoology **54**:97-105.
Chirgwin, E., D. J. Marshall, C. M. Sgrò, and K. Monro. 2016. The other 96%: Can neglected sources of fitness variation offer new insights into adaptation to global change? Evolutionary Applications **10**:267-275.
Chown, S. L. 2012. Trait-based approaches to conservation physiology: forecasting environmental change risks from the bottom up. Philos Trans R Soc Lond B Biol Sci **367**:1615-1627.
Christman, S. P. 1974. Geographic variation for salt water tolerance in the frog *Rana sphenocephala* Copeia **1974**:773-778.
Christy, M. T., and C. R. Dickman. 2002. Effects of salinity on tadpoles of the green and golden bell frog (Litoria aurea). Amphibia-Reptilia **23**:1-11.
Church, J. A., P. U. Clark, A. Cazenave, J. M. Gregory, S. Jevrejeva, A. Levermann, M. A. Merrifield, G. A. Milne, R. S. Nerem, P. D. Nunn, A. J. Payne, W. T. Pfeffer, D. Stammer, and A. S. Unnikrishnan. 2013. IPCC Fifth Assessment Report (AR5), Climate Change 2013: The Physical Science Basis.1-124.
Craft, C., J. Clough, J. Ehman, S. Joye, R. Park, S. Pennings, H. Guo, and M. Machmuller. 2009. Forecasting the effects of accellerated sea-level rise on tidal marsh ecosystem services Frontiers in Ecology and the Environment **7**:73-78.
Crispo, E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. Evolution **61**:2469-2479.
Crother, B. I., and C. L. Fontenot. 2006. Amphibian and Reptile Monitoring in the Ponchartrain-Maurepas Region. Lake Pontchartrain Basin Research Program (PBRP):35.
DaLaune, R. D., and S. R. Pezeshki. 1994. The influence of subsidence and saltwater intrustion on coastal marsh stability: Louisiana gulf coast Journal of Coastal Research **12**:77-89.
Darwin, C. 1859. On The Origin of Species by Means of Natural Selection, or Preservation of Favoured Races in the Struggle for Life. John Murray, London.
Davis, M. B., and R. G. Shaw. 2001. Range shifts and adaptive responses to Quaternary climate change. Science **292**:673-679.
Davis, M. B., R. G. Shaw, and J. R. Etterson. 2005. Evolutionary responses to changing climate. Ecology **86**:1704-1714.
Dawson, T. P., S. T. Jackson, J. I. House, I. C. Prentice, and G. M. Mace. 2011. Beyond predictions: biodiversity conservation in a changing climate. Science **332**:53-58.
Day, J. W., R. R. Christian, D. M. Boesch, A. Yáñez-Arancibia, J. Morris, R. R. Twilley, L. Naylor, L. Schaffner, and C. Stevenson. 2008. Consequences of Climate Change on the Ecogeomorphology of Coastal Wetlands. Estuaries and Coasts **31**:477-491.
Dietz, T. H., and R. H. Alvarado. 1974. Na and Cl transport across gill chamber epithelium of Rana catesbeiana tadpoles. Am J Physiol **226**:764-770.
Domingues, C. M., J. A. Church, N. J. White, P. J. Gleckler, S. E. Wijffels, P. M. Barker, and J. R. Dunn. 2008. Improved estimates of upper-ocean warming and multi-decadal sea-level rise. Nature **453**:1090-1093.

Dougherty, C. K., and G. R. Smith. 2006. Acute effects of road de-icers on the tadpoles of three anurans. Applied Herpetology **3**:87-93.
Foden, W. B., G. M. Mace, J.-C. Vié, A. Angulo, S. H. M. Butchart, L. DeVantier, H. T. Dublin, A. Gutsche, S. N. Stuart, and E. Turak. 2009. Species susceptibility to climate change impacts. Pages 77-88 *in* J.-C. Vie, C. Hilton-Taylor, and S. N. Stuart, editors. Wildlife in a Changing World: An Analysis of the 2008 IUCN Red List of Threatened Species, Barcelona, Spain.
Forsman, A. 2015. Rethinking phenotypic plasticity and its consequences for individuals, populations and species. Heredity **115**:276-284.
Fraser, D. J., L. K. Weir, L. Bernatchez, M. M. Hansen, and E. B. Taylor. 2011. Extent and scale of local adaptation in salmonid fishes: review and meta-analysis. Heredity **106**:404-420.
Geddes, N. A., and S. Mopper. 2006. Effects of environmental salinity on vertebrate florivory and wetland communities. Natural Areas Journal **26**:31-37.
Gelman, A., J. B. Carlin, H. S. Stern, and D. B. Rubin. 2004. Bayesian data analysis. Texts in statistical science series. Chapman & Hall/CRC, Boca Raton, FL.
Gibbons, J. W., and J. W. Coker. 1978. Herpetofaunal colonization patterns of atlantic coast barrier islands American Midland Naturalist **99**:219-233.
Gibbons, J. W., C. T. Winne, D. E. Scott, J. D. Willson, X. Glaudas, K. M. Andrews, B. D. Todd, L. A. Fedewa, L. Wilkinson, R. N. Tsaliagos, S. J. Harper, J. L. Greene, T. D. Tuberville, B. S. Metts, M. E. Dorcas, J. P. Nestor, C. A. Young, T. Akre, R. N. Reed, K. A. Buhlmann, J. Norman, D. A. Croshaw, C. Hagen, and B. B. Rothermel. 2006. Remarkable amphibian biomass and abundance in an isolated wetland: implications for wetland conservation. Conserv Biol **20**:1457-1465.
Gomez-Mestre, I., and M. Tejado. 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. Evolution **57**:1889-1899.
Gomez-Mestre, I., and M. Tejado. 2005. Adaptation or Exaptation? An experimental test of hypotheses on the origin of salinity tolerance in Bufo calamita. Journal of Evolutionary Biology **18**:847-855.
Gomez-Mestre, I., M. Tejedo, and J. Merilä. 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. Evolution **57**:1889-1899.
Gonzalez, A., O. Ronce, R. Ferriere, and M. E. Hochberg. 2013. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**:20120404.
Gordon, M. S. 1962. Osmotic regulation in the Green Toad (*Bufo viridis*) Journal of Experimental Biology **39**:261-270.
Gordon, M. S. 1965. Intracellular osmoregulation in skeletal muscle during salinity adaptation in two species of toads Biological Bulletin **128**:218-229.
Gordon, M. S., K. Schmidt-Nielsen, and H. M. Kelly. 1961. Osmotic regulation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **38**:659-678.
Gordon, M. S., and V. A. Tucker. 1965. Osmotic regulation in the tadpoles of the crab-eating frog (*Rana cancrivora)* Journal of Experimental Biology **42**:437-445.
Gordon, M. S., and V. A. Tucker. 1968. Further observations on the physiology of salinity adaptation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **49**:185-193.
Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica:183-190.

Gunzburger, M. S. 2006. Reproductive ecology of the green treefrog (Hyla cinerea) in Northwestern Florida. The American midland naturalist **155**:321-328.
Hamer, A. J., and M. J. McDonnell. 2008. Amphibian ecology and conservation in the urbanising world- a review Biological Conservation **141**:2432-2449.
Haramura, T. 2007. Salinity tolerance of eggs of Buergeria japonica (Amphibia, Anura) inhabiting coastal areas. Zoolog Sci **24**:820-823.
Haramura, T. 2011. Use of oviposition sites by a Rhacophorid frog inhabiting a coastal area in Japan Journal of Herpetology **45**:432-437.
Hardy, J. D. 1953. Notes on the distribution of *Mycrohyla carolinensis* in southern Maryland. Herpetologica **8**:162-166.
Hardy, J. D. J. 1972. Amphibians of the Chesapeake Bay Region Chesapeake Science **13**:S123-S128.
Harley, C. D. 2011. Climate change, keystone predation, and biodiversity loss. Science **334**:1124-1127.
Havird, J. C., R. P. Henry, and A. E. Wilson. 2013. Altered expression of Na(+)/K(+)-ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: a meta-analysis of 59 quantitative PCR studies over 10 years. Comp Biochem Physiol Part D Genomics Proteomics **8**:131-140.
Hendry, A. P. 2015. Key Questions on the Role of Phenotypic Plasticity in Eco-Evolutionary Dynamics. Journal of Heredity:esv060.
Heyer, R., M. A. Donnelly, M. Foster, and R. Mcdiarmid. 2014. Measuring and monitoring biological diversity: standard methods for amphibians. Smithsonian Institution.
Hintz, W. D., and R. A. Relyea. 2017. Impacts of road deicing salts on the early-life growth and development of a stream salmonid: Salt type matters. Environ Pollut.
Holt, R., and R. Gomulkiewicz. 2004. Conservation implications of niche conservatism and evolution in heterogeneous environments. Pages 244-264 Evolutionary Conservation Biology. Cambridge University Press.
Hopkins, G. R., and J. E. D. Brodie. 2015. Occurrence of Amphibians in Saline Habitats: A Review and Evolutionary Perspective. Herpetological Monographs **29**:1-27.
Hsu, W.-T., C.-S. Wu, J.-C. Lai, Y.-K. Chiao, C.-H. Hsu, and Y.-C. Kam. 2012. Salinity acclimation affects survival and metamorphosis of crab-eating frog tadpoles. Herpetologica **68**:14-21.
K.P., G., and L. L.R. 1995. Effects of ultraviolet radiation on life-history stages of anurans from Ontario, Canada. Canadian Journal of Zoology **73**:2292-2301.
Kemp, A. C., B. P. Horton, S. J. Culver, D. R. Corbett, O. v. d. Plassche, W. R. Gehrels, B. C. Douglas, and A. C. Parnell. 2009. Timing and magnitude of recent accelerated sea-level rise Geology **37**:1035-1038.
Kemp, A. C., B. P. Horton, J. P. Donnelly, M. E. Mann, M. Vermeer, and S. Rahmstorf. 2011. Climate related sea level variations over the past two millennia. PNAS **108**:11017-11022.
Kiesecker, J. M., and A. R. Blaustein. 1997. Influences of egg laying behavior on pathogenic infection of amphibian eggs. Conservation BIology **11**:214-220.
Kirkpatrick, M., and R. Lande. 1989. The evolution of maternal characters. Evolution **1989**:485-503.
Knighton, A. D., K. Mills, and C. D. Woodroffe. 1991. Tidal-creek extension and saltwater intrusion in northern Australia. Geology **19**:831-834.

Konno, N., S. Hyodo, K. Matsuda, and M. Uchiyama. 2006. Effect of osmotic stress on expression of a putative facilitative urea transporter in the kidney and urinary bladder of the marine toad, Bufo marinus. Journal of Experimental Biology **209**:1207-1216.

Kopp, R. E., B. P. Horton, A. C. Kemp, and C. Tebaldi. 2015. Past and future sea-level rise along the coast of North Carolina, USA. Climatic Change **132**:693-707.

Lamichhaney, S., A. M. Barrio, N. Rafati, G. Sundström, C.-J. Rubin, E. R. Gilbert, J. Berglund, A. Wetterbom, L. Laikre, and M. T. Webster. 2012. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. Proceedings of the National Academy of Sciences **109**:19345-19350.

Lande, R. 2009. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. Journal of Evolutionary Biology **22**:1435-1446.

Langhans, M., B. Peterson, A. Walker, G. R. Smith, and J. E. Rettig. 2009. Effects of salinity on survivorship of wood frog (Rana sylvatica) tadpoles.

Lawler, J. J., S. L. Shafer, B. A. Bancroft, and A. R. Blaustein. 2010. Projected climate impacts for the amphibians of the Western hemisphere. Conserv Biol **24**:38-50.

Lewis, O. T. 2006. Climate change, species-area curves and the extinction crisis. Philos Trans R Soc Lond B Biol Sci **361**:163-171.

Lillywhite, H. B. 2006. Water relations of tetrapod integument. J Exp Biol **209**:202-226.

Loaiciga, H. A. 2003. Climate change and ground water Annals of the Association of American Geographers **93**:30-41.

Maclean, I. M., and R. J. Wilson. 2011. Recent ecological responses to climate change support predictions of high extinction risk. Proc Natl Acad Sci U S A **108**:12337-12342.

Marshall, D. J., and T. Uller. 2007. When is a maternal effect adaptive? . Oikos **116**:1957-1963.

Martin, G., R. Aguile, J. Ramsayer, O. Kaltz, and O. Ronce. 2013. The probability of evolutionary rescue: towards a quantitative comparison between theory and evolution experiments. Phil. Trans. R. Soc. B **368**:20120088.

McClanahan, L., Jr., J. N. Stinner, and V. H. Shoemaker. 1978. Skin lipids, water loss, and energy metabolism in a South American tree frog (*Phyllomedusa sauvagei*). Physiological Zoology **51**:179-187.

McCoy, M. W., M. Barfield, and R. D. Holt. 2009. Predator shadows: complex life histories as generators of spatially patterned indirect interactions across ecosystems. Oikos **118**:87-100.

Meehl, G. A., W. M. Washington, W. D. Collins, J. M. Arblaster, A. Hu, L. E. Buja, W. G. Strand, and H. Teng. 2005. How much more climate change and sea level rise? Science **307**:1769-1772.

Merilä, J., and A. P. Hendry. 2014. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. Evolutionary Applications **7**:1-14.

Meteyer, C. U., R. A. Cole, K. A. Converse, D. E. Docherty, M. Wolcott, J. C. Helgen, R. Levey, L. Eaton-Poole, and J. G. Burkhart. 2000. Defining anuran malformations in the context of a developmental problem. The Journal of the Iowa Academy of Science **107**:72-78.

Meyssignac, B., and A. Cazenave. 2012. Sea Level- A review of present day and recent past changes and variability Journal of Geodynamics **58**:96-109.

Michener, W. K., E. R. Blood, K. L. Bildstein, M. M. Brinson, and L. R. Gardner. 1997. Climate change, hurricanes and tropical storms, and rising sea level in coastal wetlands. Ecological Applications **7**:770-801.

Mopper, S., and S. Y. Strauss. 2013. Genetic structure and local adaptation in natural insect populations: effects of ecology, life history, and behavior. Springer Science & Business Media.
Moritz, C., and R. Agudo. 2013. The future of species under climate change: resilience or decline? Science **341**:504-508.
Morris, J. T., P. V. Sundarshwar, C. T. Nietch, B. Kjerfve, and D. R. Cahoon. 2002. Responses of coastal wetlands to rising sea level. Ecology **83**:2869-2877.
Munsey, L. D. 1972. Salinity tolerance of the african Pipid Frog. *Xenopus laevis* Copeia **1972**:584-586.
Murren, C. J., J. R. Auld, H. Callahan, C. K. Ghalambor, C. A. Handelsman, M. A. Heskel, J. Kingsolver, H. J. Maclean, J. Masel, and H. Maughan. 2015. Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. Heredity **115**:293-301.
Nakkrasae, L. i., S. Phummisutthigoon, and N. Charoenphandhu. 2015. Low salinity increases survival, body weight and development in tadpoles of the Chinese edible frog Hoplobatrachus rugulosus. Aquaculture Research.
Neill, W. T. 1958. The occurrence of amphibians and reptiles in saltwater areas, and a bibliography. Bulletin of Marine Science **8**:1-97.
Nicholls, R. J., and A. Cazenave. 2010. Sea-level rise and its impact on coastal zones. Science **328**:1517-1520.
Nicholls, R. J., and R. S. Tol. 2006. Impacts and responses to sea-level rise: a global analysis of the SRES scenarios over the twenty-first century. Philos Trans A Math Phys Eng Sci **364**:1073-1095.
Nonaka, E., R. Svanbäck, X. Thibert-Plante, G. Englund, and Å. Brännström. 2015. Mechanisms by Which Phenotypic Plasticity Affects Adaptive Divergence and Ecological Speciation. The American Naturalist **186**:E126-143.
Parkinson, R. W. 1994. Sea-level rise and the fate of tidal wetlands Journal of Coastal Research **10**:987-989.
Parmesan, C. 2006. Ecological and evolutionary responses to recent climate change. Annual Review of Ecology, Evolution, and Systematics:637-669.
Petranka, J. W., and E. J. Doyle. 2010. Effects of road salts on the composition of seasonal pond communities: can the use of road salts enhance mosquito recruitment? Aquatic Ecology **44**:155-166.
Pfennig, D. W., M. A. Wund, E. C. Snell-Rood, T. Cruickshank, C. D. Schlichting, and A. P. Moczek. 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends in Ecology & Evolution **25**:459-467.
Plummer, M. 2015. rjags: Bayesian Graphical Models using MCMC. R package version 3-15.
Plummer, M., N. Best, K. Cowles, and K. Vines. 2006. coda: Convergence Diagnosis and Output Analysis for MCMC. R News.
Rader, R. B., D. P. Batzer, and S. A. Wissinger. 2001. Bioassessment and management of North American freshwater wetlands. John Wiley & Sons.
Rahmstorf, S., M. Perrette, and M. Vermeer. 2012. Testing the robustness of semi-empirical sea level projections Climate Dynamics **39**:861-875.
Räsänen, K., and L. Kruuk. 2007. Maternal effects and evolution at ecological timescales. Functional Ecology **21**:408-421.

Reed, T. E., D. E. Schindler, and R. S. Waples. 2011. Interacting effects of phenotypic plasticity and evolution on population persistence in a changing climate. Conservation BIology **25**:56-63.

Reed , T. E., R. S. Waples, D. E. Schindler, J. J. Hard, and M. T. Kinnison. 2010. Phenotypic plasticity and population viability: the importance of environmental predictability. Proceedings of the Royal Society of London B: Biological Sciences **277**.

Refsnider, J. M., and F. J. Janzen. 2010. Putting eggs in one basket: ecological and evolutionary hypotheses for variation in oviposition-site choice. Annual Review of Ecology, Evolution and Systematics **41**:39-57.

Reznick, D. N., and C. K. Ghalambor. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. Genetica **112**:183-198.

Rieger, J. F., C. A. Binckley, and W. J. Resetarits Jr. 2004. Larval performance and oviposition site preference along a predation gradient. Ecology **85**:2094-2099.

Rios-López, N. 2008. Effects of increased salinity on tadpoles of two anurans from a Caribbean coastal wetland in relation to their natural abundance. Amphibia-Reptilia **29**:7-18.

Roberts, J. 1970. Variations in salinity tolerance in the Pacific Treefrog, Hyla regilla. Oregon [dissertation]. Corvallis, OR: Oregon State University.

Rohr, J. R., and K. A. McCoy. 2010. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environmental Health Perspectives **2010**:20-32.

Ruibal, R. 1959. The ecology of a brackish water population of *Rana pipiens* Copeia **1959**:315-322.

Sanzo, D., and S. J. Hecnar. 2006. Effects of road de-icing salt (NaCl) on larval wood frogs (Rana sylvatica). Environ Pollut **140**:247-256.

Savolainen, O., M. Lascoux, and J. Merila. 2013. Ecological genomics of local adaptation. Nat Rev Genet **14**:807-820.

Scavia, D., J. C. Field, D. F. Boesch, R. W. Buddemeier, V. Burkett, D. R. Cayan, M. Fogarty, M. A. Harwell, R. W. Howarth, C. Mason, D. J. Reed, T. C. Royer, A. H. Sallenger, and J. G. Titus. 2002. Climate change impacts on U.S. coastal and marine ecosystems Estuaries **25**:149-164.

Schwartz, M. W., L. R. Iverson, A. M. Prasad, S. N. Matthews, and R. J. O'Connor. 2006. Predicting extinctions as a result of climate change. Ecology **87**:1611-1615.

Senior, C. A., R. G. Jones, J. A. Lowe, C. F. Durman, and D. Hudson. 2002. Predictions of extreme precipitation and sea-level rise under climate change Philos Trans A Math Phys Eng Sci **360**:1301-1311.

Shoemaker, V., S. Hillman, S. Hillyard, D. Jackson, L. McClanahan, P. Withers, and M. Wygoda. 1992. Exchange of water, ions, and respiratory gases in terrestrial amphibians. Environmental physiology of the amphibians:125-150.

Smith, M. J., E. S. G. Shreiber, M. P. Scroggie, M. Kohout, K. Ough, J. Potts, R. Lennie, D. Turnbull, C. Jin, and T. Clancy. 2006. Associations between anuran tadpoles and salinity in a landscape mosaic of wetlands impacted by secondary salinisation Freshwater Biology **52**:75-84.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science **306**:1783-1786.

Su, Y.-S., and M. Yajima. 2015. R2jags: Using R to Run 'JAGS'. . R package version 0.5-7.
Thirion, J.-M. 2014. salinity of the reproduction habitats of the Western spadefoot Toad Pelobates cultripes (cuvier, 1829), along the atlantic coast of France. Herpetozoa **27**:13-20.
Thomas, C. D., A. Cameron, R. E. Green, M. Bakkenes, L. J. Beaumont, Y. C. Collingham, B. F. Erasmus, M. F. De Siqueira, A. Grainger, and L. Hannah. 2004. Extinction risk from climate change. Nature **427**:145-148.
Titus, J. G., and C. Richman. 2001. Maps of lands vulnerable to sea level rise: modeled elevations along the US Atlantic and Gulf coasts. Climate Research **18**:205-228.
Touchon, J. C. 2006. Hatching plasticity in two temperate anurans: responses to pathogen and predation cues. Canadian Journal of Zoology **84**:556-563.
Touchon, J. C., I. Gomez-Mestre, and K. M. Warkentin. 2006. Hatching plasticity in two temperate anurans: responses to a pathogen and predation cues. Canadian Journal of Zoology **84**:556-563.
Traill, L. W., M. L. Lim, N. S. Sodhi, and C. J. Bradshaw. 2010. Mechanisms driving change: altered species interactions and ecosystem function through global warming. J Anim Ecol **79**:937-947.
Tuberville, T. D., J. D. Willson, M. E. Dorcas, and J. W. Gibbons. 2005. Herpetofaunal species richness of southeastern national parks Southeastern Naturalist **4**:537-569.
Uchiyama, M., and N. Konno. 2006. Hormonal regulation of ion and water transport in anuran amphibians. Gen Comp Endocrinol **147**:54-61.
Uchiyama, M., and H. Yoshizawa. 1992. Salinity tolerance and structure of external and internal gills in tadpoles of the crab-eating frog, Rana cancrivora. Cell and Tissue Research **267**:35-44.
Urban, M. C., G. Bocedi, A. P. Hendry, J.-B. Mihoub, G. Pe'er, A. Singer, J. R. Bridle, L. G. Crozier, L. De Meester, W. Godsoe, A. Gonzalez, J. J. Hellmann, R. D. Holt, A. Huth, K. Johst, C. B. Krug, P. W. Leadley, S. C. F. Palmer, J. H. Pantel, A. Schmitz, P. A. Zollner, and J. M. J. Travis. 2016. Improving the forecast for biodiversity under climate change. Science **353**.
Urban, M. C., J. L. Richardson, and N. A. Reidenfelds. 2014. Plasticity and genetic adaptation mediate amphibian and reptile responses to climate change. Evolutionary Applications **7**:88-103.
Vitt, L. J., J. P. Caldwell, H. M. Wilbur, and D. C. Smith. 1990. Amphibians as harbingers of decay. BioScience **40**:418-418.
Wallace, A. R. 1859. On the Tendency of Varieties to depart indefinitely from the Original Type. John Murray, London.
Walther, G.-R., E. Post, P. Convey, A. Menzel, C. Parmesan, T. J. C. Beebee, J.-M. Fromentin, O. Hoegh-Guldberg, and F. Bairlein. 2002. Ecological responses to recent climate change. Nature **416**:389-395.
Wells, K. D. 2007. The Ecology and Behavior of Amphibians. The University of Chicago Press, Chicago, Illinois.
Whitman, D. W., and A. A. Agrawal. 2009. What is Phenotypic Plasticity and Why is it important? .1-63.
Wilbur, H. M. 1980. Complex Life Cycles. Annual Review of Ecology and Systematics **11**:67-93.

Wilder, A. E., and A. M. Welch. 2014. Effects of Salinity and Pesticide on Sperm Activity and Oviposition Site Selection in Green Treefrogs, Hyla cinerea. Copeia **2014**:659-667.
Williams, K., K. C. Ewel, R. P. Stumpf, F. E. Putz, and T. W. Workman. 1999. Sea-level rise and coastal forest retreat on the west coast of florida, USA Ecology **80**:2045-2063.
Williams, S. J. 2013. Sea Level Rise Implications for Coastal Regions Journal of Coastal Research **63**:184-196.
Wood, L., and A. M. Welch. 2015. Assessment of interactive effects of elevated salinity and three pesticides on life history and behavior of southern toad (Anaxyrus terrestris) tadpoles. Environmental Toxicology and Chemistry **34**:667-676.
Wu, C.-S., and Y.-C. Kam. 2009a. Effects of salinity on the survival, growth, development, and metamorphosis of Fejervarya limnocharis tadpoles living in brackish water. Zoolog Sci **26**:476-482.
Wu, C. S., I. Gomez-Mestre, and Y. C. Kam. 2012. Irreversibility of a bad start: early exposure to osmotic stress limits growth and adaptive developmental plasticity. Oecologia **169**:15-22.
Wu, C. S., and Y. C. Kam. 2009b. Effects of salinity on the survival, growth, development, and metamorphosis of Fejervarya limnocharis tadpoles living in brackish water. Zoolog Sci **26**:476-482.
Wu, C. S., W. K. Yang, T. H. Lee, I. Gomez-Mestre, and Y. C. Kam. 2014. Salinity acclimation enhances salinity tolerance in tadpoles living in brackish water through increased Na(+) , K(+) -ATPase expression. J Exp Zool A Ecol Genet Physiol **321**:57-64.
Wund, M. A. 2012. Assessing the impacts of phenotypic plasticity on evolution. Integr Comp Biol **52**:5-15.

Figures and tables

Figure 1-1. Map of Survey Sites along eastern North Carolina. Locations of survey sites are denoted by white circles. During surveys, the relative abundance of anurans was recorded, along with salinity of the wetland along with other environmental data.

Figure 1-2. Anuran survival across life stages. The predicted survival for each life stage across the anuran amphibian clade as a function of salinity (in parts per thousand).

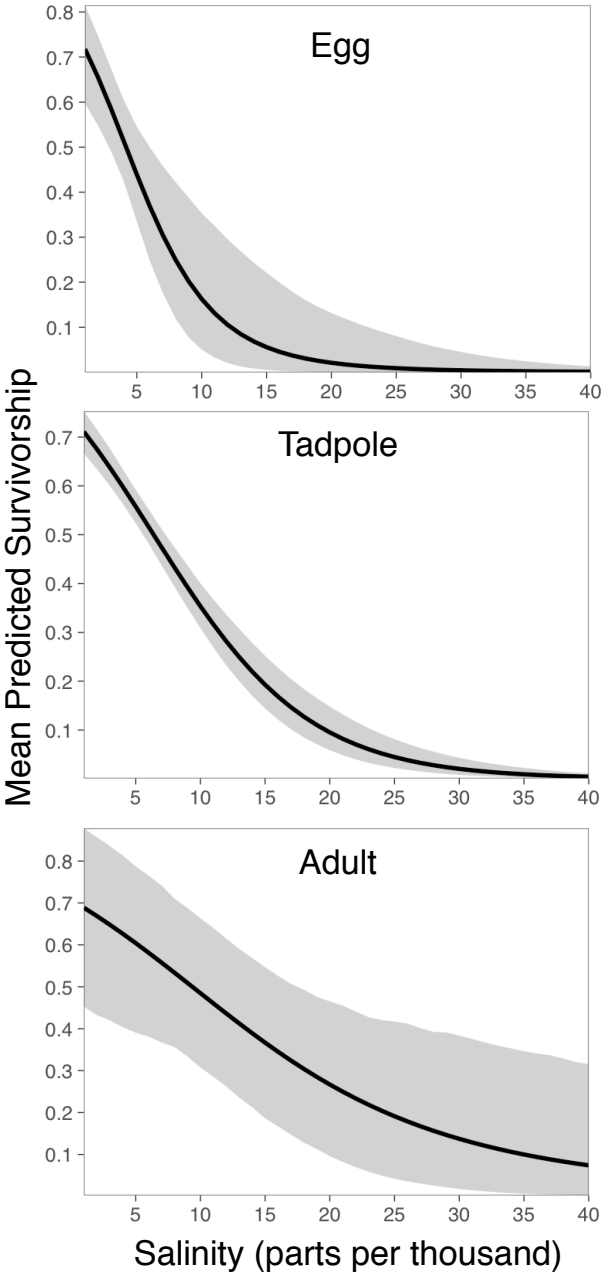


Figure 1-3. **Predicted probability of oviposition** according to salinity and population location with 95% credibility envelopes. Green denotes the oviposition patterns from inland populations; blue indicates the oviposition patterns from coastal populations.

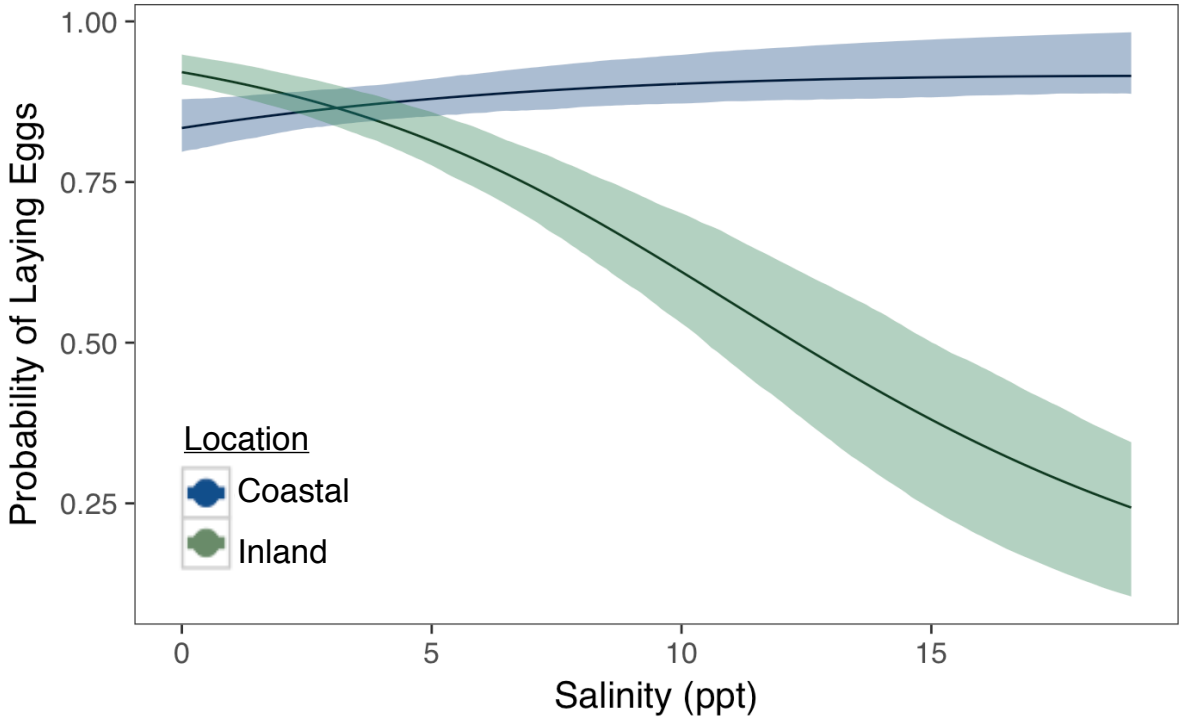


Figure 1-4. The proportion of eggs laid in freshwater according to salinity and population location with 95% credible envelopes. Green denotes the proportion of eggs laid in freshwater by inland populations; blue indicates the proportion of eggs laid in freshwater from coastal populations.

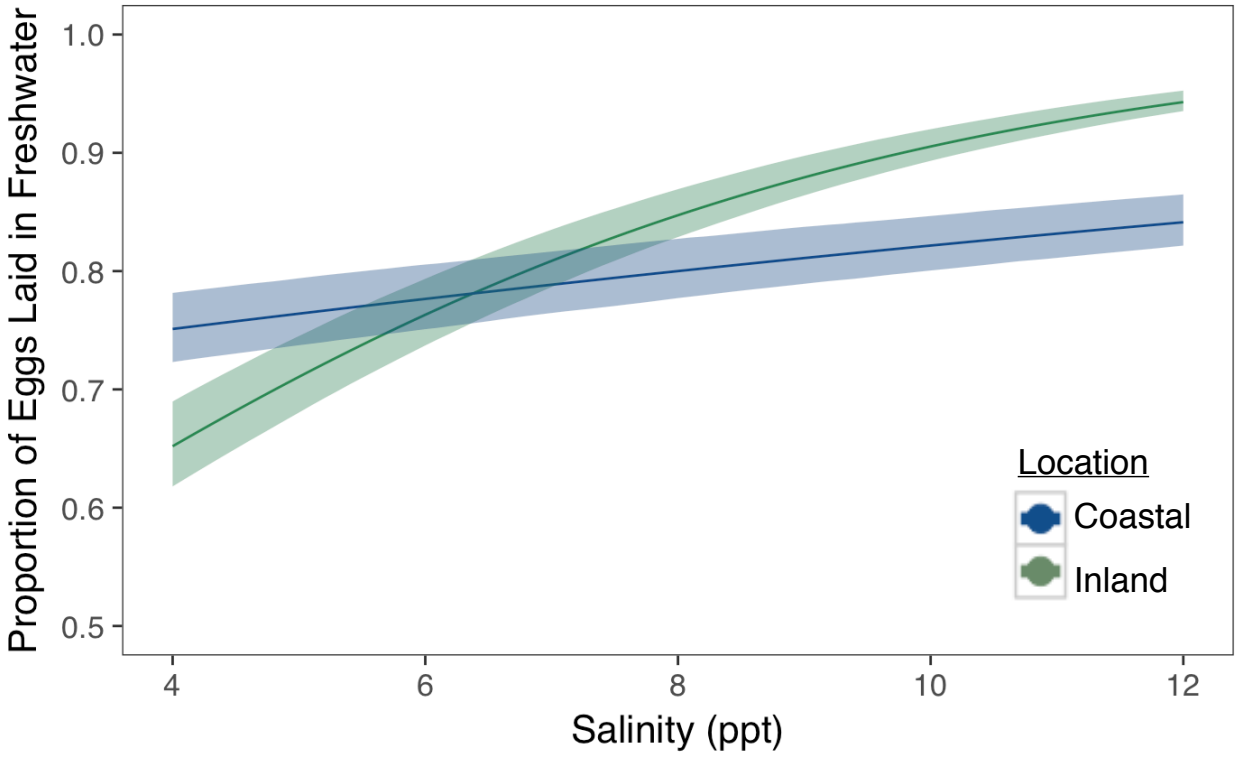


Figure 1-5. Predicted probability of egg hatching according to salinity and population location with 95% credible envelopes. Green denotes the hatching patterns from inland populations; blue indicates hatching patterns from coastal populations.

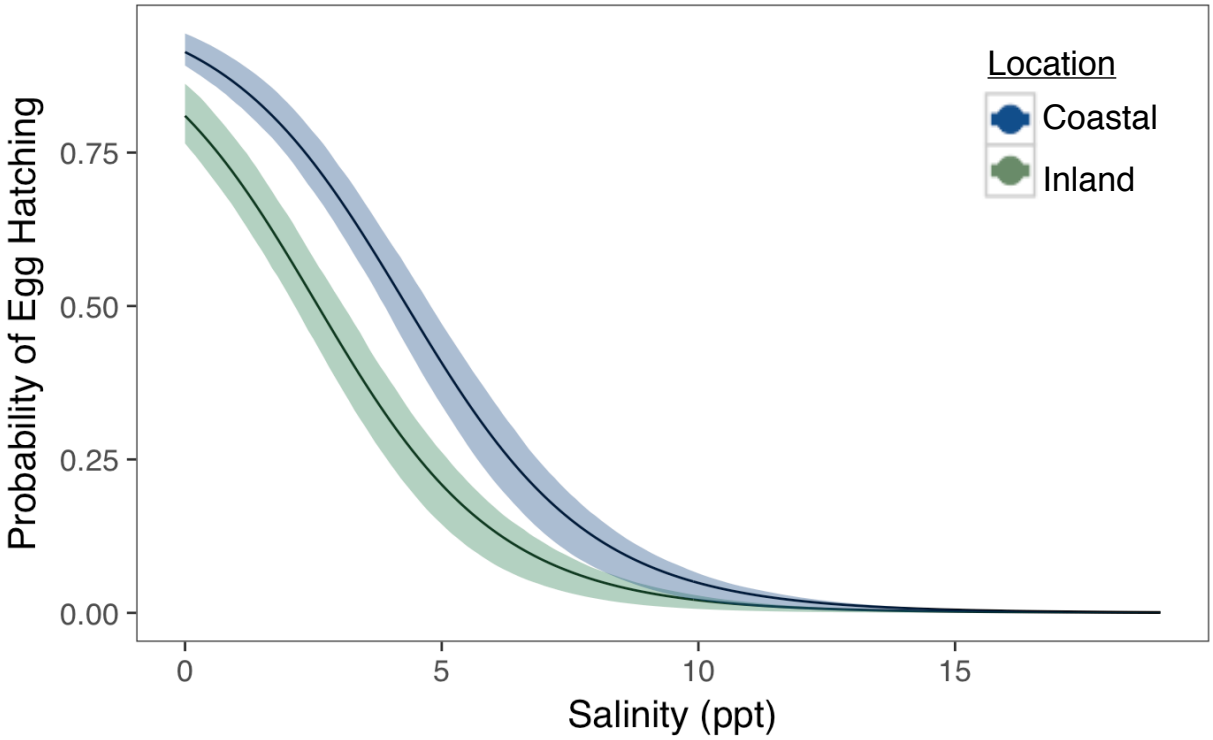


Figure 1-6. The proportion of eggs that hatched according to salinity and population location with 95% confidence envelopes. Green denotes the proportion of eggs hatched from inland populations; blue indicates the proportion of eggs hatched from coastal populations.

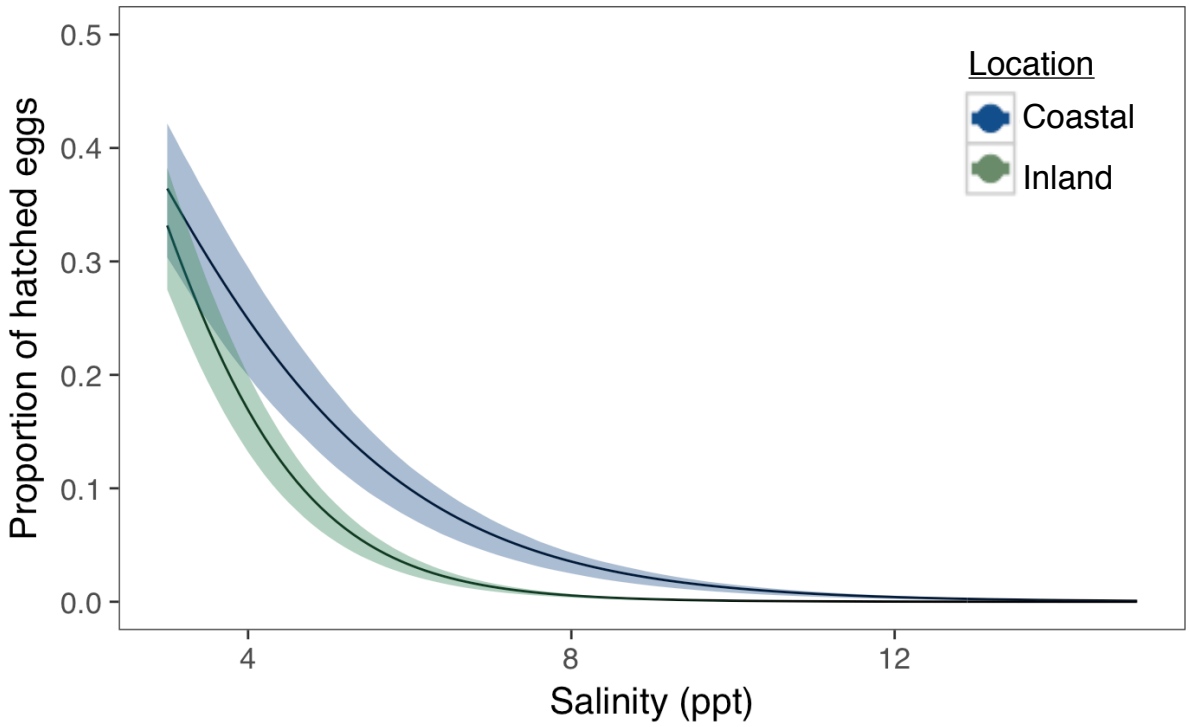


Figure 1-7. Mean probability of tadpole survivorship according to salinity and population location with 95% credible envelopes. Green denotes the proportion of tadpoles sourced from inland populations; blue indicates tadpoles sourced from coastal populations.

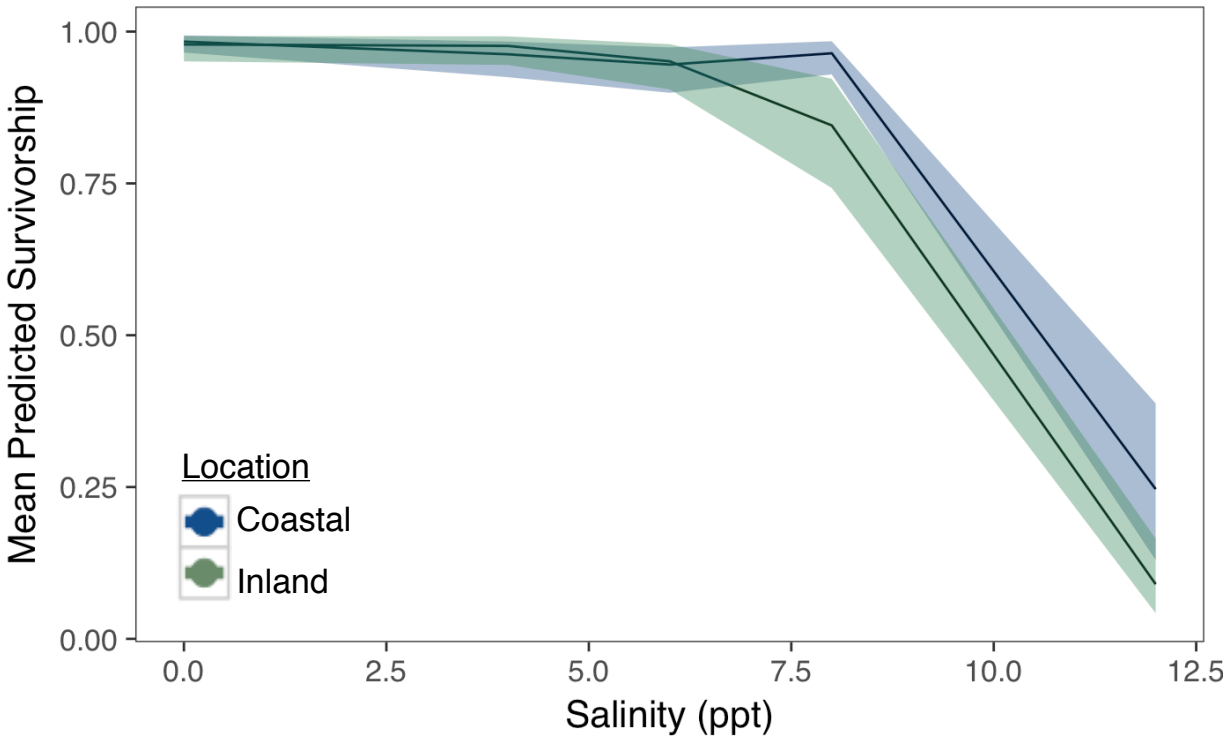


Table 1-1. The location and identity of the four anuran species observed in coastal, brackish wetlands along with the highest salinity in which each species was observed.

Species	*Highest Salinity Observed*	*Occurrence*	*Location*
Lithobates sphenocephalus	11ppt	Abundant	Alligator River NWR
Hyla cinerea	23.4ppt	Abundant	Cape Hatteras National Seashore
Gastrophryne carolinensis	3.9ppt	Abundant	Alligator River NWR
Lithobates catesbeianus	6.2ppt	Rare	Pea Island NWR

Table 1-2. Predicted survivorship (and Bayesian Credible Intervals) of anurans in various salinities based on the findings of the meta-analysis (Fig. 1-2). Each salinity concentration represents the salinity of a wetland in which frogs were observed along North Carolina's coast.

Salinity (ppt) in which anurans were observed:	*Predicted Egg Survivorship (+95% BCIs)*	*Predicted Larval Survivorship (+95% BCIs)*	*Predicted Adult Survivorship (+95% BCIs)*
3.9	0.52 (0.39-0.66)	0.60 (0.51-0.68)	0.62 (0.39-0.84)
8.3	0.25 (0.09-0.45)	0.32 (0.23-0.39)	0.53 (0.32 – 0.74)
11	0.15 (0.03-0.35)	0.18 (0.12-0.25)	0.46 (0.25 – 0.70)
16.9	0.04 (0.002-0.19)	0.04 (0.02-0.07)	0.35 (0.13-0.61)
23.4	0.01 (0.00-0.008)	0.01 (0.002-0.02)	0.25 (0.05-0.57)

Supporting Materials

Table 1-3. Predicted *Hyla cinerea* tadpole survivorship after a six-day exposure to one of five salinity concentrations, along with slope and intercept estimates, each with 95% Bayesian credible intervals (L.C.I = Lower Credible Interval, U.C.I = Upper Credible Interval) (Fig. 1-7)

	Coastal										Inland									
Salinity (ppt)	**Survivorship**	**L.C.I**	**U.C.I**	**Slope Estimate**	**L.C.I**	**U.C.I**	**Intercept Estimate**	**L.C.I**	**U.C.I**	**Survivorship**	**L.C.I**	**U.C.I**	**Slope Estimate**	**L.C.I**	**U.C.I**	**Intercept Estimate**	**L.C.I**	**U.C.I**		
0	0.98	0.97	0.99	-0.38	-0.67	-0.13	6.09	4.95	7.46	0.97	0.96	0.99	-0.14	-0.24	-0.06	7.42	6.77	8.14		
4	0.93	0.91	0.95	-0.39	-0.55	-0.23	4.96	4.26	5.75	0.97	0.95	0.98	-0.17	-0.27	-0.07	3.33	2.93	3.78		
6	0.94	0.90	0.95	-0.42	-0.58	-0.27	5.07	4.35	5.84	0.94	0.92	0.96	-0.20	-0.29	-0.12	3.06	2.71	3.43		
8	0.94	0.92	0.96	-0.23	-0.36	-0.09	4.12	3.61	4.80	0.84	0.80	0.87	-0.39	-0.47	-0.30	3.72	3.31	4.12		
12	0.25	0.22	0.29	-1.42	-1.56	-1.29	7.42	6.78	8.14	0.10	0.08	0.12	-1.57	-1.71	-1.52	5.59	5.15	6.04		

II. THE ROLE OF ENVIRONMENTALLY INDUCED PHENOTYPIC PLASTICITY IN THE EVOLUTION OF SALTWATER-TOLERANCE IN AN AMPHIBIAN

Abstract

Environmental change linked to anthropogenic activities is affecting the quality and quantity of ecosystems and the organisms they support. Currently we do not fully understand the mechanisms that drive adaptive evolutionary responses in response to environmental change. Phenotypic plasticity, or the ability of a single genotype to produce different phenotypes in response to the environment, is a common and important biological mechanism that can either drive or inhibit adaptation to environmental change. In this study, we compare how highly plastic developmental endpoints differ between coastal and inland frog populations after exposing embryos and tadpoles to either freshwater or saltwater. We show that regardless of developmental environment, coastal frogs demonstrate a canalized developmental pattern to grow faster and initiate metamorphosis sooner than inland populations, but at a smaller size at metamorphosis. This pattern is exhibited even in the lowest stress environments, which supports the hypothesis that genetic assimilation (e.g., when phenotypes become fixed across environments) is contributing to adaptive divergence across frog populations. We also show that the salinity within coastal wetlands is highly variable across the breeding season and across years. We suggest that high variability in salinity may drive the decoupling of environmental cue (e.g., salinity) to individual fitness, which may be driving character displacement in coastal populations.

Introduction

Environmental change is triggering a proliferation of new and intensified selective pressures on organisms around the world (Bellard et al. 2012, Brysse et al. 2013, Moritz and Agudo 2013, Urban 2015). While some affected biota will adapt to the novel conditions, others may be extirpated. Recently there has been a surge of studies documenting local adaptation across strong environmental clines (Reznick and Ghalambor 2001, Fraser et al. 2011, Brady 2012, Lamichhaney et al. 2012a, Mopper and Strauss 2013, Anderson et al. 2015, Reid et al. 2016), but few studies provide mechanistic insights about how adaptation to novel stressors arises in natural systems (Merilä and Hendry 2014, Urban et al. 2016). For example, some populations may exhibit distinct phenotypes that appear adaptive, yet these populations may have no detectable genetic differences (Gienapp et al. 2008, Merilä and Hendry 2014, Cattau et al. 2018). We have limited capacity to make realistic predictions about whether a species will adapt or be extirpated after an environmental shift because we do not fully understand the etiological mechanisms that promote or inhibit adaptive evolutionary responses (Murren et al. 2015a, Nunney 2015, Urban et al. 2016).

One common and important process that can affect adaptive evolution is phenotypic plasticity, which occurs when a single genotype can express different phenotypes (e.g., behavior, morphology, development, etc.) in response to the environment (Travis 1994, West-Eberhard 2003). Adaptive phenotypic plasticity is a well-studied phenomenon that allows organisms to inhabit a wider range of environments by better matching phenotypes to environmental optima (Whitman and Agrawal 2009, Whitehead et al. 2011b, Wund 2012, Urban et al. 2014, Forsman 2015, Hendry 2015, Murren et al. 2015a, Nonaka et al. 2015, Cattau et al. 2018). Following a

shift in the environment, adaptive plasticity should decrease extinction risk as long as the plasticity is not too costly to maintain, whereas costly or non-adaptive plasticity may increase extinction risk by diminishing overall fitness (Bradshaw 1965, Pigliucci and Murren 2003, Schlichting 2004, Ghalambor et al. 2007, Murren et al. 2015a, Scheiner et al. 2017). Moreover, plasticity itself has a genetic basis and therefore may be subject to evolutionary change (Scheiner 1993, West-Eberhard 2003, Chevin et al. 2010). Because plasticity can promote adaptation, inhibit adaptation, or be the adaptive response itself, understanding the role of phenotypic plasticity in adaptive evolution remains a key challenge for understanding and predicting adaptation to environmental change (Gienapp et al. 2008, Whitman and Agrawal 2009, Chevin et al. 2010, Pfennig et al. 2010, Wund 2012, Chevin et al. 2013, Urban et al. 2014, Hendry 2015, Murren et al. 2015a, Nonaka et al. 2015, Cattau et al. 2018).

Two key processes through which plasticity can contribute to adaptive evolution are the Baldwin effect and genetic assimilation (Pigliucci and Murren 2003, West-Eberhard 2003, Crispo 2007, Ghalambor et al. 2007, Lande 2009). The Baldwin effect suggests that if an organism can improve its fitness via an induced plastic response to a change in the environment, then selection will favor a progressive and directional shift that enhances heritable plasticity toward the environmental optimum (Baldwin 1896, Crispo 2007). Because the reaction norm is the evolving feature, the product of Baldwin's effect is increased plasticity within an adapting population (Sarkar 2004) (Fig. 2-1-Panel B). Conversely, genetic assimilation occurs when plastic phenotypes that initially require an environmental trigger for induction eventually become genetically fixed and no longer require the trigger because they only express the induced phenotype) (Fig. 2-1 – Panel C). Populations that exhibit genetic assimilation should have a

reaction norm that is reduced or lost across different environments compared to non-assimilated populations) (Fig. 2-1 – Panels C and D).

Genetic assimilation and Baldwin's effect are not mutually exclusive processes and can occur simultaneously to drive adaptive evolution within a single trait, or they may act independently and influence the evolution of different traits within the same population. Nonetheless, certain environmental conditions are predicted to favor each process. Genetic assimilation is predicted if the environmental cue is decoupled from the adaptive phenotype (Lande 2009). If the environmental cue is unreliable, yet the fitness benefits of generating an induced phenotype are high, the best strategy may be to generate the induced phenotype constitutively. Genetic assimilation is also expected when the costs of phenotypic plasticity outweigh the fitness benefits (Via and Lande 1985, Relyea 2002a, Lande 2009, Murren et al. 2015a). Costs may include any energetic costs associated with producing and maintaining phenotypes, or possibly genetic costs if the alleles that allow for plasticity are antagonistically correlated with disadvantageous traits via pleiotropy or genetic linkage (Relyea 2002a, Murren et al. 2015a). Conversely, the Baldwin effect should be favored when costs of plasticity are low and the environment is variable (DeWitt et al. 1998, Crispo 2007). Although there are other patterns of evolution associated with phenotypic plasticity, we chose to focus on Baldwin's effect and genetic assimilation as umbrella concepts from which other evolutionary patterns can be understood (West-Eberhard 2003, Crispo 2007, Lande 2009, Renn and Schumer 2013, Levis et al. 2017, Scheiner et al. 2017).

In order to advance our ability to understand and forecast organismal responses to global climate change, studies should disentangle how mechanisms such as plasticity contribute to adaptive

responses (Gienapp et al. 2008, Merilä and Hendry 2014, Urban et al. 2016). Current methods that predict organismal responses to climate change are based on statistical correlations between species ranges and future climate forecasts, but they are increasingly inapplicable as environmental change continues to create new and unique conditions that fall outside the scope of historic correlations (Urban et al. 2016). A powerful way to improve upon our predictive capacity is to identify populations that are persisting in novel or stressful conditions and use these populations to understand how factors such as plasticity contribute to their persistence. Here, we capitalize on populations of a freshwater organism known to be inhabiting and persisting in atypical brackish marshes to understand how plasticity (via genetic assimilation or the Baldwin effect) contributes to the adaptive evolution of organismal traits in a changed environment.

We focus on the evolution of salt tolerance in an amphibian, which may occur response to secondary salinization. Secondary salinization is the rapid increase of soluble salts into freshwater due to a variety of anthropogenic causes and global climate change (Araujo and Rahbek 2006, Harley 2011, Bellard et al. 2012, Herbert et al. 2015, Kaushal et al. 2018). In coastal areas, salt concentrations are progressively increasing due to canal dredging, storm surges, saltwater intrusion, modified riverine flow, agricultural activities, and sea level rise (SLR) (Montagna et al. 2002, Mulligan et al. 2012, Church et al. 2013, Manda et al. 2014, Schuler and Relyea 2018). Many coastal freshwater species will be affected by increased osmotic stress in coastal regions including anuran amphibians (frogs and toads) (DaLaune et al. 1987, Parkinson 1994, Moorhead and Brinson 1995, Michener et al. 1997, Williams et al. 1999, Nicholls and Cazenave 2010, Williams 2013). Anurans are particularly vulnerable to rising

salinities due to their permeable skin, a life history tied to freshwater wetlands, and an inability to concentrate and excrete excess salts (Neill 1958, Balinsky 1981, Hillyard et al. 2009b, Hopkins and Brodie 2015). However, some species, such as the American green treefrog (*Hyla cinerea*), have been documented in low to moderate salinity wetlands, including coastal swamps and saltmarshes (McNab 2002, Hillyard et al. 2009a, Albecker and McCoy 2017). Further, research comparing coastal, chronically salt-exposed *H. cinerea* populations against inland saltwater-naive populations found divergent and adaptive responses to saltwater exposure among coastal green tree frog populations (Albecker and McCoy 2017). Therefore, coastal *H. cinerea* is an ideal model to better understand the biological mechanisms that drive the ability of some species to adapt and persist in a brackish environment.

The egg and larval stages for pond-breeding frog species, like those found along the southeastern coast of the United States, are obligatorily aquatic for the duration of the larval period and are sensitive to the biotic and abiotic features of their environment, especially salinity (Gomez-Mestre and Tejado 2003, Haramura 2007, Karraker 2007, Karraker and Ruthig 2009, Albecker and McCoy 2017). Many North American frog species lay eggs in large, gelatinous masses. As eggs are extruded, they are covered in a thin, jelly matrix that allows sperm to inseminate the egg. Upon entering the water, the thin matrix surrounding the fertilized embryo absorbs water from the environment and inflates to provide each embryo with a protective jelly coat. Because the water contacts the egg directly and contemporaneously with fertilization, the quality of the water can impact developmental pathways occurring within the embryo and alter phenotypes throughout development that include anti-predator behavior and morphological deformities (Allran and Karasov 2001, Ferrari and Chivers 2009).

Upon hatching, exposure to low to moderate levels of salt stress (i.e., 1 to 6 ppt) during larval development has been shown to decrease growth rates and to increase mortality and the time to reach metamorphosis (Rios-López 2008, Langhans et al. 2009, Hsu et al. 2012, Kearney et al. 2012, Brown and Walls 2013, Wijethunga et al. 2016). Because body size in frogs is considered an important indicator of fitness (e.g., larger females will produce a larger clutch, larger males typically have greater reproductive success), reduced growth rates, a smaller size at metamorphosis, increased mortality, and longer time to metamorphosis are typically considered to be maladaptive outcomes (Berven 1990, Harris 1999). Therefore, we may expect that adaptive responses for individuals that develop under salt stress should resemble the growth rates, survival, size at, and time to metamorphosis as individuals raised in freshwater, low abiotic stress environments.

We explore the role of plasticity by comparing how life history endpoints differ between coastal and inland frog populations after exposing early developmental stages to either freshwater or saltwater. We investigate multiple outcomes throughout two key life history stages: egg and larval development through metamorphosis from coastal and inland populations. Specifically, we test whether early-life exposure to saltwater from fertilization through the egg stage induces downstream differences in larval growth rates, size at metamorphosis, the length of time to reach metamorphosis, and survival to metamorphosis between coastal and inland *Hyla cinerea* populations.

If Baldwin's effect underlies divergence between coastal and inland populations, we expected to observe a change in the degree of plasticity in phenotype expression across both populations in response to saltwater exposure, with a much steeper reaction norm in coastal *H. cinerea* populations (Fig. 2-1- Panel B). Conversely, if genetic assimilation is occurring, we expected to observe little influence of the environment on coastal frog populations with the characteristic loss plasticity (Fig. 2-1- Panel C). Indeed, both Baldwin's effect and genetic assimilation could be contributing to the ability of coastal frogs to persist in saline wetlands (Fig. 2-1- Panel D).

Experimental Methods

Experimental Methods: To initiate the common-garden experiment, we collected 2 amplexed green tree frogs from 4 coastal populations (i.e., populations chronically exposed to saltwater) and 4 inland populations (i.e., populations chronically exposed to freshwater) between May 23, 2017 and July 17, 2017. Because the coastal and inland populations are separated by approximately 200 kilometers as well as the Croatan and Pamlico sounds, so we assumed that pairs collected from populations in these two regions are sufficiently distant geographically and environmentally. Therefore, potential differences that emerge between coastal and inland frogs may be interpreted as being produced by the historically different properties of the habitats.

Each of the pairs was randomly assigned to a 5.7-liter (L) Sterilite ® container holding either 2 L of freshwater (<0.5ppt) or moderate concentration of saltwater (4ppt) water where they were allowed to deposit eggs overnight. By placing pairs into a container holding freshwater or saltwater, hereafter referred to as "egg environment", we ensure that eggs from populations across both locations are exposed to saltwater or freshwater from the moment they are fertilized.

To control for possible differences in source tap water across both locations, we used Greenville's municipal tap water treated with label recommended amounts of API® Tap Water Conditioner (Chalfont, PA) with salinities experimentally raised using InstantOcean Sea Salt® (Blacksburg, VA). We chose 4ppt because this salinity is regularly observed in coastal wetlands and has been shown to induce effects(e.g. reduced growth) in these frogs without prohibitively high rates of egg mortality (Albecker and McCoy 2017). After completing oviposition, the adults were released at the site of capture, and the egg clutches were transported back to the laboratory. Eggs were allowed to develop and hatch at room temperature (25C), which took approximately 48 hours. All experimental protocols were approved by ECU's animal care and use committee (IACUC #D314), and animals were collected under NC wildlife collection license (17-SC00840).

Approximately two days post-hatching, hatchlings transition from yolk absorption to active foraging (Gosner stage 25) (Gosner 1960). At this time, we subsampled 200 hatchlings from each clutch. These individuals were then divided into four groups of 50 individuals. Dividing the clutches allows us to control for genetic relatedness within clutches (Merilä and Hendry 2014). Each of the four groups was randomly assigned to a "tadpole environment" treatment and placed into small glass containers containing 400mL of water matching the salinity of the egg environment. At this point we expanded the range of salinities that tadpoles were exposed to for the duration of development. We randomly assigned each of the groups into a salinity that either matched the egg environment, or was either freshwater, 4ppt, 6ppt, or 8ppt. The salinity was gradually increased, decreased, or maintained over 6 days until the specified target salinity was reached. Gradually adjusting anurans to elevated salinities reduces mortality (Gordon and Tucker

1965, Gordon and Tucker 1968, Hsu et al. 2012). Tadpoles were fed spirulina flakes (O.S.I.®, Coral Springs, FL) *ad libitum* each day following water changes.

On day 6, we haphazardly selected 10 individuals from the freshwater and 4ppt treatments and 5 individuals from the 6ppt and 8ppt treatments. These tadpoles were placed into individual 16oz. plastic cups holding 300mL of treated tap water mixed to match their assigned salinity. Tadpoles were housed in these cups for the duration of development. Individual housing allows for easy monitoring and eliminates the need to control for the potentially confounding effect of changes in density from mortality. The temperature of the laboratory was maintained at 27ºC, with a 12h light/dark cycle. Tadpoles were checked daily, and mortality recorded. Water changes occurred every other day. Tadpoles were weighed and measured (total length) once per week using Neiko® digital calipers and GeneMate® digital balance, and water temperatures were also checked at that time. Upon reaching stage 42, defined as the point that forelimbs emerge (Gosner 1960), metamorphs were weighed and measured. Water levels were reduced to 50mL, lids with breathing holes fastened, and cups were tilted to allow tadpoles to climb out of the water. Upon full emergence from the water, the water was replaced with a moist paper towel, and metamorphs were checked daily for tail resorption. When tails were fully resorbed, individuals were weighed, and snout-vent length was measured. Froglets were then released at the site from which they were collected as eggs as required by IACUC. In total, we reared 480 tadpoles (4 populations x 2 locations [coastal, inland] x 2 egg environments [FW or 4ppt] x 4 developmental environments [FW, 4ppt, 6ppt, 8ppt]).

Conductivity Dataloggers: To characterize the seasonal salinity fluctuations across the frog breeding season, we installed three U-24 HOBO® Conductivity loggers into three coastal wetlands on September 30, 2014 and intermittently logged data at these sites through January 24, 2018. Loggers were occasionally removed to read out data. Loggers measured conductivity and temperature at 8-hr intervals to capture daily variation. Sites were selected because they support large frog populations and were located in regions undergoing high erosion and saltwater inundation rates. One logger was placed in the marsh near the Pamlico Sound on Point Peter Road (35º46'11.7"N, 75º44'31"W), which is a part of the Albemarle peninsula and Alligator River National Wildlife Refuge. The second logger was placed in the marsh near Bodie Island Lighthouse on Cape Hatteras National Seashore (35º49'12.6"N,75º33'44.5"W). The third logger was placed in a pond adjacent to New Inlet near Rodanthe, NC (35º41'11"N,75º29'03.6"W). Loggers were left undisturbed for the duration of the field season each year. Data were downloaded from dataloggers using HOBOware® software (version 3.7.13). The 2014 data was incomplete as the sampling design had not been finalized but we included the incomplete data nonetheless. Currently data from 2016 and 2017 are missing from Alligator River National Wildlife Refuge due to displacement during Hurricane Matthew in 2016. New Inlet is also missing 2017 data because we have not yet been able to recover the logger.

Statistical Methods: We quantified initial mass of the tadpoles one week following acclimation, growth rates throughout development, survival through time, and time to and size at metamorphosis because each of these are venerated correlates of fitness for anurans (Semlitsch et al. 1988, Skelly 1992, Werner and McPeek 1994, Werner and Anholt 1996, Denver 1997, Van Buskirk and McCollum 2000, Glennemeier and Denver 2002, Laurila et al. 2002, Relyea 2002b,

Werner and Peacor 2003, Touchon and Warkentin 2008). Each of the analyses described below was conducted in the R statistical programing environment version 3.5.0 (Team 2018)

To determine differences in initial mass according to egg environment, tadpole environment, and location, we used a linear mixed effects model using the lme4 library (function lmer) assuming a log-normal error distribution (Bates et al. 2015). We treated egg environment (freshwater or 4ppt), tadpole environment (0, 4, 6 or 8 ppt), and location (coastal or inland) as fixed effects, and population as a random effect to account for non-target variation across sampling sites. Because the ages of tadpoles (in days post oviposition) were slightly different, we included age as a covariate. We use a model selection approach based on sample size-corrected Akaike Information Criterion (AICc) (Burnham and Anderson 2003, Bolker and Team 2017) to determine the relative support for our different candidate models with all combinations of possible interactive or additive relationships between egg environment, tadpole environment, and location (Table 1). Based on AICc, we then performed hypothesis tests for the fixed effects on the most parsimonious model using likelihood ratio tests.

Growth*:* We analyzed the growth data by fitting a Gompertz growth curve of the form:

$$Y = S_0^{(\frac{\gamma}{\alpha})\,(1-e^{(-\alpha x)})}$$

where S_0 is an estimate of initial size, γ (gamma) corresponds to the maximum size specific growth rate, and α (alpha) is the exponential decay of size specific growth rates, which biologically is associated with a slowed rate of cell division, cell death, or the suspension of growth as cell differentiation occurs (Harris 1999). The functional form of this model is consistent with most empirical observations of amphibian growth (Hota 1994), and is defined by

biologically meaningful parameters for determining biological processes that underlie differences in growth.

We analyze the changes in total length (in mm) through time across the different treatments as our metric of size because we are primarily interested in somatic growth and not differences in water loss/retention through time that may occur from changes in the osmotic environment. We used maximum likelihood estimation using mle2() in package "bblme" (Bolker and Team 2017) to fit 32 different parameterizations of this growth model. Our most complex model included a 2-way interaction for initial size and 3-way interactions among the treatments (e.g., egg environment, tadpole environment, and location) for the alpha and gamma parameter and our simplest was fit to the pooled data ignoring treatment effects. For the initial size parameter, we only include location and egg environment, as hatchlings had not spent much time in the tadpole environment at that point. The specific parameterizations of the 32 models are listed in Table 2. The most parsimonious model for describing differences in growth rate between location, egg environment, and tadpole environment was selected using AICc (Table 2). After selecting the best model, we used likelihood ratio tests to test specific hypotheses about the parameters within the best model.

Survival analysis*:* We tested for differences in survival across treatments using two methods. First, we analyzed whether there are differences in the proportion of individuals surviving to metamorphosis according to egg environment, tadpole environment, and location. Second, we determined how survival through time varied across treatments. The first approach tells us how overall survival differed, while the second approach uncovers whether there were differences in

the risk of mortality during development across treatments. To test for differences in the proportion of individuals that survived to metamorphosis, we used Generalized linear mixed effects models with a binomial error distribution. However, we encountered numerical instability because of several scenarios with complete separation in the data (e.g., all inland 8ppt developmental salinity treatments experienced 100% mortality). To deal with the complete separation, we used Bayesian generalized mixed-effects models using function bglmer() in package "blme" (Bolker and Team 2017) and specified the non-derivative based optimization algorithm "bobyqa" to improve optimization efficiency. We used AICc to determine the relative support for different models ranging between the most complex (full three-way interactions between egg environment, developmental environment, and location) to simple (additive model) and no effects models. After selecting the best model, significance among fixed effects in the most parsimonious model was tested using likelihood ratio tests. For these models, we treated egg environment, developmental environment, and location as fixed effects and treated the different populations as random effects.

To test for differences in risk of mortality through time we conducted the Cox-Proportional hazard regression analysis with the R package "survival" (Therneau 2015). Survival curves were plotted using package "survminer" (Kassambara and Kosinski 2018).

Age and size at metamorphosis*:* To analyze differences in the length of time (in days) that it took for tadpoles to reach metamorphosis (defined as the day of forelimb emergence), we used generalized mixed effects models using package "lme4" (Bates et al. 2015). Age was calculated as the number of days since hatching. We assumed a Poisson error distribution.

To test how the size of tadpoles varied at metamorphosis, we test for differences in length and weight separately. We used a linear mixed effects model (function lmer()) assuming a log-normal error distribution. For each of these analyses, we used AICc to determine the relative support for different models ranging between the most complex to simple and no effects models. Again, after selecting the best model, significance of fixed effects in the most parsimonious model was tested using likelihood ratio tests. For these analyses, we again treated location, egg environment, and developmental environment as fixed effects with population as a random effect.

Conductivity Dataloggers: To test for differences in salinity through time and across the three sites, we used generalized additive models (function gam()) in package "mgcv" (Wood 2011). In these models we treated day-of-year, month, year, and location as fixed effects. We used AICc to determine the relative support for different models ranging between the most complex (full four-way interactions between day, month, year, and location) to simple, additive model (Table 3). Upon selecting the most parsimonious model, we use likelihood ratio tests to determine significance of fixed effects.

Results

Mass after Initial Acclimation: The model that best fit tadpole mass one week following placement into tadpole salinities included an additive relationship between egg environment and tadpole environment and an interaction with location (dAICc = 2.9; df = 8, weight = 0.81) (Table 1). Within that model, there was a significant influence of egg environment (X^2_8 =3.82, p = 0.05),

tadpole environment (X^2_8 =264.01, p <0.0001), and location (X^2_8 =10.65, p = 0.005). After one week in the tadpole environment following acclimation, coastal tadpoles were consistently larger than inland tadpoles (Fig. 2-2). Coastal frogs laid in the freshwater egg environment that remained in freshwater were 33% heavier with a mass of 0.28g (0.15g to 0.53g 95% Confidence Interval (C.I.) compared to 0.21g (0.11g to 0.39 C.I.) for inland. Coastal individuals laid in freshwater and transitioned into 4ppt tadpole environment were 33% heavier than inland and weighed 0.26g (0.14 – 1.49g C.I.) while inland weighed 0.19g (0.10-0.36g C.I.). Coastal tadpoles laid in freshwater and transitioned into the higher salinities were also larger by approximately 50% (6ppt: 0.15g (0.08-0.28g C.I.); 8ppt: 0.12g (0.06-0.23g C.I.)) relative to inland tadpoles (6ppt: 0.08g (0.04-0.15g C.I.); 8ppt: 0.05g (0.03-0.11g C.I.)). The same trend with larger coastal individuals continued for individuals laid in 4ppt water, though both coastal and inland individuals tended to weigh less than individuals laid in freshwater. In the 4ppt egg/ freshwater tadpole treatments, coastal tadpoles were 63% heavier and weighed 0.18g (0.097-0.35g C.I.) and inland weighed 0.11g (0.06-0.21g C.I.). In the 4ppt egg/4ppt tadpole, coastal tadpoles weighed an average of 0.16g (0.09-0.32g C.I.), approximately 75% heavier than inland that weighed 0.09g (0.05-0.18g C.I.). In the higher salinities, tadpoles weighed even less, with coastal individuals weighing 0.13g (0.06-0.24g C.I.) in 6ppt tadpole treatment, and 0.10g (0.05-0.19g C.I.) in the 8ppt tadpole treatment, while inland individuals weighed about 50% less at 0.07g (0.03-0.14g C.I.) in the 6ppt tadpole treatment and 0.05g (0.02-0.09g C.I.) in the 8ppt tadpole treatment.

Growth rates: The best fit model for tadpole length included the full complement of additive relationships between main effects (dAICc = 1.1 df = 16, weight = 0.25). The best fit is model 30 in Table 2-2.

Gamma Parameter (Size specific growth rate): Egg environment had little impact on coastal tadpole growth rates (γ) (Fig. 2-3). Coastal individuals from both the freshwater egg environment and coastal environment that were raised in the freshwater tadpole environment (FW egg environment: γ = 0.157; C.I. 0.141 – 0.171; 4ppt egg environment: γ = 0.159; C.I. 0.136 – 0.182) and 4ppt tadpole environment (FW egg environment: γ = 0.159; C.I. 0.136 – 0.182; 4ppt egg environment: γ = 0.149; C.I. 0.119 – 0.179) had very similar growth rates. Likewise, egg environment had no impact on inland growth rates. Rather, coastal growth rates were higher than the growth rates of inland tadpoles in the freshwater tadpole environment (FW egg environment: γ = 0.111; C.I. 0.084 – 0.138; 4ppt egg environment: γ = 0.114; C.I. 0.079 – 0.148) and 4ppt tadpole environment (FW egg environment: γ = 0.101; C.I. 0.067 – 0.135; 4ppt egg environment: γ = 0.104; C.I. 0.062 – 0.145). Growth rate for coastal tadpoles hatched from both fresh and salt egg environments declined at 6ppt (FW egg environment: γ = 0.105; C.I. 0.082 – 0.128; 4ppt egg environment: γ = 0.108; C.I. 0.077 – 0.139) but remained higher than inland growth rates across both egg environments (FW egg environment: γ = 0.060; C.I. 0.025 – 0.094; 4ppt egg environment: γ = 0.063; C.I. 0.020 – 0.105).

Alpha Parameter (exponential decay of size specific growth rates): We observed very little impact of the egg environment on exponential decay of size specific growth rates (α) (Fig. 2-4). Coastal individuals from both egg environments had a higher alpha value compared to inland

populations in both the freshwater tadpole environments (Coastal FW egg environment: α = 0.128; C.I. 0.118 – 0.138; Coastal 4ppt egg environment: α = 0.134; C.I. 0.117 – 0.151; Inland FW egg environment: α = 0.077; C.I. 0.059 – 0.096; Inland 4ppt egg environment: α = 0.083; C.I. 0.058 – 0.109) and 4ppt tadpole environments (Coastal FW egg environment: α = 0.116; C.I. 0.099 – 0.133; Coastal 4ppt egg environment: α = 0.121; C.I. 0.096 – 0.147; Inland FW egg environment: α = 0.066; C.I. 0.039 – 0.092; Inland 4ppt egg environment: α = 0.071; C.I. 0.038 – 0.104). In the 6ppt tadpole environment treatments, alpha declined for both inland and coastal similarly across both egg environments, but coastal retained a higher alpha (Coastal FW egg environment: α = 0.082; C.I. 0.063 – 0.101; Coastal 4ppt egg environment: α = 0.087; C.I. 0.062 – 0.113; Inland FW egg environment: α = 0.031; C.I. 0.043 – 0.058; Inland 4ppt egg environment: α = 0.037; C.I. 0.0032 – 0.071).

Survival: The most parsimonious model describing the proportional survival of individuals involved a three-way interaction between egg environment, tadpole environment, and location (dAICc = 139.1, df = 9, weight = 1) (Fig. 2-5). Within that model, survival according to egg salinity, developmental salinity, and location were statistically significant (egg environment: Z=-3.1, p = 0.001; tadpole environment: Z = -4.19, p <0.001; location: Z=-3.5, p = 0.0004). All interactions were also statistically significant (egg*tadpole: Z = 3.14, p=0.002; Egg * location: Z = 3.35, p = 0.0008; tadpole * location: Z = 3.31, p = 0.0009; egg * tadpole * location: Z = -3.26, p = 0.001). The lethal concentration required to impose 50% mortality (LC_{50}) in inland individuals from the freshwater egg environment was 4.5ppt (C.I. 3.51-5.63ppt). LC_{50} for inland individuals from the 4ppt egg environment was slightly higher at 5.6ppt (C.I. 4.76-6.68). Coastal individuals hatched in the 4ppt egg environment had an LC_{50} of 6.83ppt (C.I. 5.91-8.21). The

highest survival was observed in the coastal individuals hatched from freshwater with an LC_{50} of 7.16ppt (C.I. 6.56-7.65). Only 2 individuals out of the original 40 assigned to the 8ppt treatment survived to metamorphosis. Both of these individuals were coastal individuals laid in the freshwater egg environment (Fig. 2-6).

Cox proportional hazards analysis showed that longevity was also different according to egg environment, tadpole environment salinity, and location (X^2_4 =198, p <0.0001). Increasing the salinity in the tadpole environment increased mortality rates by 1.47-fold (standard error (SE): +/- 0.037; Z = 10.5, p <0.0001) across all treatments. The environment had less of an impact on coastal survival, as coastal individuals laid in freshwater had the lowest mortality rates through time. Compared to coastal individuals laid in the freshwater egg environment, coastal individuals laid in 4ppt egg environment had 1.9-fold increase in daily mortality rates (SE +/- 0.306; Z = 2.18, p = 0.029). Inland tadpoles fared worse than coastal tadpoles. Inland tadpoles laid in 4ppt egg environment had a 5.25-fold increase in daily mortality risk relative to coastal individuals (SE +/- 0.284; Z = 5.83, p <0.001). Inland individuals from the freshwater egg environment had the highest risk with a 6.99-fold increase in risk of death compared to coastal/freshwater egg environment (SE +/- 0.278; Z = 6.96, p < 0.001).

Time to Metamorphosis: The model that best describes the length of time in days for individuals to reach metamorphosis did not include effects of the egg environment as an important predictor but did include the additive effects of location and tadpole environment (dAICc = 1.6, df = 4, weight = 0.39) (Fig. 2-7). Within this model, we find a significant effect of tadpole environment salinity (X^2_4 = 13.18, p = 0.0002), with a trend towards an effect of location (X^2_4 =2.64, p =

0.10). Inland tadpoles raised in the freshwater tadpole environment from both the freshwater and 4ppt egg environment took approximately 43 days to reach metamorphosis (FW egg: 42.78 days (95% Confidence Interval (C.I.) 40.34 – 45.39; 4ppt egg: 43.22, C.I. 40.75-45.82), which was about 3 days longer than freshwater coastal tadpoles that metamorphosed after approximately 40 days (FW egg: 39.97 days, C.I. 37.71 – 42.36; 4ppt egg: 40.35, C.I. 38.06-42.79). Both coastal and inland tadpoles increased the time to metamorphosis as salinities increased. In the 4ppt tadpole environment, inland tadpoles reached metamorphosis after approximately 45 days (FW egg: 45.02 days, C.I. 42.46 – 47.73; 4ppt egg: 45.44, C.I. 42.92-48.15), while coastal tadpoles took approximately 42 days to reach metamorphosis (FW egg: 42.06 days, C.I. 39.79 – 44.45; 4ppt egg: 42.45, C.I. 40.13 – 44.89). In the 6ppt tadpole salinity, inland tadpoles took approximately 46 days to reach metamorphosis (FW egg: 46.17 days, C.I. 43.35 – 49.17; 4ppt egg: 46.61, C.I. 43.81-49.59) compared to coastal individuals that took approximately 43 days (FW egg: 43.11 days, C.I. 40.65 – 45.76; 4ppt egg: 43.54, C.I. 41.04 – 46.22). The two coastal individuals that survived to metamorphosis in the 8ppt treatment metamorphosed at 55 days and 63 days.

Size at Metamorphosis: The model describing both mass at metamorphosis and length at metamorphosis included additive effects of egg environment and tadpole environment and an interaction between tadpole environment and location (Mass: dAICc = 13.3 df = 7, weight = 0.998; Length: dAICc = 18.7, df = 7, weight = 1). Within this model, there was a significant effect of egg salinity (Mass: $X^2_7 = 13.29$, $p = 0.0002$; Length: $X^2_7 = 29.26$, $p < 0.0001$), developmental salinity (Mass: $X^2_7 = 20.22$, $p = 0.0001$; Length: $X^2_7 = 40.59$, $p < 0.0001$), and location (Mass: $X^2_7 = 18.61$, $p < 0.0001$; Length: $X^2_7 = 38.55$, $p < 0.0001$).

As salinity of the tadpole environment increased, inland tadpoles from both freshwater and 4ppt treatments were smaller at metamorphosis in both weight and length as tadpole environment salinities increased, while the coastal tadpoles metamorphosed at slightly larger mass and length despite increasing salinities (Mass: Fig. 2-8; Length: Fig. 2-9). In the freshwater tadpole environments, inland individuals weighed approximately 0.84g with an approximate length of 52mm at metamorphosis (Mass: FW egg environment: 0.86g, C.I. 0.72-1.03; 4ppt egg environment: 0.83g, C.I. 0.69-0.99; Length: FW egg environment: 53.50mm, C.I. 49.29-58.15; 4ppt egg environment: 51.63mm, C.I. 47.48-56.05). Coastal individuals metamorphosed smaller, with a mass of approximately 0.69g and a length of 47mm (Mass: FW egg environment: 0.71g, C.I. 0.59-0.85; 4ppt egg environment: 0.68g, C.I. 0.57-0.82; Length: FW egg environment: 48.17mm, C.I. 44.37-52.38; 4ppt egg environment: 46.48mm, C.I. 42.77-50.47). In the 6ppt tadpole environment, inland metamorphs were approximately 16% smaller in mass and 6% shorter in length by approximately 6% (Mass: FW egg environment: 0.74g, C.I. 0.61-0.89; 4ppt egg environment: 0.69g, C.I. 0.58-0.84; Length: FW egg environment: 50.50mm, C.I. 46.47-54.86; 4ppt egg environment: 48.82mm, C.I. 44.92-53.10). In the 6ppt tadpole environment, coastal frogs were slightly larger in both mass and length by 2% and 6%, respectively (Mass: FW egg environment: 0.72g, C.I. 0.60-0.86; 4ppt egg environment: 0.70g, C.I. 0.58-0.84; Length: FW egg environment: 51.02mm, C.I. 46.96-55.46; 4ppt egg environment: 49.24mm, C.I. 45.31-53.50).

Conductivity Dataloggers: AICc revealed two models with equivalent likelihoods, AICc weights (0.5) and degrees of freedom (Table 3). Because both models included the same main effects and

only differed in the structure of interactions, the choice between them had little effect on overall inferences. As a result, we arbitrarily selected one of these models to use for hypothesis testing. Thus, we base our inferences on the model that included additive effects of year and month and an interaction between day of year and location. The equivalent model had an interaction between year and month, but an additive relationship between month, day of year, and location. We found that salinity was highly variable with differences in salinity for each parameter including year (X^2_3 <1000, p < 0.0001), month (X^2_{33} <1000, p < 0.0001), day of year (X^2_{17} <1000, p < 0.0001), and location (X^2_{22} <1000, p < 0.0001) (Table 3) (Fig. 2-10).

Discussion

To improve our ability to predict outcomes of environmental change, we must gain a better mechanistic understanding of how ubiquitous biological factors such as phenotypic plasticity affect organismal responses and population persistence in changed environments. In this study, we characterize how early life exposure to saltwater modifies well-studied forms of developmental plasticity in frog populations that are diverging across a salt gradient. We found that in nearly every endpoint measured, coastal populations demonstrate different responses than inland populations with a very limited influence of early environment, which supports the hypothesis that developmental patterns are becoming fixed (e.g., genetically assimilated) in coastal populations which contributes to the adaptive divergence across frog populations. Coastal frogs grow faster (γ) and initiate metamorphosis sooner (α), but at the cost of being smaller at metamorphosis. This strategy to grow fast but metamorphose small is a well-described developmental tactic for frogs in risky or stressful environments (Werner and Gilliam 1984, Werner and Anholt 1996, Harris 1999) and is often observed in individuals experiencing an

increased risk of mortality due to predation or desiccation (Wilbur and Collins 1973, Werner and Gilliam 1984, Alford and Harris 1988, Touchon et al. 2015).

Theory predicts that developing larvae should maximize their future fitness by minimizing the ratio between current mortality risk (μ) and growth rate (g) (Werner and Gilliam 1984, Werner 1986, Touchon et al. 2015). So, tadpoles should prioritize transitioning into the next stage (e.g., metamorphosing) when the perceived risk of mortality or reduction in growth in the current life stage supersedes perceived risks of the next life stage (Istock 1967, Werner and Gilliam 1984, Werner 1986). To balance these pressures to grow rapidly and survive, many species have evolved to use information from different time points throughout development to progressively gauge risk and adjust growth rates and timing of metamorphosis (Buskirk and Saxer 2001, Vonesh and Warkentin 2006, Touchon et al. 2015). As a result, developmental traits such as growth rate, time to metamorphosis, and size at metamorphosis have been shown to be highly plastic contingent upon the quality of the larval environment.

High environmental variability leads to imperfect predictability of environmental variation, which may reduce the ability of coastal tadpoles to accurately gauge risk and adjust growth accordingly. As shown through the high degree of variability in salinity profiles in North Carolina's coastal wetlands across single breeding seasons and across years (Fig. 2-10), the environment experienced throughout the egg and larval development of coastal frogs may be an unreliable signal of future environment. Thus, the salinity encountered during hatching may be very different from the salinity encountered later in development. This decoupling of environmental cue from phenotypic response can drive decreased plasticity during genetic

assimilation (Via and Lande 1985, Crispo 2007, Lande 2009, Chevin et al. 2010). Consequentially, as theory predicts, selection favors coastal frogs that have canalized around a risk-reducing life history strategy that lessens the time spent in the larval period regardless of how saline their current environment may be. Indeed, coastal frogs consistently show a shift in developmental patterns across all egg and tadpole environments toward minimizing the amount of time spent as larvae within saline wetland – even in the least stressful, freshwater treatments.

Impact of Location: The changes in life history strategy were evident through increased γ (size-specific growth rates) (Fig. 2-3), increased α (exponential decay of growth rate) (Fig. 2-4), trends toward decreased time to metamorphosis (Fig. 2-7), and decreased size at metamorphosis (Figs. 2-8 & 2-9) in coastal populations across both egg environments. Increased α in coastal frogs indicates that coastal frogs are ceasing growth earlier as metamorphosis is initiated (Harris 1999). It is possible that a higher α may also signify increased cell death or slowed cell division, but the trend towards earlier metamorphosis in coastal frogs lends support to the hypothesis that coastal frogs reduce growth rates earlier to initiate metamorphosis sooner than inland frogs. Interestingly, a smaller size at metamorphosis is considered maladaptive and may affect coastal frog population dynamics, because smaller size at metamorphosis is correlated with older age of maturation, smaller size at first reproduction, and reduced fecundity, and reduced terrestrial performance (e.g., reduced foraging ability and predator-escape response) (Berven and Gill 1983, Smith 1983, Pough and Kamel 1984, Taigen and Pough 1985, Emerson 1986, Ficetola and De Bernardi 2006). However, previous experiments that collected *H. cinerea* adults from breeding populations show no differences in the size or mass of breeding adults across locations (Albecker et al. 2018) suggesting that post-metamorphic coastal frogs may be engaging in

compensatory growth to offset any potential fitness costs of a stressful larval period (Squires et al. 2010).

Egg Environment Impacts: We observed very few impacts of embryonic exposure to saltwater across both coastal and inland populations. With inland populations, we did observe that early exposure to saltwater reduces the risk of mortality and improves survivorship to metamorphosis in ways consistent with the predictions of Baldwin's effect. A greater proportion of inland individuals from the 4ppt egg environment survived and had lower risks of mortality compared to individuals hatched in freshwater (Fig. 2-5). This response is consistent with the predictions of Baldwin's effect as early life exposure to saltwater generates a downstream improvement in ability to survive in higher salinities. It is possible that differential survivorship due to early life environment may have contributed to the original ability of *H. cinerea* to invade and persist in saline environments, but further research is required (Albecker and McCoy 2017, Barrow et al. 2017). The cellular mechanisms that underpin improved survivorship are yet unidentified but may be key in understanding how and why this species is able to persist in coastal habitats. Interestingly, we observed the opposite pattern in coastal tadpole survival, where individuals hatched in 4ppt egg environment had lower survival to metamorphosis with higher risk of mortality compared to individuals hatched in freshwater. It is important to note that even though survival was reduced for coastal individuals from the 4ppt egg environment compared to coastal individuals from freshwater egg environment, survival of both coastal treatments was still higher than inland populations from both egg environments. Nonetheless, reduced survival can be expected when embryos are exposed to some elevated stressor, so reduced coastal survival is consistent with life history plasticity theory (Werner and Gilliam 1984), as well as other studies

that demonstrate that early exposure to stress (e.g., low resource availability, predator presence) can lead to reduced survival and growth (Wilbur & Collins 1973, Alford and Harris 1988, Hensley 1993, Leips and Travis 1994, Audo et al. 1995, Beck 1997, Newman 1994, Tejedo and Reques 1994).

Tadpole Environment Impacts: Across nearly all endpoints, we observed an effect of tadpole environment which suggests that saltwater exposure during development impacts developmental outcomes for both coastal and inland tadpoles. For example, growth rate was reduced across coastal and inland populations in the 6ppt treatment, although coastal growth rate remained higher than inland. Although both coastal and inland populations were affected by tadpole salinity, the nature of responses sometimes differed according to location. For example, inland tadpoles metamorphosed at a smaller size (e.g., weight and length) as tadpole salinity increased, but coastal individuals metamorphosed at a marginally increased size as tadpole salinity increased (Figs. 2-8 & 2-9). This location by environment pattern is consistent with patterns expected with genetic assimilation (Crispo 2007).

Our findings differ from a study by Wu et al. (2012) investigating developmental impacts of early salt-exposure in the salt-specialist frog species, *Fejervarya cancrivora.* This study found that individuals that were initially exposed to low salinities but switched to higher salinities later in development were smaller at metamorphosis. Although metamorphosing frogs from coastal populations were smaller than inland populations, the size differences were produced regardless of the salinity of the environment suggesting a stronger influence of environment on *F. cancrivora* development (Wu et al. 2012). Differences between *H. cinerea* and *F. cancrivora*

may be due to different phylogenetic histories of these species (Gordon et al. 1961, Gordon and Tucker 1965, Gordon and Tucker 1968).

We are yet uncertain of the genetic mechanisms that underlie the observed phenotypic patterns, but the consistency in phenotypic differences regardless of egg and tadpole environment suggests that genetic differences do exist between coastal and inland frog populations (Gienapp et al. 2008, Merilä and Hendry 2014). We expect genetic changes to be reflected in genetic and physiological pathways that regulate development and metabolism because coastal frogs have shifted towards a constitutive strategy that involves faster growth and development. Additionally, since we observed significantly higher survivorship in coastal individuals across tadpole salinities, it is likely that some genetic differences also exist in osmoregulatory pathways that allow for increased salt tolerance.

Conclusions: We show evidence that coastal *H. cinerea* frog populations are diverging from inland counterparts via genetic assimilation. Genetic assimilation has been demonstrated in laboratory settings (Waddington 1953, Walworth et al. 2016) and there is a growing list of genetic assimilation observed in wild populations (Wund et al. 2008, Losos 2009., Lamichhaney et al. 2016, Parsons et al. 2016, Levis et al. 2017). This suggests that genetic assimilation may be a more common mechanism of character displacement in evolving populations than previously appreciated (Levis et al. 2017), and should be considered in future research on adaptation to environmental change. However, few have provided mechanisms that drive the loss of plasticity across assimilated populations. In this study, we suggest that the mechanism underlying the canalization of tadpole development is the decoupling of environmental cue (e.g., salinity) to

individual fitness, based on theoretical principles (Lande 2009) and the high degree of salinity variability as revealed by long-term environmental monitoring.

Literature Cited

Albecker, M. A., W. B. Brantley Jr, and M. W. McCoy. 2018. Diet and Nematode Infection Differ Between Coastal and Inland Populations of Green Treefrogs (*Hyla cinerea*). Southeastern Naturalist **17**:155-165.

Albecker, M. A., and M. W. McCoy. 2017. Adaptive responses to salinity stress across multiple life stages in anuran amphibians. Frontiers in Zoology **14**:40.

Alford, R. A., and R. N. Harris. 1988. Effects of larval growth history on anuran metamorphosis. The American Naturalist **131**:91-106.

Allran, J. W., and W. H. Karasov. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environmental Toxicology and Chemistry, **20**:769-775.

Anderson, J. T., N. Perera, B. Chowdhury, and T. Mitchell-Olds. 2015. Microgeographic patterns of genetic divergence and adaptation across environmental gradients in Boechera stricta (Brassicaceae). American Naturalist **186**:S60-S73.

Araujo, M. B., and C. Rahbek. 2006. Ecology. How does climate change affect biodiversity? Science **313**:1396-1397.

Baldwin, J. M. 1896. A new factor in evolution. American Naturalist **30**:441-451.

Balinsky, J. B. 1981. Adaptation of Nitrogen metabolism to hyperosmotic environment in amphibia. Journal of Experimental Zoology **215**:335-350.

Barrow, L. N., J. A. Soto-Centeno, A. R. Warwick, A. R. Lemmon, and E. Moriarty Lemmon. 2017. Evaluating hypotheses of expansion from refugia through comparative phylogeography of south-eastern Coastal Plain amphibians. Journal of Biogeography, **44**:2692-2705.

Bates, D., M. Martin, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models using lme4. Journal of Statistical Software **2015**:1-48.

Bellard, C., C. Bertelsmeier, P. Leadley, W. Thuiller, and F. Courchamp. 2012. Impacts of climate change on the future of biodiversity. Ecology Letters **15**:365-377.

Berven, K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology **71**:1599-1608.

Berven, K. A., and D. E. Gill. 1983. Interpreting geographic variation in life-history traits. American Zoologist **23**:85-97.

Bolker, B., and C. D. Team. 2017. bbme: Tools for general maximum likelihood estimation. R

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity in plants. Advances in Genetics **13**:115-155.

Brady, S. P. 2012. Road to evolution? Local adaptation to road adjacency in an amphibian (Ambystoma maculatum). Scientific reports **2**.

Brown, M. E., and S. C. Walls. 2013. Variation in salinity tolerance among larval anurans: Implications for community composition and the spread of an invasive, non-native species. Copeia **2013**:543-551.

Buskirk, J. V., and G. Saxer. 2001. Delayed costs of an induced defense in tadpoles? Morphology, hopping, and development rate at metamorphosis. Evolution **55**:821-829.

Cattau, C. E., R. J. Fletcher Jr, R. T. Kimball, C. W. Miller, and W. M. Kitchens. 2018. Rapid morphological change of a top predator with the invasion of a novel prey. Nature Ecology and Evolution **2**:108.

Chevin, L. M., R. Gallet, R. Gomulkiewicz, R. D. Holt, and S. Fellous. 2013. Phenotypic plasticity in evolutionary rescue experiments. . Phil. Trans. R. Soc. B **368**:p.20120089.

Chevin, L. M., R. Lande, and G. M. Mace. 2010. Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. PLoS Biol **8**:e1000357.

Church, J. A., P. U. Clark, A. Cazenave, J. M. Gregory, S. Jevrejeva, A. Levermann, M. A. Merrifield, G. A. Milne, R. S. Nerem, P. D. Nunn, A. J. Payne, W. T. Pfeffer, D. Stammer, and A. S. Unnikrishnan. 2013. IPCC Fifth Assessment Report (AR5), Climate Change 2013: The Physical Science Basis.1-124.

Crispo, E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. Evolution **61**:2469-2479.

DaLaune, R. D., S. R. Pezeshki, and W. H. P. Jr. 1987. Response of Coastal plants increase in submergence and salinity. Journal of Coastal Research **3**:535-546.

Denver, R. J. 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis American Zoologist **37**:172-184.

DeWitt, T. J., A. Sih, and D. S. Wilson. 1998. Costs and limits of phenotypic plasticity. Trends in Ecology & Evolution **12**:77-81.

Emerson, S. B. 1986. Heterochrony and frogs: the relationship of a life history trait to morphological form. The American Naturalist **127**:167-183.

Ferrari, M. C., and D. P. Chivers. 2009. Sophisticated early life lessons: threat-sensitive generalization of predator recognition by embryonic amphibians. Behavioral Ecology **20**:1295-1298.

Ficetola, G. F., and F. De Bernardi. 2006. Trade-off between larval development rate and post-metamorphic traits in the frog Rana latastei. Evolutionary Ecology **20**:143-158.

Forsman, A. 2015. Rethinking phenotypic plasticity and its consequences for individuals, populations and species. Heredity **115**:276-284.

Fraser, D. J., L. K. Weir, L. Bernatchez, M. M. Hansen, and E. B. Taylor. 2011. Extent and scale of local adaptation in salmonid fishes: review and meta-analysis. Heredity **106**:404-420.

Ghalambor, C. K., J. K. McKay, S. P. Carroll, and D. N. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Functional Ecology **21**:394-407.

Gienapp, P., C. Teplitsky, J. S. Alho, J. A. Mills, and J. Merilä. 2008. Climate change and evolution: disentangling environmental and genetic responses. Molecular ecology **17**:167-178.

Glennemeier, K. A., and R. J. Denver. 2002. Developmental changes in interrenal responsiveness in anuran amphibians. Integr Comp Biol **42**:565-573.

Gomez-Mestre, I., and M. Tejado. 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. Evolution **57**:1889-1899.

Gordon, M. S., K. Schmidt-Nielsen, and H. M. Kelly. 1961. Osmotic regulation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **38**:659-678.

Gordon, M. S., and V. A. Tucker. 1965. Osmotic regulation in the tadpoles of the crab-eating frog (*Rana cancrivora)* Journal of Experimental Biology **42**:437-445.

Gordon, M. S., and V. A. Tucker. 1968. Further observations on the physiology of salinity adaptation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **49**:185-193.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica:183-190.

Haramura, T. 2007. Salinity tolerance of eggs of Buergeria japonica (Amphibia, Anura) inhabiting coastal areas. Zoolog Sci **24**:820-823.

Harley, C. D. 2011. Climate change, keystone predation, and biodiversity loss. Science **334**:1124-1127.

Harris, R. N. 1999. The Anuran Tadpole: Evolution and maintenance. Pages 279-294 *in* R. Mcdiarmid and R. Altig, editors. Tadpole. University of Chicago Press, Chicago, IL.

Hendry, A. P. 2015. Key Questions on the Role of Phenotypic Plasticity in Eco-Evolutionary Dynamics. Journal of Heredity:esv060.

Herbert, E. R., P. Boon, A. J. Burgin, S. C. Neubauer, R. B. Franklin, M. Ardón, K. N. Hopfensperger, L. P. Lamers, and P. Gell. 2015. A global perspective on wetland salinization: ecological consequences of a growing threat to freshwater wetlands. Ecosphere **6**:1-43.

Hillyard, S., N. Møbjerg, S. Tanaka, and E. Larsen. 2009a. Osmotic and ion regulation in amphibians. Osmotic and ionic regulation: Cells and animals:367-441.

Hillyard, S. D., N. Møbjerg, S. Tanaka, and E. H. Larsen. 2009b. Osmotic Regulation in Amphibians.*in* D. H. Evans, editor. Osmotic and ionic Regulation. CRC Press, Boca Raton, FL.

Hopkins, G. R., and J. E. D. Brodie. 2015. Occurrence of Amphibians in Saline Habitats: A Review and Evolutionary Perspective. Herpetological Monographs **29**:1-27.

Hota, A. K. 1994. Growth in Amphibians. Gerontology **40**:147-160.

Hsu, W.-T., C.-S. Wu, J.-C. Lai, Y.-K. Chiao, C.-H. Hsu, and Y.-C. Kam. 2012. Salinity acclimation affects survival and metamorphosis of crab-eating frog tadpoles. Herpetologica **68**:14-21.

Istock, C. A. 1967. The evolution of complex life cycle phenomena: an ecological perspective. Evolution **21**:592-605.

Karraker, N. E. 2007. Are embryonic and larval green frogs (Rana clamitans) insensitive to road deicing salt. Herpetological Conservation and Biology **2**:35-41.

Karraker, N. E., and G. R. Ruthig. 2009. Effect of road deicing salt on the susceptibility of amphibian embryos to infection by water molds. Environmental Research **109**:40-45.

Kassambara, A., and M. Kosinski. 2018. survminer: Drawing survival curves using 'ggplot2'. R, R statistical environment.

Kaushal, S. S., G. E. Likens, M. L. Pace, R. M. Utz, S. Haq, J. Gorman, and M. Grese. 2018. Freshwater salinization syndrome on a continental scale. PNAS:p.201711234.

Kearney, B. D., P. G. Byrne, and R. D. Reina. 2012. Larval tolerance to salinity in three species of Australian anuran: an indication of saline specialisation in Litoria aurea. PLOS one **7**:e43427.

Lamichhaney, S., A. M. Barrio, N. Rafati, G. Sundström, C.-J. Rubin, E. R. Gilbert, J. Berglund, A. Wetterbom, L. Laikre, and M. T. Webster. 2012. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. Proceedings of the National Academy of Sciences **109**:19345-19350.

Lamichhaney, S., F. Han, J. Berglund, C. Wang, M. S. Almén, M. T. Webster, B. R. Grant, P. R. Grant, and L. Andersson. 2016. A beak size locus in Darwin's finches facilitated character displacement during a drought. Science **352**:470-474.

Lande, R. 2009. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. Journal of Evolutionary Biology **22**:1435-1446.

Langhans, M., B. Peterson, A. Walker, G. R. Smith, and J. E. Rettig. 2009. Effects of salinity on survivorship of wood frog (Rana sylvatica) tadpoles.

Laurila, A., S. Karttunen, and J. Merilä. 2002. Adaptive phenotypic plasticity and genetics of larval life histories in two Rana temporaria populations. Evolution **56**:617-627.

Levis, N. A., A. Serrato-Capuchina, and D. W. Pfennig. 2017. Genetic accommodation in the wild: evolution of gene expression plasticity during character displacement. Journal of evolutionary biology.

Losos, J. B. 2009. Lizards in an Evolutionary Tree: Ecology and

Adaptive Radiation of Anoles. University of California Press, Berkeley, CA.

Manda, A. K., A. S. Giuliano, and T. R. Allen. 2014. Influence of artificial channels on the source and extent of saline water intrusion in the wind tide dominated wetlands of southern Albemarle estuarine system (USA). Environmental Earth Science **71**:4409-4419.

McNab, B. K. 2002. The physiological ecology of vertebrates: a view from energetics. Cornell University Press.

Merilä, J., and A. P. Hendry. 2014. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. Evolutionary Applications **7**:1-14.

Michener, W. K., E. R. Blood, K. L. Bildstein, M. M. Brinson, and L. R. Gardner. 1997. Climate change, hurricanes and tropical storms, and rising sea level in coastal wetlands. Ecological Applications **7**:770-801.

Montagna, P. A., M. Alber, P. Doering, and M. S. F. Connor. 2002. Freshwater inflow: science, policy, and management. Estuaries:1243-1245.

Moorhead, K. K., and M. M. Brinson. 1995. Responses of wetlands to rising sea level in the lower coastal plain of North Carolina. Ecological Applications **5**:261-271.

Mopper, S., and S. Y. Strauss. 2013. Genetic structure and local adaptation in natural insect populations: effects of ecology, life history, and behavior. Springer Science & Business Media.

Mulligan, R. P., J. P. Walsh, and H. M. Wadman. 2012. Storm surge and surface waves in a large and shallow estuarine system during the passage of a hurricane Journal of Waterway, Port, Coastal, and Ocean Engineering.

Murren, C. J., J. R. Auld, H. Callahan, C. K. Ghalambor, C. A. Handelsman, M. A. Heskel, J. Kingsolver, H. J. Maclean, J. Masel, and H. Maughan. 2015. Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. Heredity.

Neill, W. T. 1958. The occurrence of amphibians and reptiles in saltwater areas, and a bibliography. Bulletin of Marine Science **8**:1-97.

Nicholls, R. J., and A. Cazenave. 2010. Sea-level rise and its impact on coastal zones. Science **328**:1517-1520.

Nonaka, E., R. Svanbäck, X. Thibert-Plante, G. Englund, and Å. Brännström. 2015. Mechanisms by Which Phenotypic Plasticity Affects Adaptive Divergence and Ecological Speciation. The American Naturalist **186**:E126-143.

Nunney, L. 2015. Adapting to a Changing Environment: Modeling the Interaction of Directional Selection and Plasticity. Journal of Heredity.

Parkinson, R. W. 1994. Sea-level rise and the fate of tidal wetlands Journal of Coastal Research **10**:987-989.

Parsons, K. J., M. Concannon, D. Navon, J. Wang, I. Ea, K. Groveas, C. Campbell, and R. C. Albertson. 2016. Foraging environment determines the genetic architecture and evolutionary potential of trophic morphology in cichlid fishes. Molecular ecology **25**:6012-6023.

Pfennig, D. W., M. A. Wund, E. C. Snell-Rood, T. Cruickshank, C. D. Schlichting, and A. P. Moczek. 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends in Ecology & Evolution **25**:459-467.

Pigliucci, M., and C. J. Murren. 2003. Perspective: Genetic assimilation and a possible evolutionary paradox: can macroevolution sometimes be so fast as to pass us by?. Evolution **57**:1455-1464.

Pough, F. H., and S. Kamel. 1984. Post-metamorphic change in activity metabolism of anurans in relation to life history. Oecologia **65**:138-144.

Reid, N. M., D. A. Proestou, B. W. Clark, W. C. Warren, J. K. Colbourne, J. R. Shaw, S. I. Karchner, M. E. Hahn, D. Nacci, M. F. Oleksiak, and D. L. Crawford. 2016. The genomic landscape of rapid repeated evolutionary adaptation to toxic pollution in wild fish. Science **354**:1305-1308.

Relyea, R. A. 2002a. Costs of phenotypic plasticity. The American Naturalist **159**:272-282.

Relyea, R. A. 2002b. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs **72**:77-93.

Renn, S. C. P., and M. E. Schumer. 2013. Genetic accommodation and behavioural evolution: insights from genomic studies. Animal Behavior **85**.

Reznick, D. N., and C. K. Ghalambor. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. Genetica **112**:183-198.

Rios-López, N. 2008. Effects of increased salinity on tadpoles of two anurans from a Caribbean coastal wetland in relation to their natural abundance. Amphibia-Reptilia **29**:7-18.

Sarkar, S. 2004. From the Reaktionsnorm to the evolution of adaptive plasticity. Phenotypic plasticity. Pages 10-30 *in* T. J. DeWitt and S. M. Scheiner, editors. Functional and conceptual approaches. Oxford University Press, NY., New York.

Scheiner, S. M. 1993. Genetics and evolution of phenotypic plasticity. Annual Review of Ecology and Systematics **24**:35-68.

Scheiner, S. M., M. Barfield, and R. D. Holt. 2017. The genetics of phenotypic plasticity: Genetic assimilation, the Baldwin effect, and evolutionary rescue. Ecology and evolution **7**:8788-8803.

Schlichting, C. D. 2004. The role of phenotypic plasticity in diversification. Phenotypic plasticity: functional and conceptual approaches:191-200.

Schuler, M. S., and R. A. Relyea. 2018. A Review of the Combined Threats of Road Salts and Heavy Metals to Freshwater Systems. BioScience **68**:327-335.

Semlitsch, R. D., D. E. Scott, and J. H. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in Ambystoma talpoideum. Ecology **69**:184-192.

Skelly, D. K. 1992. Field evidence for a cost of behavioral antipredator response in a larval amphibian. Ecology:704-708.

Smith, D. C. 1983. Factors controlling tadpole populations of the chorus frog (Pseudacris triseriata) on Isle Royale, Michigan. Ecology:501-510.

Taigen, T. L., and F. H. Pough. 1985. Metabolic correlates of anuran behavior. American Zoologist **25**:987-997.

Team, R. C. 2018. R: A Language and Environment for Statistical Computing.

Therneau, T. 2015. survival: A package for survival analysis in S. R statistical environmnet.

Touchon, J. C., M. W. McCoy, T. Landberg, J. R. Vonesh, and K. M. Warkentin. 2015. Putting μ/g in a new light: plasticity in life history switch points reflects fine-scale adaptive responses. Ecology **96**:2192-2202.

Touchon, J. C., and K. M. Warkentin. 2008. Reproductive mode plasticity: Aquatic and terrestrial oviposition in a treefrog. PNAS **105**:7495-7499.

Travis, J. 1994. Evaluating the adaptive role of morphological plasticity. Ecological Morphology:99-122.

Urban, M. C., G. Bocedi, A. P. Hendry, J.-B. Mihoub, G. Pe'er, A. Singer, J. R. Bridle, L. G. Crozier, L. De Meester, W. Godsoe, A. Gonzalez, J. J. Hellmann, R. D. Holt, A. Huth, K. Johst, C. B. Krug, P. W. Leadley, S. C. F. Palmer, J. H. Pantel, A. Schmitz, P. A. Zollner, and J. M. J. Travis. 2016. Improving the forecast for biodiversity under climate change. Science **353**.

Urban, M. C., J. L. Richardson, and N. A. Reidenfelds. 2014. Plasticity and genetic adaptation mediate amphibian and reptile responses to climate change. Evolutionary Applications **7**:88-103.

Van Buskirk, J., and S. A. McCollum. 2000. Influence of tail shape on tadpole swimming performance. Journal of Experimental Biology **203**:2149-2158.

Via, S., and R. Lande. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution:505-522.

Vonesh, J. R., and K. M. Warkentin. 2006. Opposite shifts in size at metamorphosis in response to larval and metamorph predators. Ecology **87**:556-562.

Waddington, C. H. 1953. Genetic assimilation of an acquired character. Evolution **7**:118-126.

Walworth, N. G., M. D. Lee, F. X. Fu, D. A. Hutchins, and E. A. Webb. 2016. Molecular and physiological evidence of genetic

assimilation to high CO2 in the marine nitrogen fixer Trichodesmium. Proc. Natl. Acad. Sci. **113**:E7367–E7374.

Werner, E. E. 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. The American Naturalist **128**:319-341.

Werner, E. E., and B. R. Anholt. 1996. Predator-induced behavioral indirect effects: consequences to competitive interactions in anuran larvae. Ecology **77**:157-169.

Werner, E. E., and J. F. Gilliam. 1984. The ontogenetic niche and species interactions in size-structured populations. . Annual Review of Ecology and Systematics **15**:393-425.

Werner, E. E., and M. A. McPeek. 1994. Direct and indirect effects of predators on two anuran species along an environmental gradient. Ecology:1368-1382.

Werner, E. E., and S. D. Peacor. 2003. A review of trait-mediated indirect interactions in ecological communities. Ecology **84**:1083-1100.

West-Eberhard, M. J. 2003. Developmental plasticity and evolution. Oxford University Press, Oxford, England.

Whitehead, A., J. L. Roach, S. Zhang, and F. Galvez. 2011. Genomic mechanisms of evoloved physiological plasticity in killifish distributed along an environmental salinity gradient. Proceedings of the National Academy of Science **108**:6193-6198.

Whitman, D. W., and A. A. Agrawal. 2009. What is Phenotypic Plasticity and Why is it important? .1-63.

Wijethunga, U., M. Greenlees, and R. Shine. 2016. Living up to its name? The effect of salinity on development, growth, and phenotype of the "marine" toad (Rhinella marina). Journal of Comparative Physiology B **186**:205-213.

Wilbur, H. M., and J. P. Collins. 1973. Ecological aspects of amphibian metamorphosis: nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. Science **182**:1305-1314.

Williams, K., K. C. Ewel, R. P. Stumpf, F. E. Putz, and T. W. Workman. 1999. Sea-level rise and coastal forest retreat on the west coast of florida, USA Ecology **80**:2045-2063.

Williams, S. J. 2013. Sea Level Rise Implications for Coastal Regions Journal of Coastal Research **63**:184-196.

Wood, S. N. 2011. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. Journal of the Royal Statistical Society **73**:3-36.

Wund, M. A. 2012. Assessing the impacts of phenotypic plasticity on evolution. Integr Comp Biol **52**:5-15.

Wund, M. A., J. A. Baker, B. Clancy, J. L. Golub, and S. A. Foster. 2008. A test of the "flexible stem" model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. American Naturalist **172**.

Figures and tables:

Figure 2-1. Possible phenotypic outcomes given varying influence of plasticity in the adaptive evolution of coastal and inland frog populations in response to saltwater. The egg environment salinity refers to the pool into which eggs are laid and hatched, and the salinity of the tadpole environment is the salinity of the environment into which tadpoles are transferred and spend the majority of development. Panel A describes expected outcomes if phenotypes are produced entirely via environmentally induced phenotypic plasticity. Panel B shows outcomes if plasticity resembling Baldwin's effect drives the adaptive evolution of coastal frog populations. Panel C demonstrates the loss of plasticity expected if genetic assimilation is occurring. Panel D shows expected patterns if both Baldwin's effect and genetic assimilation contribute to adaptive evolution.

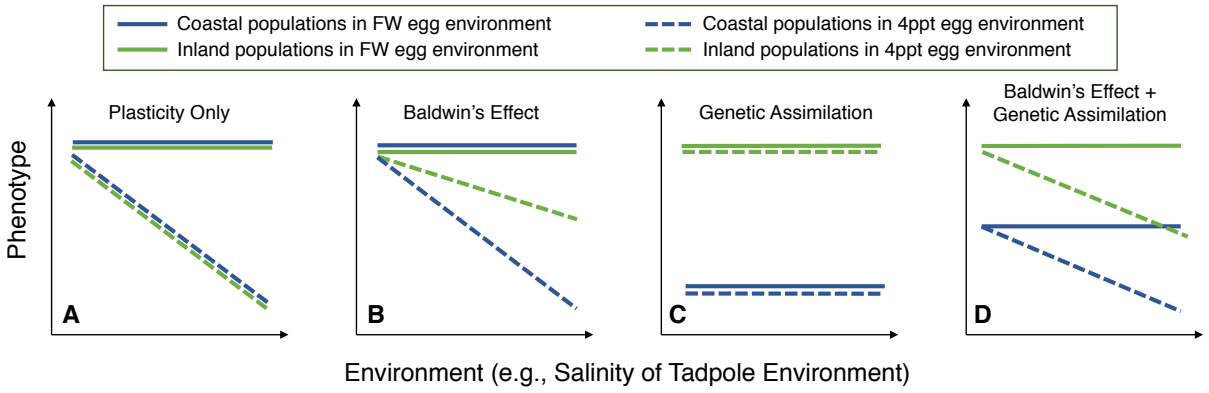


Figure 2-2. Tadpole mass after spending approximately one week in the tadpole environment according to egg environment, tadpole environment, and location. Each panel denotes a different tadpole environment while colors refer to the different egg environments (green = freshwater egg environment, blue = 4ppt egg environment). Error bars represent 95% confidence intervals.

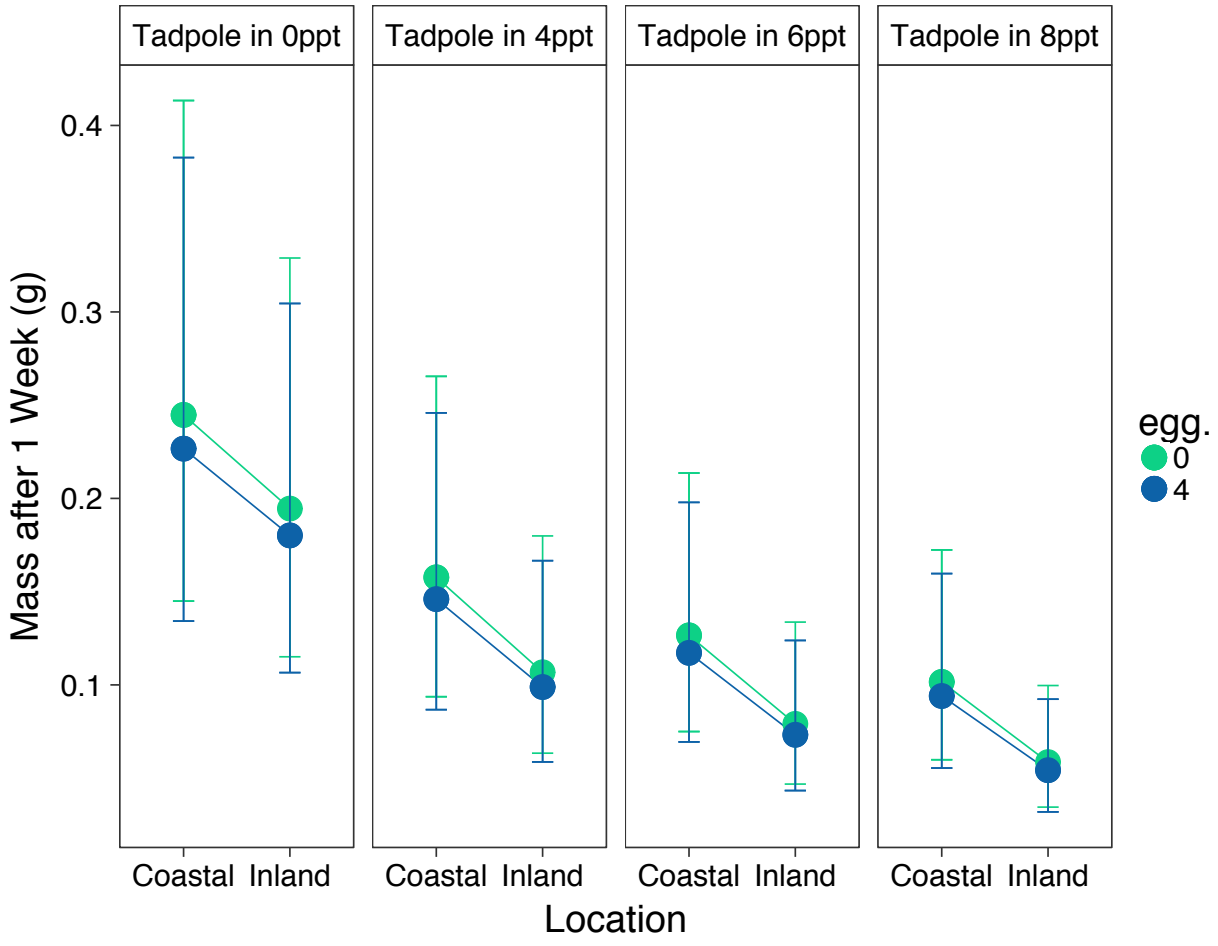


Figure 2-3. Parameter for γ predicted by Gompertz growth model. Larger γ indicates greater size specific growth rates. Each panel refers to a different tadpole environment while colors refer to the different egg environments (green = freshwater egg environment, blue = 4ppt egg environment). Since only coastal individuals survived long enough (see Fig. 2-6) to gather growth rate data from the 8ppt tadpole environment, we excluded all 8ppt tadpole environments from this analysis to avoid bias. Error bars represent 95% confidence intervals.

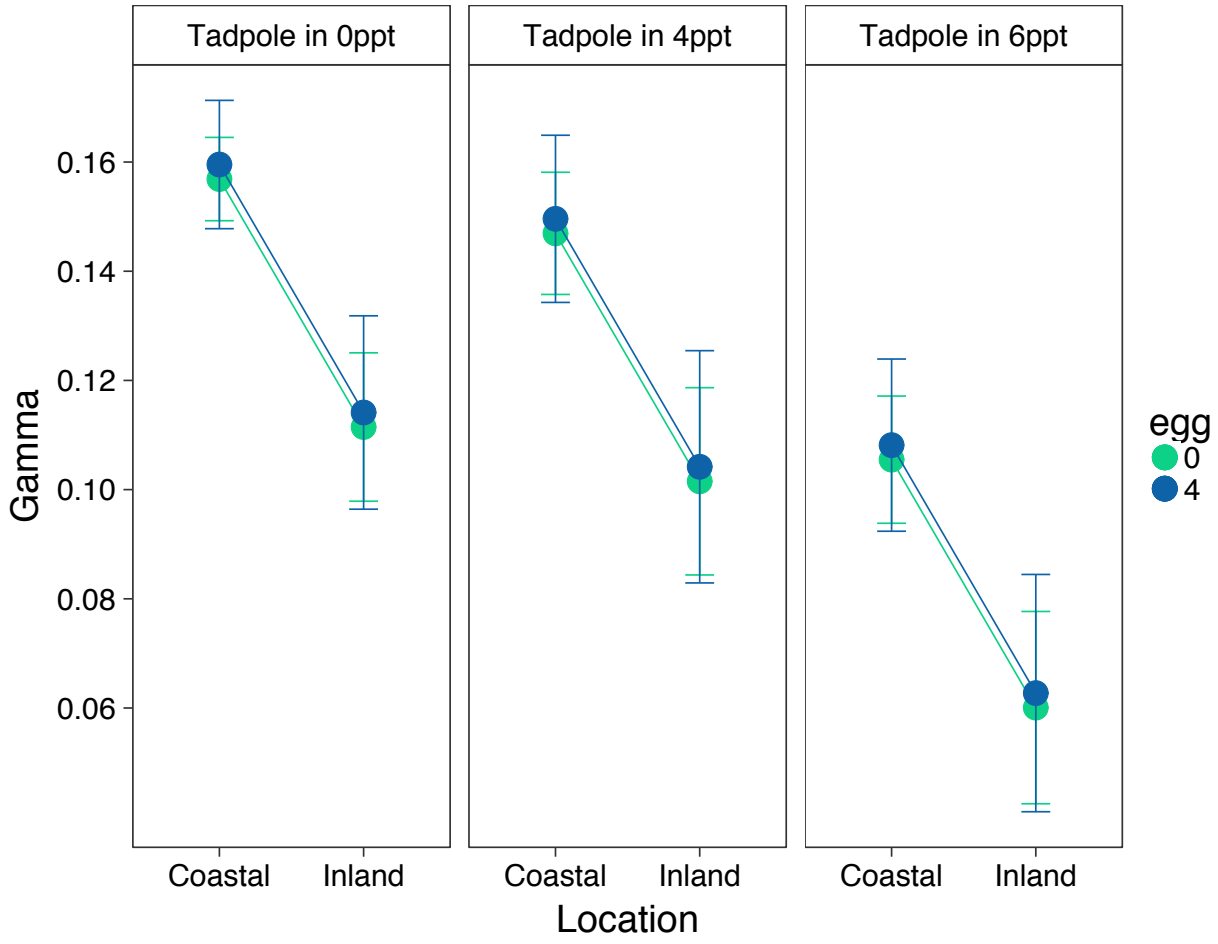


Figure 2-4. Parameter for α predicted by Gompertz growth model. Larger α indicates exponential decay of size specific growth rates, which biologically is associated with a slowed rate of cell division, cell death, or the suspension of growth as cell differentiation occurs. Each panel refers to a different tadpole environment while colors refer to the different egg environments (green = freshwater egg environment, blue = 4ppt egg environment). Since only coastal individuals survived long enough (see Fig. 2-6) to gather growth rate data from the 8ppt tadpole environment, we excluded all 8ppt tadpole environments from this analysis to avoid bias. Error bars represent 95% confidence intervals.

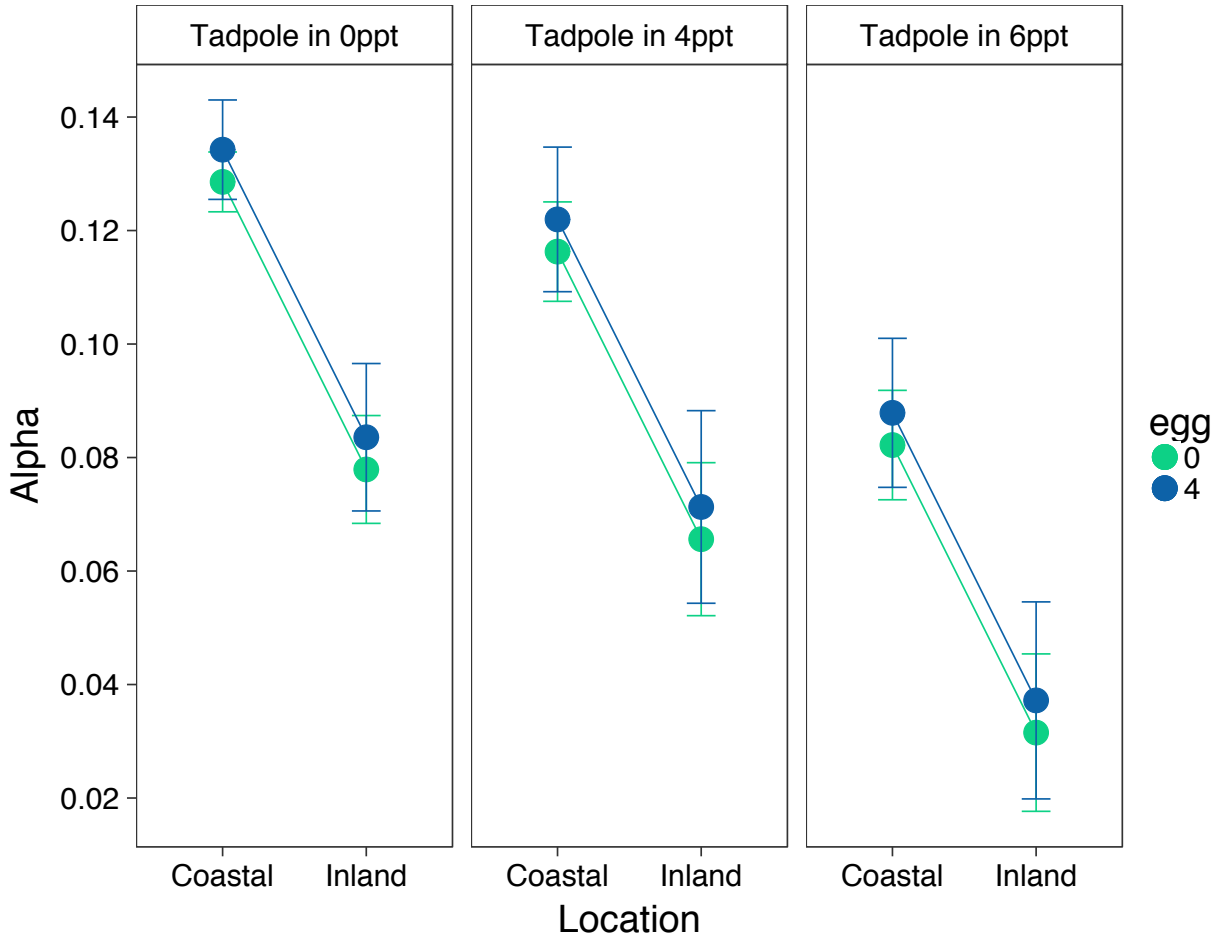


Figure 2-5. The proportion of tadpoles that survived to metamorphosis. Each panel refers to a different tadpole environment while colors refer to the different egg environments (green = freshwater egg environment, blue = 4ppt egg environment). Error bars represent 95% confidence intervals.

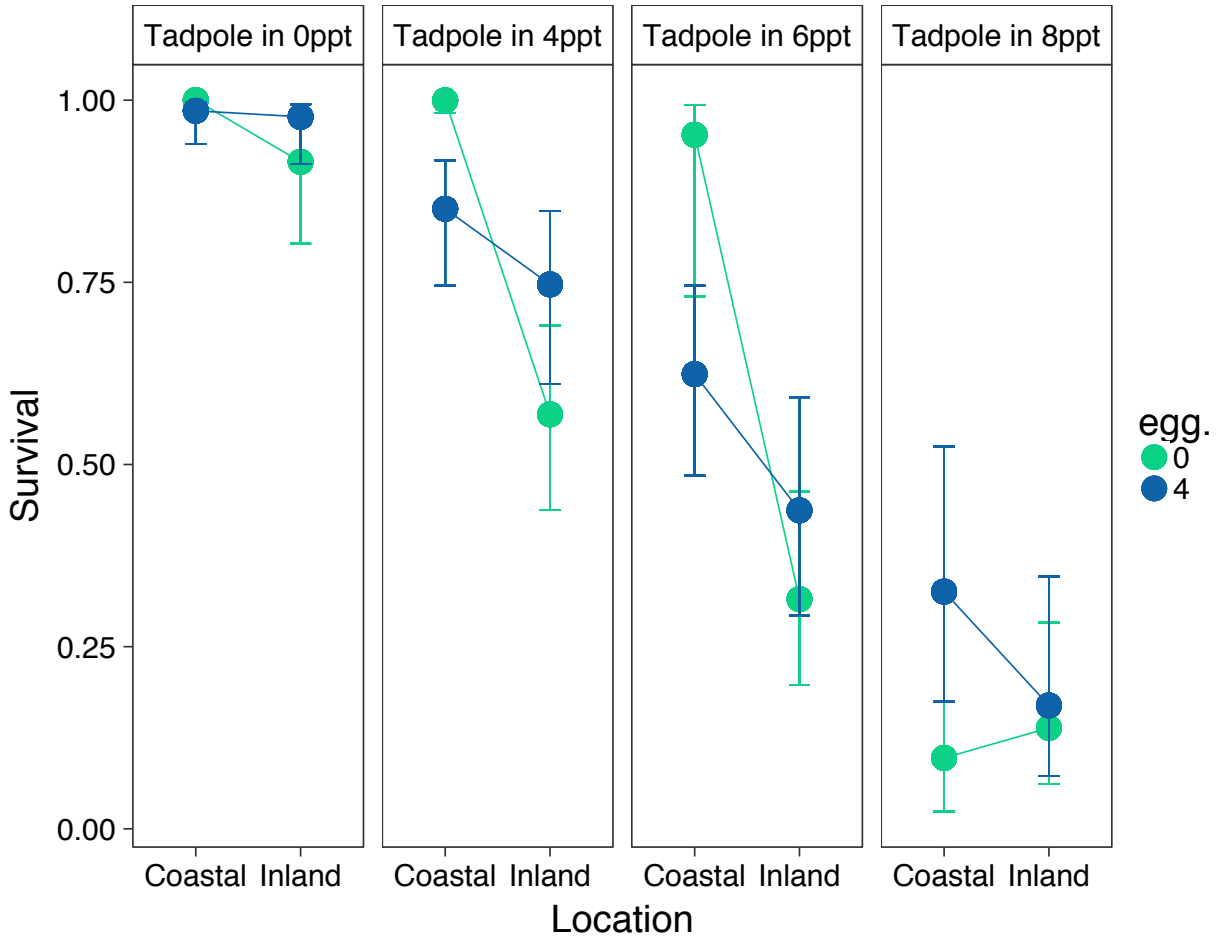


Figure 2-6. Kaplan-Meier survival curves through time (in days) for tadpoles from each location and environment combination. Each panel refers to a different tadpole environment while colors refer to the different egg environments and location (light green = inland tadpoles laid in freshwater egg environment, light blue = coastal tadpoles laid in freshwater egg environment, dark green = inland tadpoles laid in 4ppt egg environment, dark blue = coastal tadpoles laid in 4 ppt egg environment.

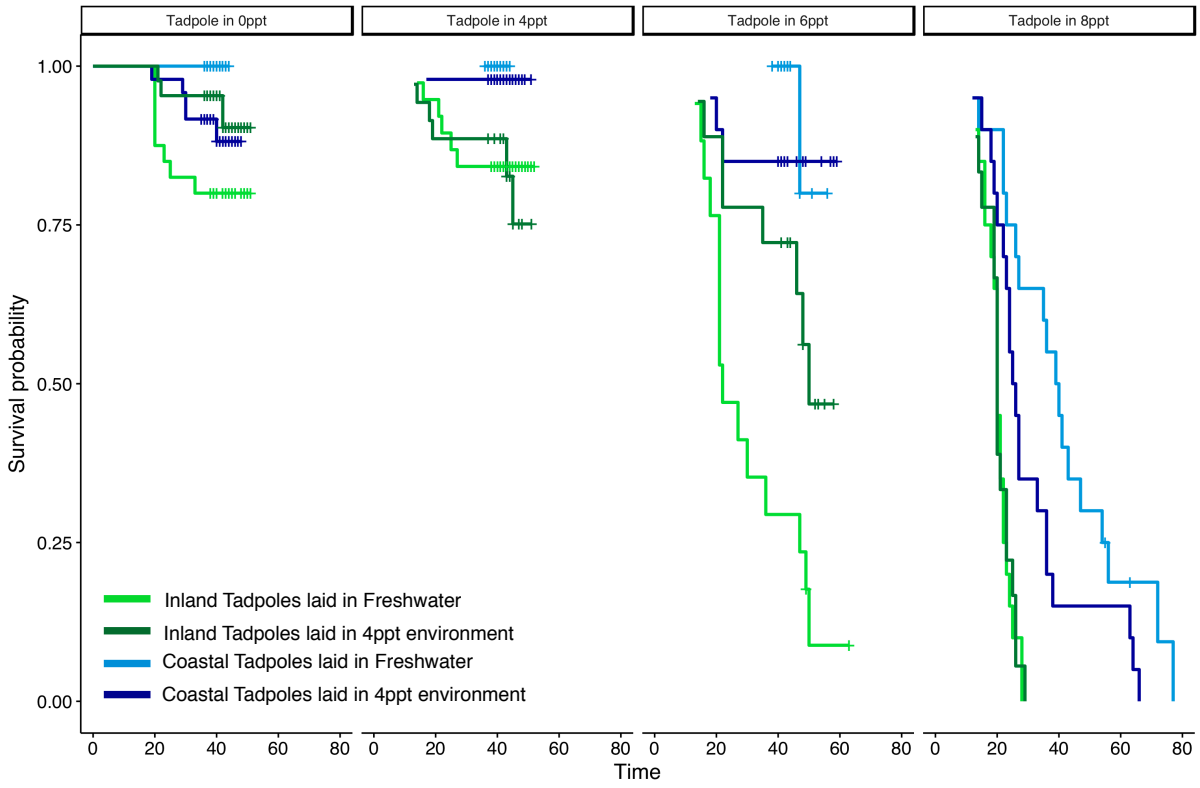


Figure 2-7. The amount of time in days to reach metamorphosis. Each panel denotes a different tadpole environment while colors refer to the different egg environments (green = freshwater egg environment, blue = 4ppt egg environment). Since only 2 coastal individuals metamorphosed from the 8ppt tadpole environment, 8ppt tadpole treatment was excluded from this analysis. Error bars represent 95% confidence intervals.

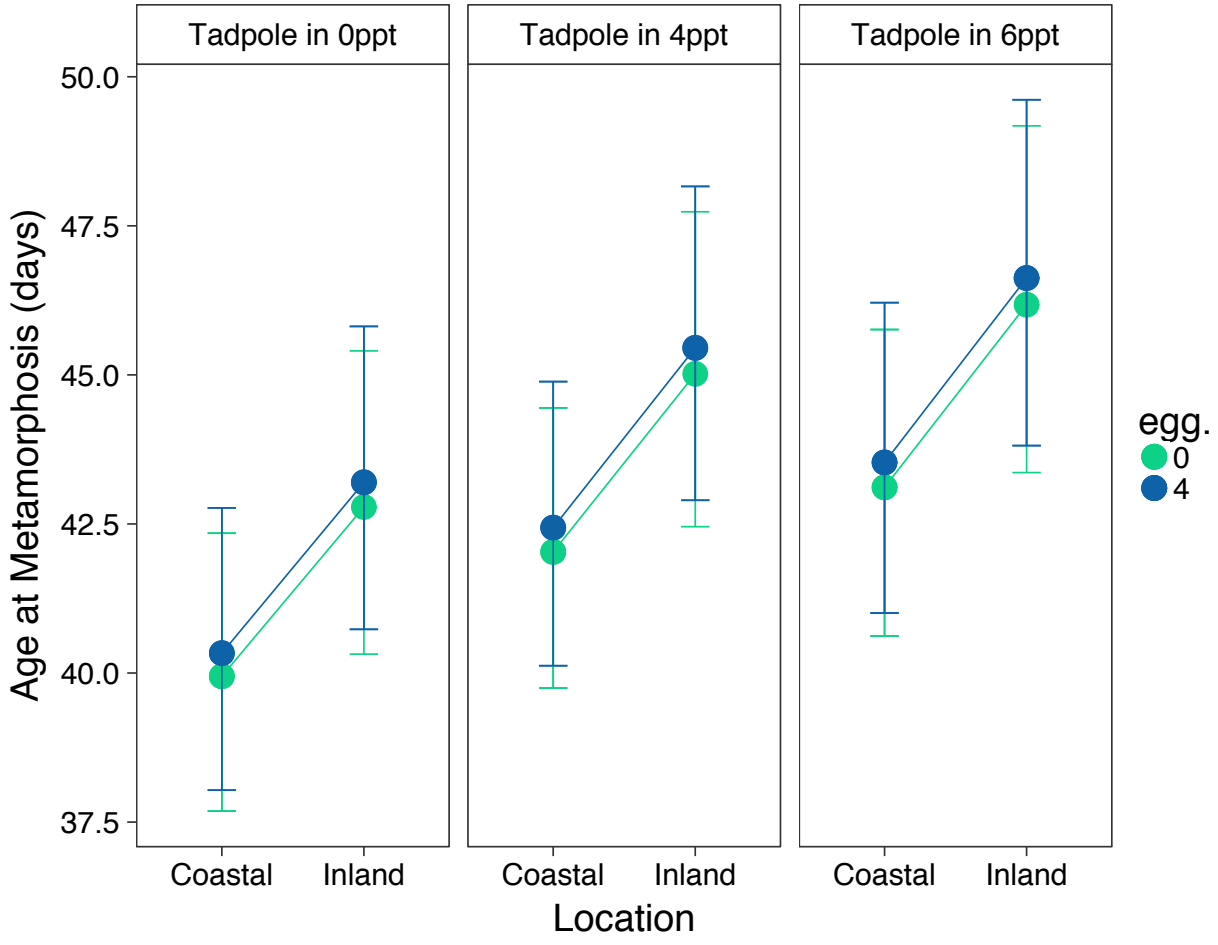


Figure 2-8. Mass at metamorphosis according to egg environment, tadpole environment, and location. Each panel denotes a different tadpole environment while colors refer to the different egg environments (green = freshwater egg environment, blue = 4ppt egg environment). Since only 2 coastal individuals metamorphosed from the 8ppt tadpole environment, 8ppt tadpole treatment was excluded from this analysis. Error bars represent 95% confidence intervals.

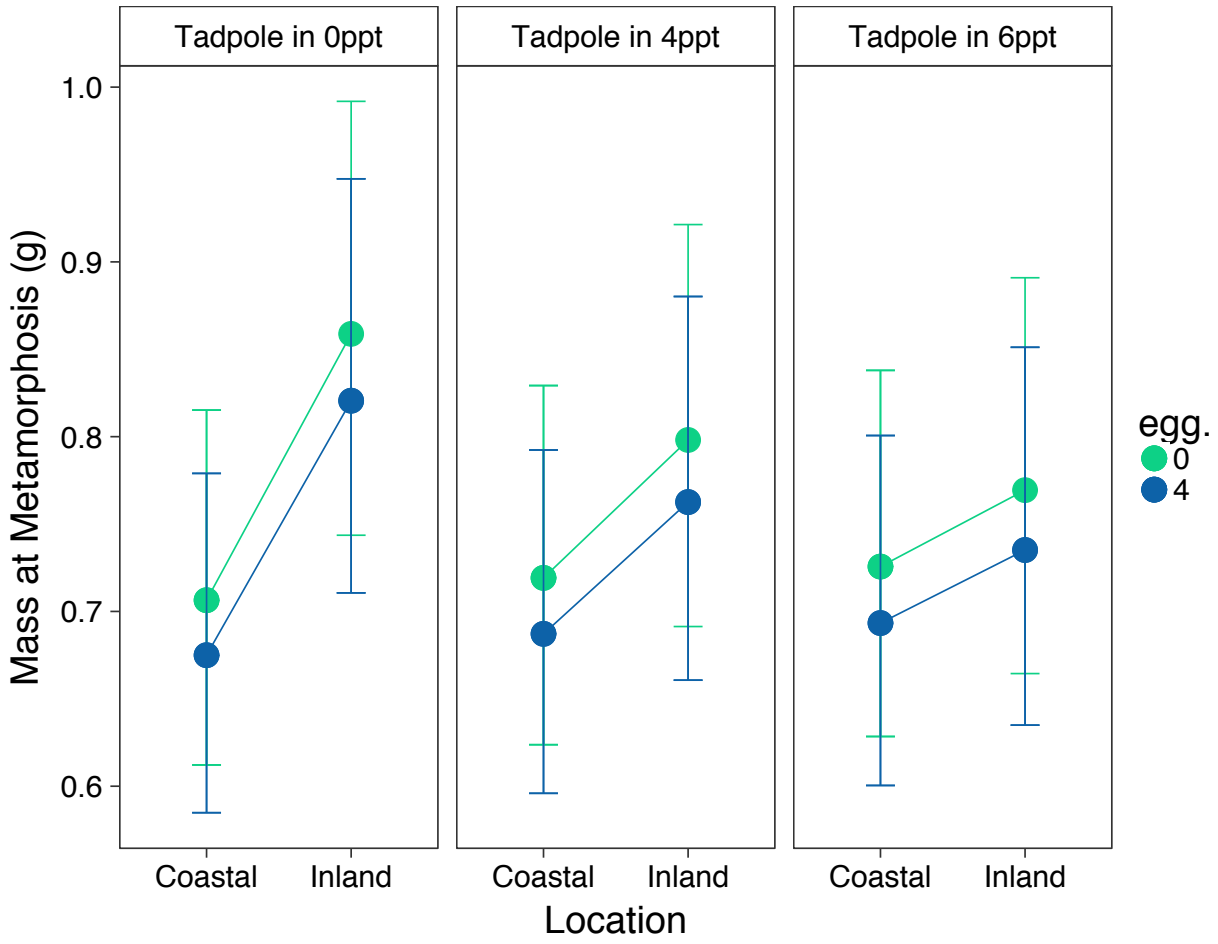


Figure 2-9. Total length (snout to vent in mm) at metamorphosis according to egg environment, tadpole environment, and location. Each panel denotes a different tadpole environment while colors refer to the different egg environments (green = freshwater egg environment, blue = 4ppt egg environment). Since only 2 coastal individuals metamorphosed from the 8ppt tadpole environment, 8ppt tadpole treatment was excluded from this analysis. Error bars represent 95% confidence intervals.

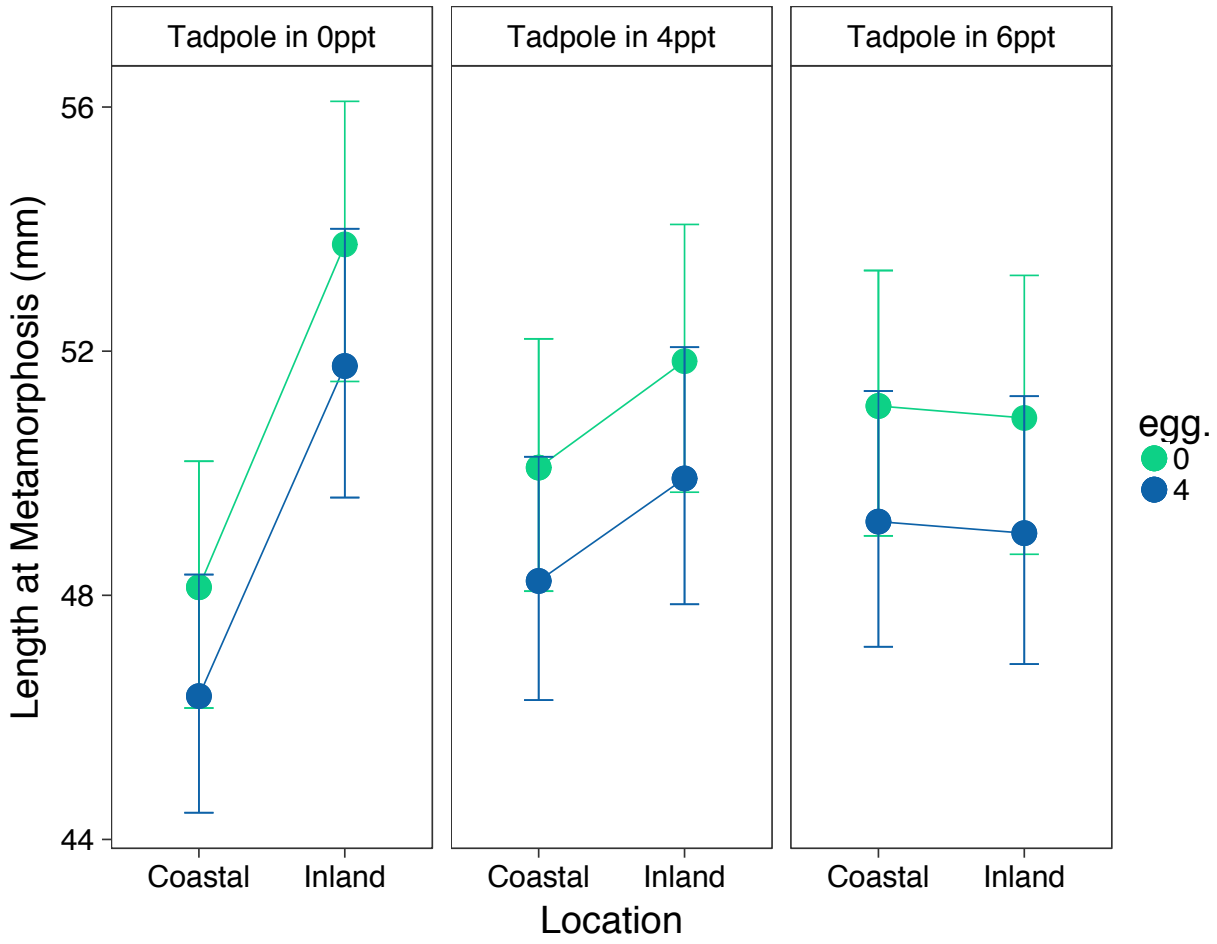


Figure 2-10. Salinity profiles for three coastal wetlands (Bodie Island, New Inlet, and Alligator River National Wildlife Refuge) over the frog breeding season for four years. The different panels denote different sites (top = Bodie Island at Cape Hatteras National Seashore, middle = New Inlet near Rodanthe, NC, bottom = Point Peter Road in Alligator River National Wildlife Refuge). Salinity is shown in partial salinity units (psu), and colors show salinity profiles for each year (blue = 2014, red = 2015, green = 2016, yellow = 2017). Data is missing from 2014 because dataloggers were not installed for the entirety of the breeding season. Alligator River NWR are missing some years because Hurricane Matthew displaced the datalogger.

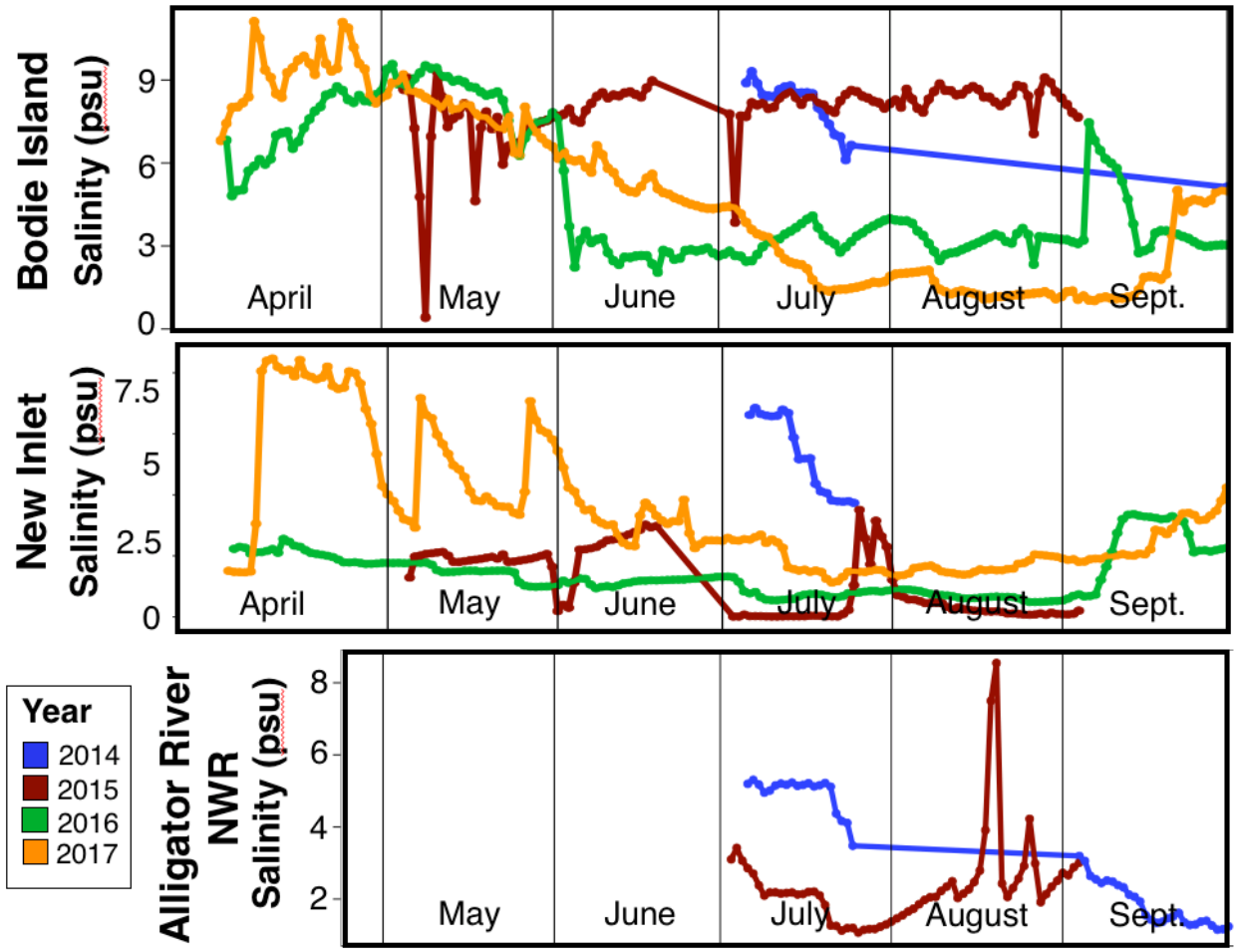


Table 2-1. Basic models used in sample-size corrected Akaike Information Criterion (AICc) model comparisons. These models were used to determine best fit for tests on response variables that include initial size in length, time to metamorphosis, size at metamorphosis (both weight and length), and survival. Because the same models were used for multiple tests, no dAICc, ranks, or weight data are provided here.

Model:	Formula:
Model 1:	Response ~ egg environment * tadpole environment * location + (1|Population)
Model 2:	Response ~ egg environment + tadpole environment * location + (1|Population)
Model 3:	Response ~ egg environment * tadpole environment + location + (1|Population)
Model 4:	Response ~ egg environment + tadpole environment + location + (1|Population)
Model 5:	Response ~ tadpole environment + location + (1|Population)
Model 6:	Response ~ egg environment + location + (1|Population)
Model 7:	Response ~ egg environment + tadpole environment + (1|Population)

Table 2-2. Models and results used in sample-size corrected Akaike Information Criterion (AICc) model comparisons to determine best fit for tests on tadpole length (in mm) based on the Gompertz growth equation. All 32 possible combinations of interactive and additive relationships are shown.

Model	S_o	γ	α	Rank	dAICc	Df	weight
1	Egg * location	Egg * Location * Tadpole	Egg * Location * Tadpole	31	58.3	37	<0.001
2	Egg * location	Egg + Location + Tadpole	Egg * Location * Tadpole	14	13.3	27	<0.001
3	Egg * location	Egg + Location * Tadpole	Egg * Location * Tadpole	20	20.1	30	<0.001
4	Egg * location	Egg * Location + Tadpole	Egg * Location * Tadpole	28	45.3	28	<0.001
5	Egg + location	Egg * Location * Tadpole	Egg * Location * Tadpole	32	60.2	36	<0.001
6	Egg + location	Egg + Location + Tadpole	Egg * Location * Tadpole	27	41.9	26	<0.001
7	Egg + location	Egg + Location * Tadpole	Egg * Location * Tadpole	24	32.3	29	<0.001
8	Egg + location	Egg * Location + Tadpole	Egg * Location * Tadpole	29	46.6	27	<0.001
9	Egg * location	Egg * Location * Tadpole	Egg + Location * Tadpole	15	17.0	30	<0.001
10	Egg * location	Egg + Location + Tadpole	Egg + Location * Tadpole	6	2.4	20	0.0765
11	Egg * location	Egg + Location * Tadpole	Egg + Location * Tadpole	22	25.2	23	<0.001
12	Egg * location	Egg * Location + Tadpole	Egg + Location * Tadpole	17	17.8	21	<0.001
13	Egg + location	Egg * Location * Tadpole	Egg + Location * Tadpole	26	41.5	29	<0.001
14	Egg + location	Egg + Location + Tadpole	Egg + Location * Tadpole	30	47.1	19	<0.001
15	Egg + location	Egg + Location * Tadpole	Egg + Location * Tadpole	23	29.0	22	<0.001
16	Egg + location	Egg * Location + Tadpole	Egg + Location * Tadpole	9	3.3	20	0.0472
17	Egg * location	Egg * Location * Tadpole	Egg * Location + Tadpole	25	39.8	28	<0.001
18	Egg * location	Egg + Location + Tadpole	Egg * Location + Tadpole	18	6.0	18	0.0123
19	Egg * location	Egg + Location * Tadpole	Egg * Location + Tadpole	3	1.3	21	0.128
20	Egg * location	Egg * Location + Tadpole	Egg * Location + Tadpole	11	6.0	19	0.0125
21	Egg + location	Egg * Location * Tadpole	Egg * Location + Tadpole	21	23.1	27	<0.001
22	Egg + location	Egg + Location + Tadpole	Egg * Location + Tadpole	8	2.6	17	0.0689
23	Egg + location	Egg + Location * Tadpole	Egg * Location + Tadpole	7	2.5	20	0.0711
24	Egg + location	Egg * Location + Tadpole	Egg * Location + Tadpole	10	5.1	18	0.0198
25	Egg * location	Egg * Location * Tadpole	Egg + Location + Tadpole	17	19.0	27	<0.001
26	Egg * location	Egg + Location + Tadpole	Egg + Location + Tadpole	4	2.1	17	0.088
27	Egg * location	Egg + Location * Tadpole	Egg + Location + Tadpole	2	1.1	20	0.141
28	Egg * location	Egg * Location + Tadpole	Egg + Location + Tadpole	13	7.0	18	0.0075
29	Egg + location	Egg * Location * Tadpole	Egg + Location + Tadpole	19	19.6	26	<0.001
30	Egg + location	Egg + Location + Tadpole	Egg + Location + Tadpole	1	0	16	0.25
31	Egg + location	Egg + Location * Tadpole	Egg + Location + Tadpole	5	2.4	19	0.0768
32	Egg + location	Egg * Location + Tadpole	Egg + Location + Tadpole	16	17.8	17	<0.001

Table 2-3. Models and results for sample-size corrected Akaike Information Criterion (AICc) model comparison testing conductivity in coastal wetlands through time based on data collected over four years.

Model	Formula	Rank	dAICc	Df	Weight
1	Year * Month * Day of year * Location	2	169.2	38	<0.001
2	Year * Month + Day of year * Location	2	169.2	38	<0.001
3	Year * Month * Day of year + Location	2	169.2	38	<0.001
4	Year + Month * Day of year * Location	1 **	0	25	0.5
5	Year * Month + Day of year + Location	1	0	25	0.5
6	Year + Month + Day of year * Location	3	383.9	15	<0.001
7	Year + Month * Day of year + Location	3	383.9	15	<0.001
8	Year + Month + Day of year + Location	3	383.9	15	<0.001

** Model arbitrarily chosen of two most parsimonious models for likelihood ratio tests

III. DENSITY DEPENDENCE, STAGE-SPECIFIC SELECTION, AND THE GOLDILOCKS EFFECT: EVOLUTIONARY RESCUE IN ORGANISMS WITH COMPLEX LIFE HISTORIES

Abstract

Ecosystems are changing at unprecedented rates, which can reduce individual fitness and population growth rates of affected species. Although rapid evolution may rescue some populations from extirpation, the processes that govern adaptation and the spread of the adaptive alleles through populations have primarily been explored in organisms with simple life cycles. These dynamics may be different for organisms with complex life cycles especially when one or more life stages are independently regulated by density-dependence. This is because selection and density dependence may interact across discrete life stages to affect population dynamics and evolutionary outcomes. We use an agent-based model to explore how stage-specific density dependence and selection interact to influence whether environmental change will cause extinction or evolutionary rescue in organisms with complex life cycles. We show that adults and larvae that experience strong selection pressure and undergo weak or strong density dependence have higher rates of extinction after environmental change. However, at intermediate levels of density dependence and selection, more populations persist. Additionally, the timing of selection and density dependence is also important since strong selection and density dependence can induce extinction or adaptation depending upon which stage they are imposed. The scenario demonstrating the highest potential for evolutionary rescue and least amount of extinction occurred when selection acted on the stage preceding the stage regulated by density-dependent

processes. In general, we found that evolutionary rescue is most likely to occur at intermediate levels of selection and density dependence—a “Goldilocks effect”.

Introduction

Human activities are causing rapid and drastic changes to natural habitats at a global scale. The extreme rate and degree of change have led some researchers to suggest that human activities have changed the geological environment sufficiently to move the Earth from the Holocene epoch into the Anthropocene (Crutzen 2002, Steffen et al. 2011). Environmental changes include increases in rates of climate change, sea level rise, habitat destruction and fragmentation, pollution, spread of invasive species, and disease and epidemics. Each of these changes imposes new selection pressures on organisms causing reductions in individual fitness and population growth rates. Consequentially, population extirpation rates are accelerating and predicted to continue accelerating through time (Thomas et al. 2004, Malcolm et al. 2006, Foden et al. 2013).

To persist, organisms must either migrate into suitable habitats or adapt to the new conditions (Parmesan 2006, Moritz and Agudo 2013, Urban et al. 2016). However, for many organisms migration may be limited by increased habitat fragmentation (Kubisch et al. 2014), and further complicated by lesser-understood dynamics such as maintaining genetic diversity across dispersal clines or competition with established species in the new range (Pearson 2006, Aitken et al. 2008, Nadaeu et al. 2017). Evolutionary adaptation *in situ* may be key for some populations to persist. Evolutionary adaptation can occur over ecological timescales (e.g., within several dozen generations), and in some cases, can rescue maladapted populations from extinction (Gomulkiewicz and Holt 1995, Bell and Gonzalez 2009).

Evolutionary rescue occurs when adaptive alleles that improve fitness and facilitate positive population growth rate in a maladapted population (Carlson et al. 2014, Bell 2017). For example,

after a change in the environment, most individuals may be maladapted causing an initial decline in abundance followed by a period of stabilization and subsequent increase in population abundance as adaptive alleles increase in frequency in the population (Fig. 3-1). However, several interacting factors can affect the probability of evolutionary rescue such as initial population size (Lynch and Lande 1993, Gomulkiewicz and Holt 1995, Bell 2013), genetic variation present within the population (Barrett and Schluter 2008), rates of immigration and emigration (Bolnick and Nosil 2007, Holt 2011), degree of initial maladaptation (Bell and Gonzalez 2009), and the rate and degree of environmental change (Boeye et al. 2013, Scheiner et al. 2017) -- see Carlson et al. (2014) and Bell (2017) for reviews).

Density-dependent population regulation can also influence likelihoods of evolutionary rescue (Gomulkiewicz et al. 1999, Holt et al. 2004b). On the one hand, positive density dependence (e.g., allee effects) coupled with immigration can indirectly improve likelihoods of evolutionary rescue by increasing population size through immigration. Immigration boosts the effective population size, which can enhance population growth via allee effects which ultimately can facilitate the spread of adaptive alleles in recipient populations (Holt et al. 2004b). Negative density dependence, on the other hand, may reduce population sizes to levels that make evolutionary rescue and population persistence increasingly unlikely (Lynch and Lande 1998, Gomulkiewicz et al. 1999). Plasticity, by allowing individuals to induce phenotypes that better match the environment, can buffer individuals from selective pressure which can slow adaptation. However, we propose that under some conditions negative density dependence might also facilitate evolutionary rescue. When population sizes are small, changes in the frequency of adaptive alleles can have greater proportional impact on population level fitness (Gomulkiewicz

and Holt 1995). Because these individuals may be released from density-dependent processes, population growth is unhindered and the distribution of adaptive alleles throughout the population may be enhanced (Vonesh and De la Cruz 2002, Holt and Gomulkiewicz 2004).

Evolutionary rescue can also depend on the life history strategy of organisms. For instance, many organisms have complex life cycles in which they undergo rapid and significant changes in their morphology, physiology, and behavior through ontogeny, which are typically accompanied with changes in habitats (Wilbur 1980). Complex life cycles evolved because they allow organisms to respond to selective pressures in different habitats via ontogenetic habitat shifts (Moran 1994), yet may hinder the ability of taxa to evolve to novel environmental stressors compared to organisms with simpler life cycles (Schluter et al. 1991, Marshall et al. 2016). Because complex life cycles allow for independent adaptation to different selective pressures in each life stage, changes to the environment across life stages may delay adaptation (but see Barfield et al. (2011)).

It is estimated that upwards of 80% of higher-order taxa have a complex life history (Wilbur 1980, Werner and Gilliam 1984), and strong density dependence is pervasive in at least one life history stage for most taxa (Brook and Bradshaw 2006). However, we know little about how evolutionary rescue will be affected when selection and density dependence act independently across discrete life stages. There are three ways that evolutionary responses to environmental change could differ in organisms with complex life cycles in which one or more life stages are regulated by density dependence. First, the environmental change may affect life stages differently. For example, early life stages (free glochidia) of freshwater bivalves have different

exposure to aquatic pollutants at the surface of the water because glochidia are only exposed through brief contact with surface water, while sessile adults can face years of exposure to contaminated surface water (Cope et al. 2008). Second, if all life stages are affected by a common environmental perturbation, each life stage could have different vulnerabilities to the same environmental stressor. For example, adult frogs can survive in saltwater concentrations that are twice the lethal concentration for frog eggs (Albecker and McCoy 2017). Third, density dependence can strongly influence population level dynamics that result from complex life cycle organisms in a changing environment. For example, high egg mortality reduces the number of emerging juveniles but those few eggs that do survive are released from density-dependent mortality in subsequent stages. Thus the number of adults recruited from earlier life stages may go unchanged (Crouse et al. 1987, Vonesh and De la Cruz 2002).

Although the impacts of selection and density dependence have been explored extensively in a theoretical framework in organisms with simple life cycles, less is understood about how both selection and density dependence will interact across life stages in organisms with complex life cycles. To enhance our ability to predict extinction or evolutionary rescue for organisms with complex life cycles, we must understand how the strength of selection and density dependence that occur across different life stages affect adaptation and persistence following an environmental change. Here, we investigate evolutionary rescue for organisms with complex life cycles and density-dependent population regulation using an individual based modeling approach with the goal of understanding how the impacts of selection and density dependence affect extinction and evolution when they occur in different life stages.

The Model

We base our model on a four-stage amphibian life cycle that is representative of many organisms with distinct egg, larval, juvenile, and adult life stages (Fig. 2). In our model, the number of eggs is a function of adult fecundity (clutch size) and the number of breeding pairs. The model proceeds in annual time steps during which the eggs hatch into the larval stage, which emerge as juveniles, eventually becoming full adults, at which point breeding and egg laying can occur to begin the next annual cycle and generation (i.e. our model assumes an annual, semelparous species). Each individual has a transition probability (σ) that they will survive to the next stage of the life cycle, which can be affected by either selection or density dependence (Fig. 2).

Density-dependent Regulation -- Density dependence can occur across stages (e.g., large egg clutches can reduce hatching due to lack of available oxygen, high densities of adults may facilitate breeding opportunities, etc.). However, it is a common characteristic of many organisms with complex life cycles (e.g., amphibians, invertebrates) to produce many eggs that subsequently experience high larval mortality rates due in part to density-dependent processes. Therefore, we incorporate density dependence into the larval stage by calculating the proportion of larvae that survive through the larval stage (σ_t) as a function of larval density using a classic recruitment function (Beverton and Holt 1957):

$$\sigma_t = \frac{\sigma_{tmax}}{(1+dN)^{\gamma}} \qquad \text{(Equation 1)}$$

where larval survival is a function of σ_{tmax} which sets the maximum larval survival, the density-dependent coefficient, *d*, which functions primarily as a scaling term (m^2/larvae), the initial density of larvae (*N*), and the density dependence exponent, γ, which determines the strength of density dependence. For $\gamma = 0$, larval survival would be density independent, while γ between 0

and 1 indicates weak density dependence. Compensation occurs when γ approaches unity, and any γ value over 1 indicates over compensation via strong density-dependent population regulation.

Imposing Selection -- To model selection for traits in any given life stage, we use a standard Gaussian fitness function (Scheiner 2014):

$$\omega_{ij} = e^{-\left(\frac{(\theta_i - \theta_{opt})}{\rho}\right)^2} \qquad \text{(Equation 2)}$$

In our model, fitness (ω) is the probability of survival to the next stage for a given phenotype for each individual (i) in every environment (j). An individual's fitness is the difference between an individual's phenotype (θ_i) and the optimum phenotype (θ_{opt}) for a particular environment scaled by the level of selection (ρ) (Scheiner 2014). In this equation, the strength of selection is the inverse of ρ.

For each individual, we assume a 10-allele genotype, in which the value for each allele position in the founding population is randomly selected from a uniform distribution and could take any integer value between -2 and 2. The individual "phenotype" is the arithmetic mean of all 10 positions, thus the initial cohort has an average phenotype near the optimum value of zero. For every generation we randomly assign a "sex" and select pairs from the surviving adults to mate. The allelic value for each of the 10 allele positions is then randomly selected from one of the parents (heredity = 50%). We imposed a mutation probability of 5% at each allele position per generation (Scheiner and Holt 2012, Scheiner 2014). When an allele mutated, a value was randomly selected from a normal distribution with a mean of zero and standard deviation of 0.1 and added to the original allele value.

Simulating Environmental Change -- The founding populations in our model have phenotypes close to the optimum value of zero, which are maintained by moderate stabilizing selection (ρ=1.2) for 200 generations. This simulates a scenario in which populations are relatively well suited to the current environment. During these 200 generations, we impose 6 different magnitudes of negative density-dependent regulation on the number of larvae surviving to the juvenile stage. These ranged from weak (γ = 0.6), to strong (γ =1.6) density dependence. These values are based on a range of density dependence estimates calculated from amphibian populations in nature (Vonesh and De la Cruz 2002).

After 200 generations, we abruptly change to the environment by shifting the optimum (θ_{opt}) phenotype from zero to two, which effectively imposes strong maladaptation on populations. Changing the optimum phenotype value reflects a scenario commonly observed in species undergoing an abrupt environmental change, but it should be noted that an abrupt change will likely lead to different evolutionary outcomes compared to more gradual environmental shifts (Scheiner et al. 2017). Following the change in the environment, we explore the consequences of six different magnitudes of selection (ρ, equation 2) crossed with each of the six levels of density dependence. We change the levels of selection to reflect that some populations and life stages may be more or less vulnerable to a changing environment. Simulations were continued for 400 generations (total of 600 generations) or until the population went extinct. We conducted a total of 36 combinations of selection (n = 6) and density dependence (n = 6) across each life stage scenario (e.g., egg, larval, adult, egg + larval). Each scenario was replicated 1200 times and consider each simulation as an independent "population".

Results and Discussion

Influence of density dependence -- Density dependence and selection interact to produce different and emergent evolutionary outcomes dependent upon which life stage is affected as well as the strength of selection and density dependence. Across all life stages, density dependence influences the genetic variation of phenotypes across populations before the environmental change (i.e., within the first 200 generations). In our model, weak density dependence maintains the starting phenotypic distribution and variation across populations, while increased density dependence increases the variation in phenotypes across populations via drift (Fig. 3). Weak density dependence likely maintains the original distribution of phenotypes around the optimum because few individuals are removed from the population as a result of density-dependent regulation. As a result, there are no significant changes to genetic diversity across populations, which on average remains near the optimum.

Strong density dependence (e.g., γ greater or equal to 1) maintains small populations sizes such that genetic drift leads to increasing amounts of genetic variation among populations over time (Fig. 3, panels B, C). This pattern is illustrated by the inverse relationship between genetic variance among populations and population size (Fig. 4). Because density dependence is reducing population size via random filtering and because variation in allele frequencies is occurring independent of selection, some populations will by random chance drift towards average phenotypes that are predisposed to the new conditions that may follow in the aftermath of environmental change. For example, some populations may drift towards a phenotypic average that is closer to the future optimum phenotype. However, other populations may drift

toward average phenotypes that are maladapted to future optima. Consequently, density-dependent regulation may predispose certain populations to adaptation or extinction before environmental change by random chance alone (Lynch and Lande 1993, Burger and Lynch 1995).

Most research has focused on the role of genetic variation *within* populations and has found that sufficient background genetic variation and relatively large population sizes are required for population persistence following an abrupt environmental shift (Lande and Shannon 1996, Lynch and Lande 1998, Gomulkiewicz et al. 1999, Bolnick and Nosil 2007). Indeed, genetic variation is often the raw material that selection acts upon that allows for rapid adaptation to occur (Bürger 1999, Barrett and Schluter 2008). However, our simulations show increased genetic variation *across* populations as the result of density-dependent population regulation. Increased genetic variation across populations could possibly function as a genetic form of the spatial insurance hypothesis, which proposes that biological diversity across a heterogenous landscape can protect overall ecosystem function and stability by maintaining sufficient diversity that can disperse into open niche space and functionally compensate for any species losses (Loreau et al. 2003). The outcome of spatial insurance hypothesis depends on the rates of dispersal among habitats, as intermediate levels of dispersal optimally increase productivity and reduce variability (Loreau et al. 2003). Because density-dependent regulation increases genetic variation across populations, meta-populations that are connected by dispersal may be buffered against extinction as dispersing individuals continuously introduce necessary genetic diversity into populations that are experiencing a genetic bottleneck (e.g., genetic rescue) (Gomulkiewicz et al. 1999, Bolnick and Nosil 2007). If so, immigration and subsequent gene flow should behave similarly as the

spatial insurance hypothesis since previous theoretical work has established that moderate amounts of immigration have the optimal impact on the population's ability to evolve. Specifically, low rates of immigration may not supply sufficient genetic variation, but too much immigration may continuously introduce maladapted alleles into the population and reduce the ability of adaptive alleles to propagate (Gomulkiewicz et al. 1999, Holt et al. 2004b). Previous empirical work supports the theory that adaptation in a meta-population is indeed contingent upon both the rates of dispersal as well as the level of environmental deterioration (Bell and Gonzalez 2011). In our model, we consider each simulation as an independent population and do not connect populations or allow for immigration, but future theoretical work may explore the hypothesis that increasing the diversity of the regional gene pool through density-dependent processes will increase the probability of adapted alleles immigrating into populations experiencing maladaptation or genetic bottlenecks.

Selection at the Egg Stage -- We found that when selection is imposed at a stage preceding the stage experiencing density-dependent regulation, populations were less likely to be extirpated, and evolutionary rescue occurred more frequently for all levels of selection strengths (Figs. 3-5, panels A,B,C & 3-6, panel on egg selection). We propose that this pattern emerges when selection is imposed during the egg stage, because egg survivorship is significantly reduced and only the individuals with adapted phenotypes are likely to hatch. These adapted cohorts are then largely released from the deleterious effects of density-dependent processes during the larval stage, thus permitting a larger proportion of individuals with adapted phenotypes and higher fitness potential to survive to become reproductive adults (Crouse et al. 1987, Vonesh and De la Cruz 2002). Because individuals that hatch are more likely to have adapted genotypes that more

closely match the new environmental optimum, the rate in which adaptive alleles spread through the population is hastened and so these populations experience rapid adaptation and increased rates of evolutionary rescue.

Importantly, the strength of selection on the egg stage affects rates of recovery in similar ways as selection on the tadpole and adult stages. When strong selection occurs in the egg stage, fitness rebounds quickly, compensating for any lost fitness prior to the environmental change from density dependence (Gomulkiewicz and Holt 1995). But at weaker levels of selection, fitness gradually increases but does not always fully recover to initial fitness levels.

Selection at larval, adult, and at multi-stages -- We observed similar outcomes across all three-life stage-scenarios (selection imposed at the larval, adult, and egg + larval stages). Both weak and strong density dependence led to rapid, complete extinctions following the environmental change. We propose two hypotheses for why density dependence coupled with strong selection is driving rapid extinctions. First, it could be that strong density dependence kept population sizes below the critical minima needed for recovery. Further, while density dependence increased the amount of genetic variation among populations, over half of the populations were predisposed to extinction because they drifted toward maladaptation prior to environmental change. When these two factors were coupled with an abrupt change in the environment along with an increase in the strength of selection, the combined effects rapidly lowered populations below the critical abundance needed to recover and persist in the new environmental conditions. Indeed, consistent with other studies, we found that population size at the time of environmental change is a key factor influencing evolutionary rescue (Lynch and Lande 1993, 1998, Bell 2013), and declines in

abundance below a threshold critical value (sometimes referred to as the stochastic threshold – Fig. 1) can put populations at increased risk of extinction due to random chance alone (Gomulkiewicz and Holt 1995, Gomulkiewicz et al. 1999). A second hypothesis is that populations experiencing strong selection and strong density dependence did respond to selection, yet strong density dependence randomly removed such a high proportion of individuals with high fitness potential (individuals with genotypes near the new optimum) that the ability of those populations to respond adaptively was reduced (Gomulkiewicz et al. 1999, Chevin and Lande 2010).

Interestingly, we also observed extinctions in weak density dependence scenarios under strong selection. Weak density dependence maintained large population sizes and stabilizing selection kept the variation of phenotypes across populations near the initial phenotypic optimum. By extension, phenotypes near the tails of the original distribution (i.e. near the new, shifted optimum) were rare at the moment of environmental change. As a result, the lack of genetic diversity may have ultimately driven those populations to extinction. This result corroborates findings across a variety of theoretical and empirical studies that report that mutation alone is typically insufficient to provide necessary genetic variation to keep pace with rapid environmental change (Bell and Gonzalez 2009, Futuyma 2010), so in the absence of immigration, populations that persist through abrupt changes to the environment must have sufficient genetic variation already present prior to the change to buffer against extinction (Hoffmann and Sgrò 2011). This suggests that genetic variation may be more important than population size in determining population persistence, although several studies have implicated

that both population size and genetic variation are key for persistence (Lynch and Lande 1993, Gomulkiewicz and Holt 1995, Lande and Shannon 1996, Barrett and Schluter 2008, Bell 2013).

When strong selection is imposed at the larval, adult, or across multiple life stages, we find that it is just the intermediate levels of density dependence that persist and successfully recover high fitness, showing the U-shaped pattern in population trajectories over times that is indicative of evolutionary rescue (Fig. 1 & 5). This is one of the most compelling effects of density dependence identified in our model and suggests that moderate density dependence allows for population persistence and evolution through environmental change.

As expected, when selection was weak we observed very few population extirpations across all life stages and levels of density dependence (Fig. 5). However, the distribution of phenotypes and associated fitness of populations was affected by density dependence prior to the environmental change, and the subsequent rates of fitness recovery were reduced under weak selection. For example, the average phenotype of populations with strong density dependence was pushed farther from the optimum which conferred lower average fitness in those populations (Fig. 5). When weak selection was imposed after the 200th generation, the recovery rate was much lower although all populations began showing signs of positive population growth and improved fitness by the 600th generation (Fig. 5). The slowed pace of recovery likely stems from weaker selection which allowed more maladapted phenotypes to persist which can dull rates of evolutionary rescue (Lynch and Lande 1993, Lande and Shannon 1996, Holt 2003, Holt and Gomulkiewicz 2004, Lande 2009, Barfield et al. 2011, Holt 2011).

Conclusions -- Collectively, we show that density dependence and selection have significant, but predictable impacts on the evolution and extinction of complex life cycle organisms that experience varying levels of selection and density-dependence across stages. In general, patterns that emerge from our model suggest that a "Goldilock's principle" may govern outcomes following environmental change. Goldilock's principle, based on the children's fable, describes the idea that there is an optimal amount of a given entity, and deviations on either end of that optimum will have negative consequences. For adults and larvae that endure strong selection pressure, strong density dependence causes high extinction following an environmental change, as does weak density dependence. Additionally, the timing of selection and density dependence are important, since strong selection and density dependence can induce extinction or adaptation, depending when each pressure is applied. The scenario which demonstrated the highest amount of evolutionary rescue and lowest amount of extinction was when selection occurred during the stage that preceded density-dependence, but in general, the most favorable evolutionary outcomes occur at intermediate levels of selection and density dependence—a Goldilocks effect of density dependence and strength of selection.

Literature Cited

Aitken, S. N., S. Yeaman, J. A. Holliday, T. Wang, and S. Curtis-McLane. 2008. Adaptation, migration or extirpation: climate change outcomes for tree populations. Evolutionary Applications **1**:95-111.

Albecker, M. A., and M. W. McCoy. 2017. Adaptive responses to salinity stress across multiple life stages in anuran amphibians. Frontiers in Zoology **14**:40.

Barfield, M., R. D. Holt, and R. Gomulkiewicz. 2011. Evolution in Stage-structured Populations. The American Naturalist **177**:397-409.

Barrett, R. D. H., and D. Schluter. 2008. Adaptation from standing genetic variation. Trends in Ecology & Evolution **23**:38-44.

Bell, G. 2013. Evolutionary rescue and the limits of adaptation. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**:20120080.

Bell, G. 2017. Evolutionary Rescue. Annual Review of Ecology, Evolution and Systematics **48**:605-627.

Bell, G., and A. Gonzalez. 2009. Evolutionary rescue can prevent extinction following environmental change. Ecology Letters **12**:942-948.

Bell, G., and A. Gonzalez. 2011. Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration. Science **332**:1327-1330.

Beverton, R. J. H., and S. J. Holt. 1957. On the dynamics of exploited fish populations. Fishery Investigations Series II, Vol. XIX, Ministry of Agriculture. Fisheries and Food **1**:957.

Boeye, J., J. M. Travis, R. Stoks, and D. Bonte. 2013. More rapid climate change promotes evolutionary rescue through selection for increased dispersal distance. Evolutionary Applications **6**:353-364.

Bolnick, D. I., and P. Nosil. 2007. Natural selection in populations subject to migration load. Evolution **61**:2229-2243.

Brook, B. W., and C. J. Bradshaw. 2006. Strength of Evidence for Density Dependence in Abundance Time Series of 1198 Species. Ecology **87**:1445-1451.

Bürger, R. 1999. Evolution of genetic variability and the advantage of sex and recombination in changing environments. Genetics **153**:1055-1069.

Burger, R., and M. Lynch. 1995. Evolution and extinction in a changing environment: a quantitative-genetic analysis. Evolution:151-163.

Carlson, S. M., C. J. Cunningham, and P. A. H. Westley. 2014. Evolutionary rescue in a changing world. Trends in Ecology & Evolution **29**:521-530.

Chevin, L. M., and R. Lande. 2010. When do adaptive plasticity and genetic evolution prevent extinction of a density-regulated population? . Evolution **64**:1143-1150.

Cope, W. G., R. B. Bringolf, D. B. Buchwalter, T. J. Newton, C. G. Ingersoll, N. Wang, T. Augspurger, F. J. Dwyer, M. C. Barnhart, R. J. Neves, and E. Hammer. 2008. Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. Journal of the North American Benthological Society **27**:451-462.

Crouse, D. T., L. B. Crowder, and H. Caswell. 1987. A stage-based population model for Loggerhead Sea Turtles and implications for conservation. Ecology **68**:1412-1423.

Crutzen, P. J. 2002. Geology of mankind. Nature **415**:23.

Foden, W. B., S. H. Butchart, S. N. Stuart, J. C. Vié, H. R. Akçakaya, A. Angulo, L. M. DeVantier, A. Gutsche, E. Turak, L. Cao, and S. D. Donner. 2013. Identifying the world's most climate change vulnerable species: a systematic trait-based assessment of all birds, amphibians and corals. PLOS one **8**:p.e65427.

Futuyma, D. J. 2010. Evolutionary constraint and ecological consequences. Evolution **64**:1865-1884.

Gomulkiewicz, R., and R. D. Holt. 1995. When does evolution by natural selection prevent extinction? . Evolution **49**:201-207.

Gomulkiewicz, R., R. D. Holt, and M. Barfield. 1999. The effects of density dependence and immigration on local adaptation and niche evolution in a black-hole sink environment. Theoretical population biology **55**:283-296.

Hoffmann, A. A., and C. M. Sgrò. 2011. Climate change and evolutionary adaptation. Nature **470**:479-485.

Holt, R., and R. Gomulkiewicz. 2004. Conservation implications of niche conservatism and evolution in heterogeneous environments. Pages 244-264 Evolutionary Conservation Biology. Cambridge University Press.

Holt, R. D. 2003. On the evolutionary ecology of species' ranges. Evolutionary ecology research **5**:159-178.

Holt, R. D. 2011. Evolution in source-sink environments: implications for niche conservatism. . Pages 23-57 Sources, Sinks and Sustainability. Cambridge University Press, Cambridge, UK.

Holt, R. D., T. M. Knight, and M. Barfield. 2004. Allee effects, immigration, and the evolution of species' niches. The American Naturalist **163**:253-262.

Kubisch, A., R. D. Holt, H.-J. Poethke, and E. A. Fronhofer. 2014. Where am I and why? Synthesizing range biology and the eco-evolutionary dynamics of dispersal. Oikos **123**:5-22.

Lande, R. 2009. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. Journal of Evolutionary Biology **22**:1435-1446.

Lande, R., and S. Shannon. 1996. The role of genetic variation in adaptation and population persistence in a changing environment. Evolution **50**:434-437.

Loreau, M., N. Mouquet, and A. Gonzalez. 2003. Biodiversity as spatial insurance in heterogeneous landscapes. . Proceedings of the National Academy of Sciences **100**:12765-12770.

Lynch, M., and R. Lande. 1993. Evolution and Extinction in response to environmental change. Pages 234-250. *in* P. M. Kareiva, editor. Biotic interactions and global change. Sinauer Associates.

Lynch, M., and R. Lande. 1998. The critical effective size for a genetically secure population. . Animal Conservation **1**:70-72.

Malcolm, J. R., C. Liu, R. P. Neilson, L. Hansen, and L. E. E. Hannah. 2006. Global warming and extinctions of endemic species from biodiversity hotspots. Conservation BIology **20**:538-548.

Marshall, D. J., S. C. Burgess, and T. Connallon. 2016. Global change, life-history complexity and the potential for evolutionary rescue. Evolutionary Applications **9**:1189-1201.

Moran, N. A. 1994. Adaptation and constraint in the complex life cycles of animals. Annual Review of Ecology and Systematics **25**:573-600.

Moritz, C., and R. Agudo. 2013. The future of species under climate change: resilience or decline? Science **341**:504-508.

Nadaeu, C. P., M. C. Urban, and J. R. Bridle. 2017. Climates Past, Present, and Yet-to-come shape climate change vulnerabilities. Trends in Ecology & Evolution **32**:786-800.

Parmesan, C. 2006. Ecological and evolutionary responses to recent climate change. Annual Review of Ecology, Evolution, and Systematics:637-669.

Pearson, R. G. 2006. Climate change and the migration capacity of species. Trends in Ecology & Evolution **21**:111-113.

Scheiner, S. M. 2014. Bet-hedging as a complex interaction among developmental instability, environmental heterogeneity, dispersal, and life-history strategy. . Ecology and evolution **4**:505-515.

Scheiner, S. M., M. Barfield, and R. D. Holt. 2017. The genetics of phenotypic plasticity: Genetic assimilation, the Baldwin effect, and evolutionary rescue. Ecology and evolution **7**:8788-8803.

Schluter, D., T. D. Price, and L. Rowe. 1991. Conflicting serlection pressures and life history trade-offs. Proceedings of the Royal Society of London B: Biological Sciences **246**:11-17.

Steffen, W., J. Grinevald, P. Crutzen, and J. McNeill. 2011. The Anthropocene: conceptual and historical perspectives. Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, **369**:842-867.

Thomas, C. D., A. Cameron, R. E. Green, M. Bakkenes, L. J. Beaumont, Y. C. Collingham, B. F. Erasmus, M. F. De Siqueira, A. Grainger, and L. Hannah. 2004. Extinction risk from climate change. Nature **427**:145-148.

Urban, M. C., G. Bocedi, A. P. Hendry, J.-B. Mihoub, G. Pe'er, A. Singer, J. R. Bridle, L. G. Crozier, L. De Meester, W. Godsoe, A. Gonzalez, J. J. Hellmann, R. D. Holt, A. Huth, K. Johst, C. B. Krug, P. W. Leadley, S. C. F. Palmer, J. H. Pantel, A. Schmitz, P. A. Zollner, and J. M. J. Travis. 2016. Improving the forecast for biodiversity under climate change. Science **353**.

Vonesh, J. R., and De la Cruz. 2002. Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. Oecologia **133**:325-333.

Werner, E. E., and J. F. Gilliam. 1984. The ontogenetic niche and species interactions in size-structured populations. . Annual Review of Ecology and Systematics **15**:393-425.

Wilbur, H. M. 1980. Complex Life Cycles. Annual Review of Ecology and Systematics **11**:67-93.

Figures and tables:

Figure 3-1. Trademark pattern of evolutionary rescue adapted from Carlson et al. (2014). Following an environmental change, the abundance of individuals within a population declines (blue line) and recovers following an increase in the frequency of adaptive alleles (orange line). The stochastic threshold line refers to a critical threshold density below which extinction becomes increasingly likely due to random chance.

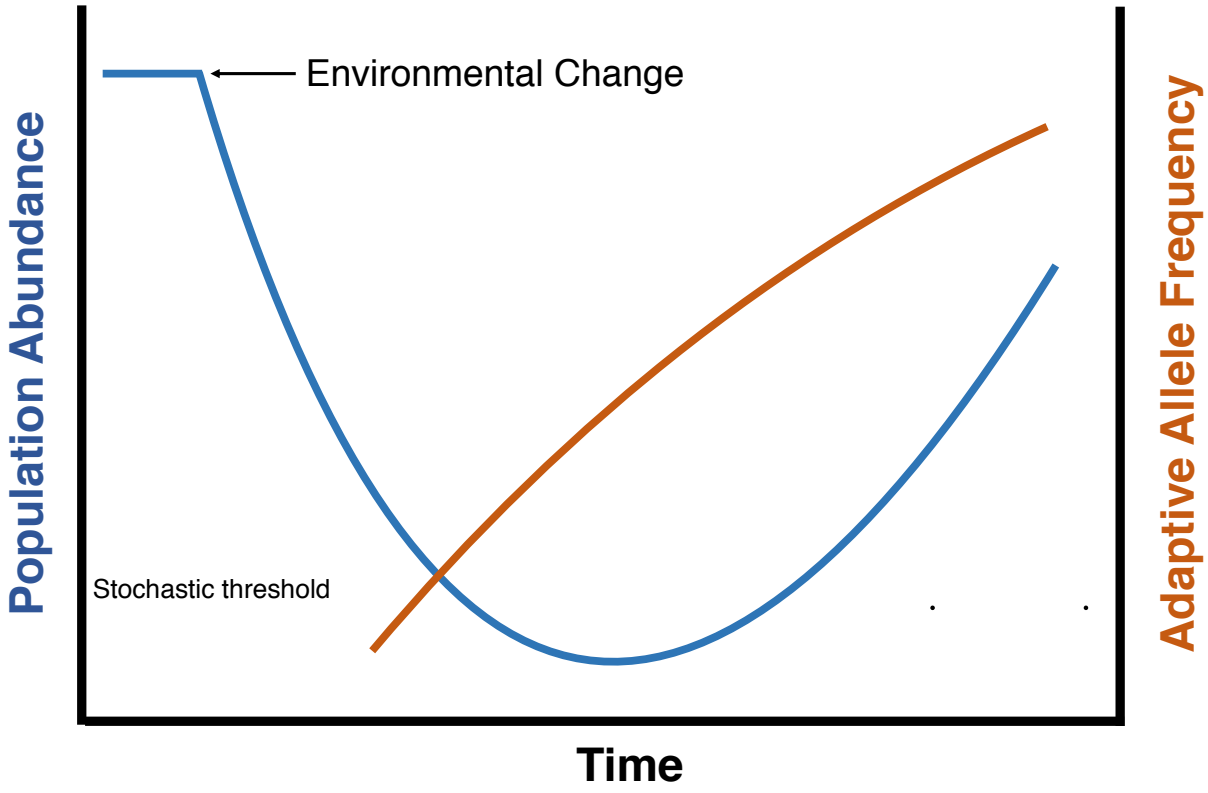


Figure 3-2. The four-stage amphibian life cycle on which the individual-based model is based. Although we use amphibians, this model can be modified to fit any organism with a complex life cycle. In this model, there is a cohort of eggs determined by clutch size and the number of breeding pairs. The eggs have some probability of hatching (σ_h). Survival through the larval period (σ_t) is determined by density-dependent regulation in which survival is a function of maximum tadpole survivorship (σ_{tmax}) over the number of incoming tadpoles (N) multiplied by a scaling term (d) and raised to the power of the density-dependent exponent (γ). When selection is imposed at each stage, the fitness of the individual (ω_{ij}) becomes the probability of survival (σ) to the next stage. The fitness of individuals is defined by a gaussian function in which fitness is defined by the difference of the individual's phenotype (θ_i) to the optimum phenotype in a particular environment (θ_{opt}) over the strength of selection (ρ).

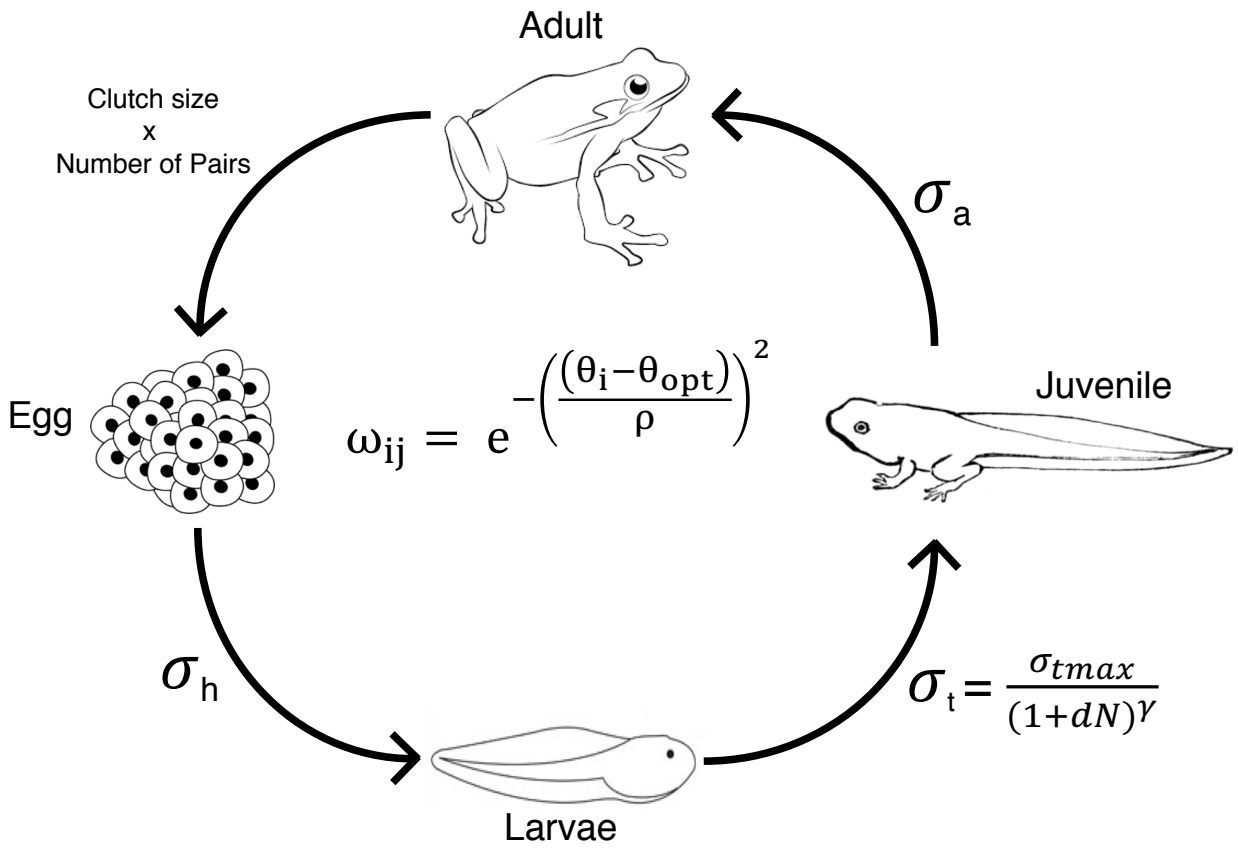


Figure 3-3. Phenotypic variation among populations according to density dependence prior to a shift in environmental optimum. Violin plots show data distributions similar to a boxplot, with the distribution of data shown along the Y-axis and the frequency of data points represented by the width along the X-axis. Each panel represents the genetic variation among populations at 50-generation intervals across three density-dependence scenarios (weak, compensation, and strong density dependence). Each of these demonstrates the impact of density dependence of genetic variation prior to environmental change, which occurred following generation 200.

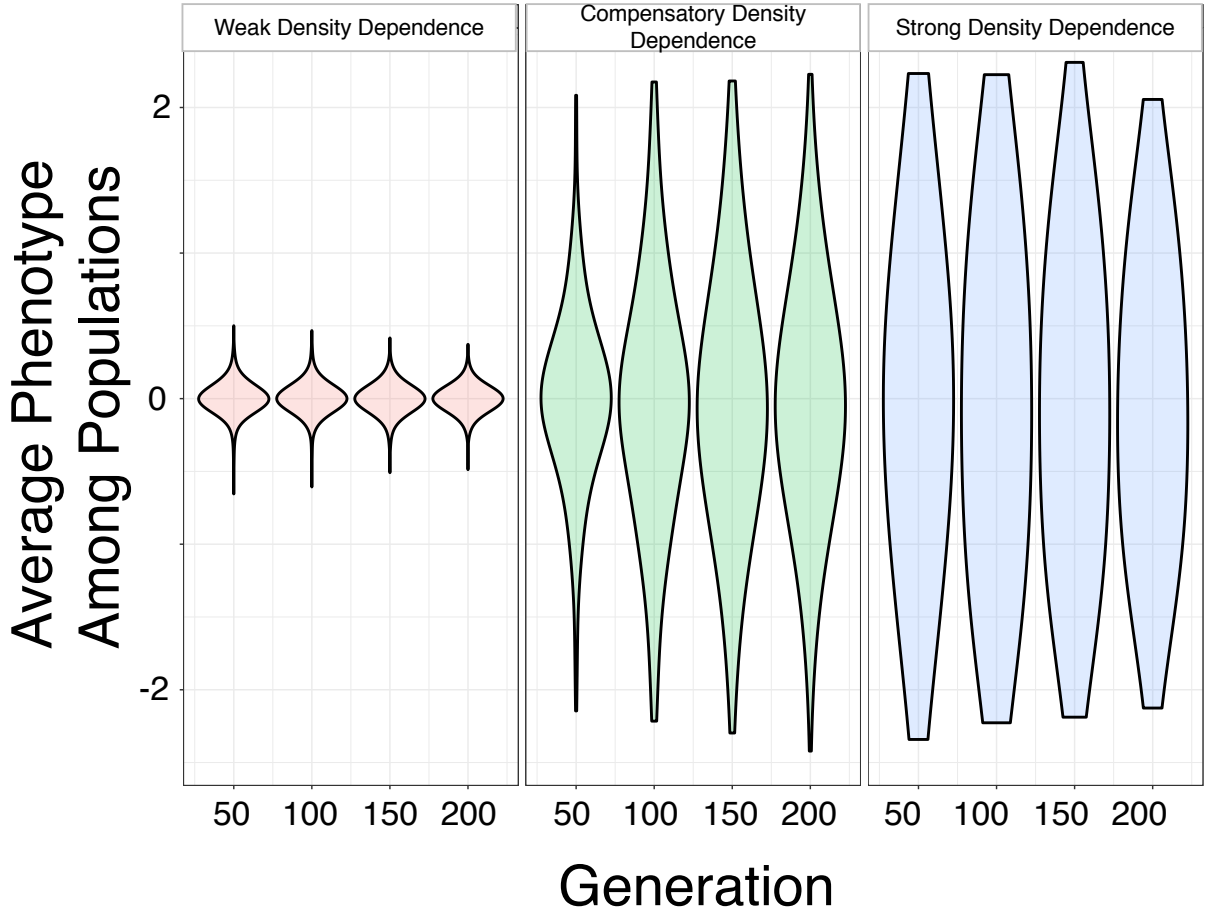


Figure 3-4. Among population genetic variance according to population size. As the population size becomes smaller, the genetic variation among populations increases independent of selection or environmental change, which supports the hypothesis that increased genetic variation among populations with strong density dependence is due to reduced population sizes.

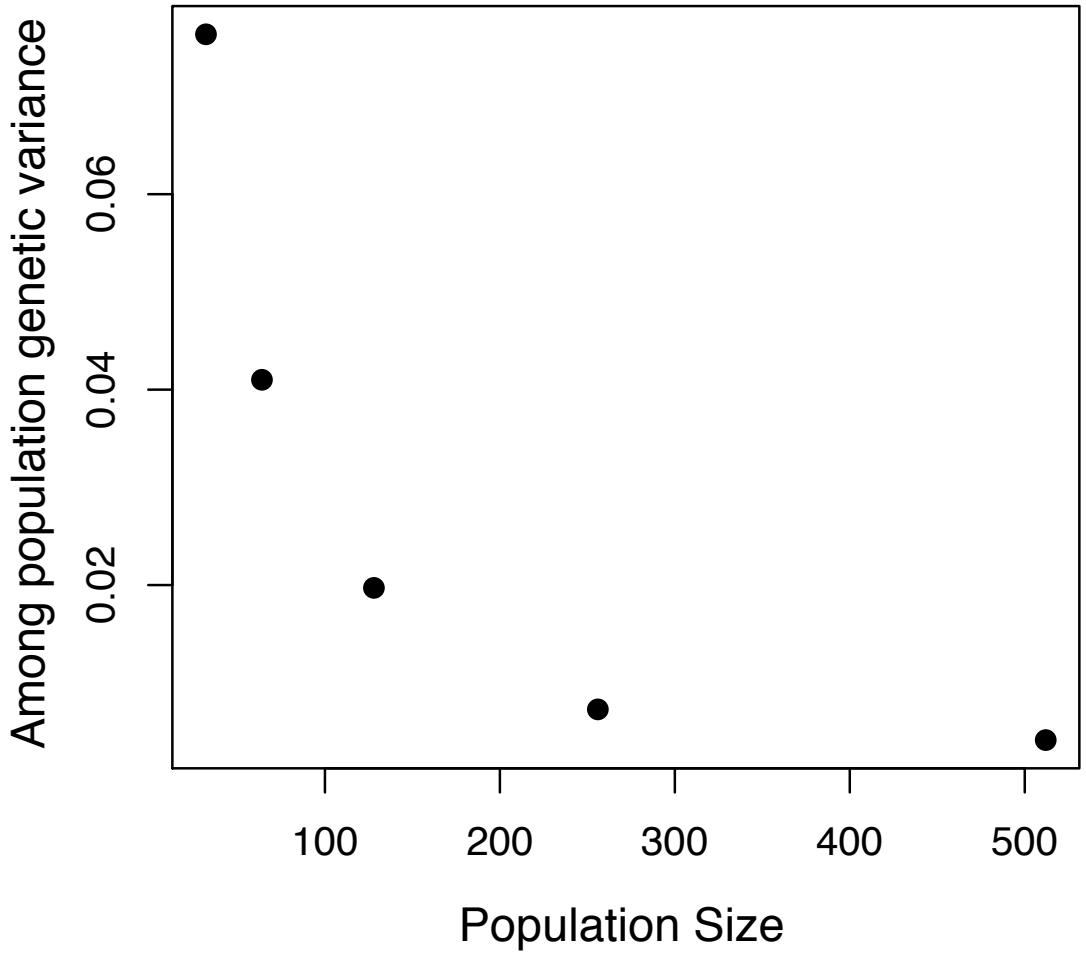


Figure 3-5. Average fitness of populations through time. Each plot shows fitness through 600 generations with different color lines denoting different levels of density dependence. For generation zero through 200, only moderate selection is imposed across all scenarios. At generation 200, the optimum phenotype shifts from zero to two and the strength of selection either grows stronger (ρ=0.5), remains the same (ρ=1.2), or weakens (ρ=1.9). Although the model simulated six different selection scenarios, only three are shown here for simplicity. The left-hand column shows the scenarios with the strongest selection (ρ=0.5), the middle column with moderate selection (ρ=1.2), and the right-hand column showing the weak selection scenarios (ρ=1.9). The top row, plots A, B, and C, show how fitness is affected when selection is imposed at the egg stage. The middle row (D, E, and F) show fitness is impacted when selection occurs at the larval stage, and the bottom row (G, H, and I) show fitness when selection occurs during the adult life stage. Lines that terminate indicate that all populations went extinct.

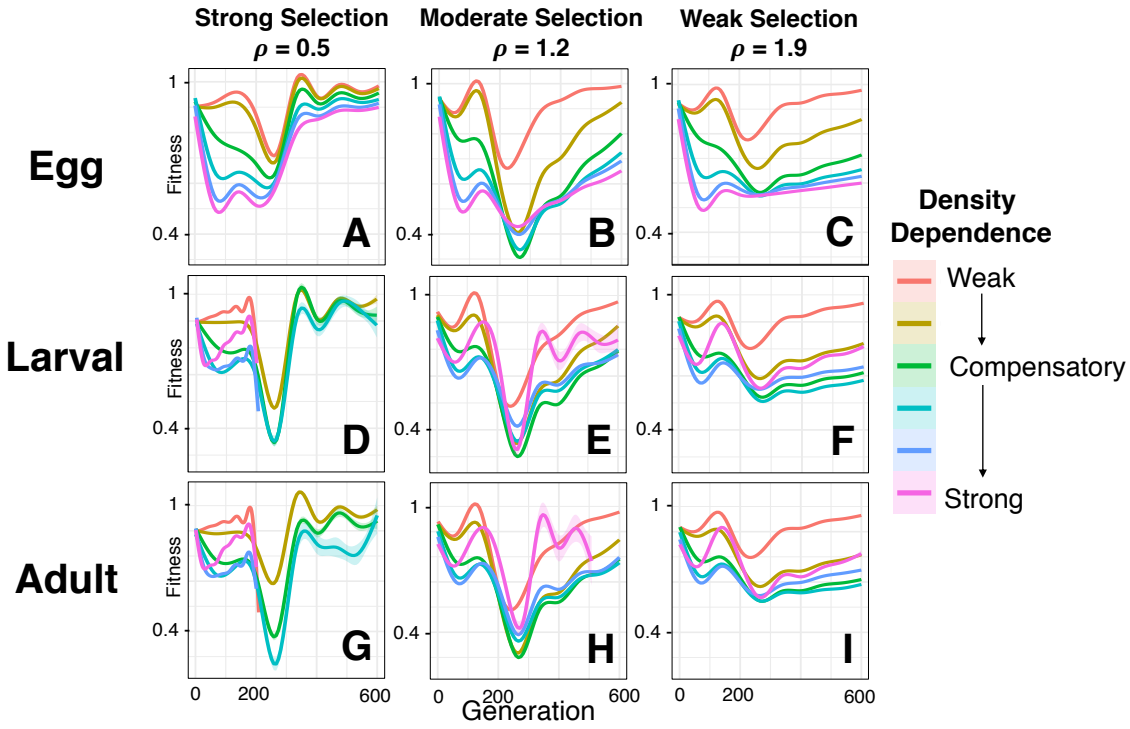
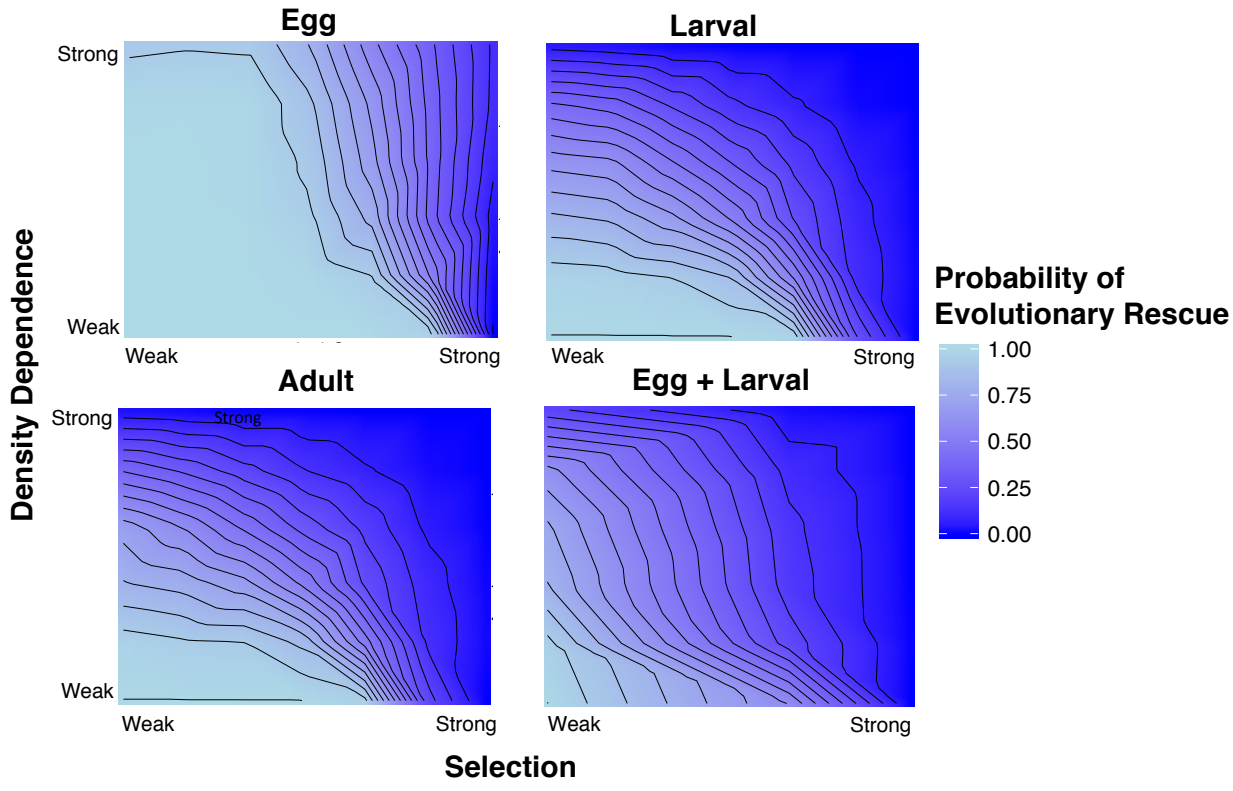


Figure 3-6. Probability of evolutionary rescue according to the strength of density dependence and selection for each of the different life stages during which selection was imposed. In all scenarios, density-dependent regulation occurred during the larval stage. In each plot, higher likelihoods of evolutionary rescue are denoted with lighter blue, while higher likelihoods of extinction are denoted by dark blue. The top left plot shows evolutionary rescue likelihoods when selection is imposed during the egg stage. Top right shows selection at the larval stage, bottom left is selection during the adult stage, and bottom right shows the impact of selection during two stage, the egg and larval stages.



IV. MOLECULAR SIGNATURES OF ADAPTATION TO SALTWATER ENVIRONMENTS IN WILD POPULATIONS OF AMPHIBIANS

Abstract

Environmental change is imposing selective pressure on a wide variety of biota, providing an ideal setting to investigate mechanisms of adaptive responses as they occur in natural systems. Salinization is a change occurring in wetland habitats globally, and it is assumed that many taxa affected by salinization will be locally extirpated. However, some coastal populations of a treefrog inhabit brackish wetlands. This study characterizes how saltwater exposure affects transcriptional responses and early developmental fitness correlates between coastal and inland populations of *Hyla cinerea*. We identify molecular mechanisms that may underlie salt tolerance in wild populations of frogs and disentangle hypotheses about the role of plasticity in the evolution of adaptive change. We found that 1,924 genes are differentially expressed between coastal and inland populations, while just 267 genes are differentially expressed across the different salinity treatments (e.g., different exposure to saltwater during egg or tadpole stages), showing clear divergence across locations that cannot be explained by phenotypic plasticity due to saltwater exposure. Moreover, individuals from coastal populations have higher tadpole survival and higher plasma osmolality which may be correlated with differences in gene expression. The genes that emerged as different between coastal and inland populations perform diverse molecular functions but include several genes that encode cellular transporter proteins and stress response pathways. Collectively, these results reveal the molecular signatures of saltwater tolerance in coastal frog populations and support the hypothesis that coastal populations of *Hyla cinerea* are locally adapted to inhabit brackish environments.

Introduction

Since the landmark studies of Charles Darwin (Darwin 1859) and Alfred Russel Wallace (Wallace 1859), understanding how organisms evolve and what factors govern species distributions across space and time remains a key focus of biological research. Current rates of environmental change have amplified the urgency to understand the conditions under which populations may evolve or decline to extinction (Lynch and Lande 1993, Bell and Gonzalez 2009, Maclean and Wilson 2011, Lindsey et al. 2013, Moritz and Agudo 2013, Nunney 2015). One changing environmental factor that determines the ecology, evolution, and distributions of aquatic species worldwide is salinity (James et al. 2003, Pinder et al. 2004, Lorenz 2014, Castillo et al. 2018, Piscart et al. 2005). However, currently, many freshwater habitats are becoming increasingly saline (Herbert et al. 2015, Kaushal et al. 2018) due to a variety of anthropogenically influenced changes including sea level rise (Church et al. 2013, Schuler and Relyea 2018), application of road de-icing salts (Schuler and Relyea 2018), reductions in riverine freshwater flow (Montagna et al. 2002), storm surges (Mulligan et al. 2012), and altered coastal geomorphology (Day et al. 2008, Manda et al. 2014).

Although there are appreciable differences in the toxicity of salinity across taxa (Castillo et al. 2018) and life-stages (Kefford et al. 2012, Albecker and McCoy 2017), increases in salt concentrations in freshwater systems are typically concomitant with declines in species richness (Hart et al. 1991, Williams et al. 1999, Williams et al. 2003, Lorenz 2014, Herbert et al. 2015). Freshwater organisms have evolved physiologies that resist the natural inflow of water and loss of ions, so increases in external salt concentrations can lead to toxic levels of ion accumulation and water loss within cells (Willmer et al. 2005, Dawson and Liu 2009). Therefore, in order to

persist in wetlands that are becoming increasingly saline, freshwater organisms must fundamentally modify osmoregulatory machinery (Willmer et al. 2005, McCormick and Bradshaw 2006, Dawson and Liu 2009).

If the rates of adaptation within the population can keep pace with the rates or degree of salinization, natural selection can rescue a population from extinction following salinization (Bell 2017). Yet there are myriad factors that affect the probability that a population will evolve in response to a changing environment (Carlson et al. 2014). For example, if the rate or degree of salinization is severe (e.g., sudden inundation via storm surge), adaptation is unlikely (Gomulkiewicz and Holt 1995, Scheiner et al. 2017). Additionally, population size (Lynch and Lande 1993, Gomulkiewicz and Holt 1995, Lynch and Lande 1998, Bell 2013), the amount of genetic variation within a population (Barrett and Schluter 2008, Vander Wal et al. 2013), and gene flow across populations (Gomulkiewicz et al. 1999, Holt et al. 2004b) can affect evolution. However, adaptation to salinity stress has been documented in a broad range of freshwater organisms including invertebrates (Riginos and Cunningham 2005), fish (Lamichhaney et al. 2012b), and amphibians (Gomez-Mestre and Tejado 2003, Brady 2012) – some emerging within the past 100 years (e.g., Spotted Salamanders, see Brady (2012)).

Adaptive phenotypic plasticity can also decrease extinction risk following salinization (Pfennig et al. 2010, Nonaka et al. 2015, Nunney 2015). Individuals that are considered plastic can alter some aspect of their phenotype (e.g., behavior, morphology, development) to better match the environment (Travis 1994, West-Eberhard 2003). Because phenotypic plasticity and natural selection can both produce phenotypic differences across a landscape that appear adaptive,

investigations into the mechanisms of adaptation should consider both plasticity and genetics in generating different phenotypes (Gienapp et al. 2008, Merilä and Hendry 2014).

Theory about rapid adaptive evolution and evolutionary rescue of populations following environmental change is well developed (Bell 2013, Gonzalez et al. 2013, Martin et al. 2013, Alexander et al. 2014, Bourne et al. 2014, Carlson et al. 2014), but logistical challenges have historically hindered efforts to empirically vet theoretical assumptions (Via and Lande 1985, Gomulkiewicz et al. 1999, Holt and Gomulkiewicz 2004, Holt et al. 2004a, Lande 2009, Chevin et al. 2010, Berdahl et al. 2015, Forsman 2015). Recent advances in genomic technologies and computing power allows empirical research to disentangle the relative contributions of plasticity and genetics and identify causal mechanisms underlying adaptive responses. Indeed, both are needed to improve our ability to understand and forecast organismal responses to environmental change (Urban et al. 2014, Urban et al. 2016).

In this study, we use a common garden experiment to quantify changes in tadpole phenotypes and gene expression in populations of an anuran amphibian that inhabit brackish habitats to determine a.) differences in growth, survival, and physiology, b.) the genetic basis for differences observed between coastal and inland populations, and c.) which genes are being differentially expressed both among locations and in response to saltwater exposure. Revealing the genetic basis of adaptation to saltwater in an amphibian is particularly compelling because amphibians are highly salt-sensitive (Albecker and McCoy 2017) with only 2% of all described amphibian species capable of occupying a brackish habitat (Hopkins and Brodie 2015). However, within that 2% are some anuran species such as *Fejervarya cancrivora*, *Rhinella marina*,

Eleutherodactylus coqui, and *Hyla cinerea* that are regularly observed in nearly full-strength seawater (Gordon and Tucker 1965, Rios-López 2008, Albecker and McCoy 2017). These salt-tolerant amphibian species may hold important clues in understanding how osmotic transitions occur and are an excellent model system for studying the evolution of salt tolerance amidst environmental change.

Despite the fact that frogs were heavily used as models for early physiological research defining basic osmoregulatory processes, we know relatively little about how frogs osmoregulate in osmotically stressful environments (Hopkins and Brodie 2015, Cunningham et al. 2016). There are several known proteins, including the Sodium-Potassium Pump (Na+/K+-ATPase or NKA) (Havird et al. 2013), Sodium-Potassium-Chloride co-transporter (Na^+-K^+-Cl^- co-transporter or NKCC), and Aquaporins (AQPs) that are central to maintaining osmotic balance (Alvarado and Moody 1970, Hildebrandt 1997, Uchiyama and Konno 2006, Ogushi 2010, Pandey et al. 2010b, Lee et al. 2011, Bernabò et al. 2013, Havird et al. 2013, Saitoh et al. 2014, Wu et al. 2014) and whose activity and abundance can change according to the environment (Havird et al. 2013, Wu et al. 2014). Yet these represent a small proportion of mechanisms known to contribute to maintaining cellular ion balance in other vertebrates. Even less is known about the genes and gene networks that initiate adaptive responses to osmotic stress. Euryhaline fish have been studied extensively (Evans and Somero 2008, Whitehead et al. 2011a, Whitehead et al. 2011b, 2012, DeFaveri and Merila 2013, Ruiz-Jarabo et al. 2016, Kusakabe et al. 2017), and larval frogs (tadpoles) breathe and osmoregulate through gills that function similar to teleosts (Hillyard et al. 2009b, Wu et al. 2014). Therefore, we also investigate whether certain genes and gene families known to contribute to osmotic responses in fish are important for frogs as well. For example, in

fish, several genes are up-regulated following saltwater exposure including ion pumps (ATP1a1, ATP1a3, ATP6V1E1), transcription factor HNF-4a, AQP3 (aquaporin3), OSTF-1 (osmotic stress transcription factor), and IGF-1 (Insulin-like growth factor) (Evans and Somero 2008, Whitehead et al. 2011b, 2012). Conversely, other genes like VCAN (versican – associated with water retention) and SBC (spermine binding protein – a polyamine regulator) are down regulated (Evans and Somero 2008). In addition to genes that regulate osmotic responses, gene expression pathways and proteins associated with cellular stress responses (e.g. HSP-60 (heat shock protein), HSP-70, Aldehyde reductase, etc.) are reliable indicators that organisms are approaching thresholds of tolerance limits, which we might also expect to differ among populations (Kültz 2005, Kassahn et al. 2009, Evans and Hofmann 2012).

Methods

Study System -- Here we focus on the American green treefrog (*Hyla cinerea*), a frog species common in wetlands in the Southeastern United States. This species has been observed in saltmarsh habitats in coastal North Carolina in salinities reaching 23 parts per thousand (ppt) which is approximately 3.8-fold higher than the lethal salinity reported for this species (McNab 2002, Hillyard et al. 2009a, Albecker and McCoy 2017). Subsequent common-garden studies strongly suggests that coastal *H. cinerea* populations are locally adapted to higher salinities (Albecker and McCoy 2017), Chapter 2 - Albecker and McCoy, *in prep*). We conducted these studies in eastern North Carolina, USA. North Carolina's coast is predicted to be among the most significantly impacted by sea level rise due to low-lying, gently sloped coastal plains and continual coastal subsidence (-1 mm ± 0.15 mm/yr.) (Titus and Richman 2001, Craft et al. 2009, Kemp et al. 2009, Williams 2013, Kopp et al. 2015). Coastal North Carolina is already

experiencing increased saltwater intrusion into coastal lowlands making it an important location for investigating the impacts of increasing salinities on coastal animal communities (Parkinson 1994, Michener et al. 1997, Morris et al. 2002, Day et al. 2008, Kopp et al. 2015).

Experimental Methods – We conducted a common-garden experiment between May 23, 2017 and July 17, 2017. We collected two pairs of breeding frogs (amplexed pairs) from four coastal populations (i.e., populations chronically exposed to saltwater) and four inland populations (i.e., populations chronically exposed to freshwater) (N = 8 pairs from each location). Each of the four pairs was randomly assigned into a plastic Sterilite® shoebox (length x width x height = 33 x 20.32 x 12.7 cm) that contained 2 liters (L) of either freshwater (<0.5ppt), or 4ppt saltwater. We used Greenville municipal tap water treated with API® Tap Water Conditioner (Chalfont, PA) with salinities prepared by mixing treated tap water with InstantOcean Sea Salt® (Blacksburg, VA). Pairs were left in the containers overnight with lids attached and allowed to deposit eggs. After completing oviposition, the parents were released at the site of capture, and the egg clutches were transported back to the laboratory.

Hyla cinerea, like many north American frog species, lay eggs in large, gelatinous masses that swell by absorbing water from the environment to provide each embryo with a protective jelly coat. Because the water contacts the egg directly contemporaneously with fertilization, the quality of the water can impact developmental pathways occurring within the embryo and alter phenotypes across development. By placing pairs into a container holding an experimental concentration of saltwater or freshwater, hereafter referred to as “egg environment”, we can

determine the extent that early life exposure contributes to genetic and phenotypic responses later in development.

At the laboratory, eggs were counted via non-invasive image analysis and then left undisturbed to develop and hatch. Image analysis involved photographing eggs against white background, and using ImageJ software to quantify eggs (Schneider et al. 2012). Approximately two days post-hatching, hatchlings transition from yolk absorption to active foraging (Gosner stage 25) (Gosner 1960). At this time, we subsampled 250 hatchlings from each clutch. The subsampled individuals were divided into 5 groups of 50 individuals and placed in a 400 mL glass container with water matching the salinity of the egg environment. Dividing the clutches allows us to control for genetic relatedness or maternal effects within clutches (Räsänen and Kruuk 2007, Merilä and Hendry 2014). Each of the five groups was randomly assigned to either a freshwater (<0.5ppt), or 4ppt, 6ppt, 8ppt, or 12ppt saltwater tadpole environment treatment. Several studies have indicated that acclimatizing anurans to elevated salinities reduces mortality (Gordon and Tucker 1965, Gordon and Tucker 1968, Hsu et al. 2012). Therefore, to mimic natural conditions, we gradually increased, decreased, or maintained the salinity over 6 days until the specified target salinity was reached. For example, tadpoles from a 4ppt egg environment assigned to the 6ppt tadpole treatment experienced salinity increases of 0.33ppt per day, while tadpoles from the 4ppt egg environment transitioning into freshwater tadpole treatment underwent salinity decreases of 0.67 ppt per day. Prior to water changes each day, we recorded tadpole survival in each container and removed deceased individuals. Hatchlings were fed Spirulina fish food flakes (Ocean Star International, Coral Springs, FL) *ad libitum* each day following water changes.

On day 6, final survival was recorded. At this time, we haphazardly subsampled 10 individuals from each cup for physiological assays and euthanized individuals via 2% MS-222 immersion (pH adjusted to 7.0). Of those 10 individuals, 5 from each container were staged, weighed, and measured, placed into 37ºC incubator for three hours and dry weight recorded. The remaining five tadpoles were dabbed dry using paper towels, placed into 2ml tubes, homogenized using mechanical mortar and pestle, and centrifuged for 2 minutes. Whole body plasma from the homogenate was pipetted into a test tube and plasma osmolality was measured using a Fiske 210 osmometer from Advanced Instruments® (Norwood, MA).

For RNA-seq, a single individual was randomly selected from the following four treatments: 1) freshwater egg/freshwater tadpole treatment, 2) freshwater egg/6ppt tadpole treatment, 3) 4ppt egg/4ppt tadpole treatment, and 4) 4ppt egg/6ppt tadpole treatment. These four treatments were specifically chosen to set up specific comparisons to understand differences according to differences in saltwater exposure at the egg stage (freshwater:6ppt vs. 4ppt:6ppt), differences following saltwater exposure at just the tadpole stage (freshwater:freshwater vs. freshwater:6ppt), and differences if salt exposure occurred during both egg and tadpole stages (freshwater: freshwater vs. 4ppt:4ppt). Although it would be best to excise gills for the genetic assays, at this early stage of development the tadpoles and their gills are extremely small. Previous attempts to dissect gill baskets at this size have indicated a high risk of dissection error that might outweigh the benefits of tissue specificity. Therefore, we removed the tail and intestines of each tadpole, and preserved the remaining tissues in 500µl RNALater®. Samples were stored at -20ºC until RNA extractions (described below). These protocols were repeated for each of the four populations from inland locations and each of the four populations from the

coastal locations, thus producing four samples to be used for RNA-seq for each treatment and location combination.

Tadpole statistical analyses – We quantified tadpole survival over the 6-day acclimation period, as well as total length, mass, and whole-body plasma osmolality of tadpoles on day 6. All analyses on tadpole phenotypes were conducted in the R statistical programing environment version 3.5.0 (Team 2018).

For the data collected on day-6 of the acclimation period including survival, mass, length, and osmolality, we used a model comparison approach using generalized linear mixed effects models to estimate parameters using package "lme4" (Bates et al. 2015) and likelihood ratio tests (Burnham and Anderson 2003) to determine levels of support for different models. We consider the salinity of the egg and tadpole environments and location (inland versus coast) as fixed effects and treat the different populations within each location as random effects. We also include the cup in which tadpoles were reared over the 6-day acclimation as a random effect to account for any non-target variation due to shared housing. For these models, we treat egg salinity as a factor, but tadpole environment as a continuous variable. In all cases, we determine support for various models ranging from the most complex (i.e., full two-way interaction between egg environment, tadpole environment, and location) to simpler (additive model) and no effects models. Because tadpole length and mass are continuous data, we assume log-normal error distributions, while with osmolality, we assume Poisson error because it is an integer (Bolker et al. 2009). We analyzed survivorship using two different approaches: First, we test whether there are differences in the proportion of individuals surviving to day 6 using

generalized mixed effects models with a binomial error family. Second, we test for differences in risk of mortality through time using Kaplan-Meier survival analysis with the R package "survival" (Therneau 2015). Survival curves were plotted using package "survminer" (Kassambara and Kosinski 2018). The first approach tells us how overall survival differed, while the second approach uncovers whether there were differences in rates of survival through time.

RNA extraction and Sequencing -- RNA was extracted using a standardized Trizol protocol and cleaned with DNAse and RNAsin. RNA was further purified using Qiagen RNeasy mini kit, quantitated using Qubit Fluorometer, and quality checked using Agilent BioAnalyzer 2100`. One sample from the coastal freshwater/freshwater treatment had low quantity and quality RNA, so this sample was excluded from sequencing, resulting in a total of 31 samples (N = 31: 4 populations x 2 location (coastal, inland) x 4 egg:tadpole treatments (fw:fw, 4ppt:4ppt, fw:6ppt, 4ppt:6ppt).

RNAseq libraries were prepared with Illumina's TruSeq Stranded mRNAseq Sample Prep kit (Illumina®, San Diego, CA). Libraries were pooled, quantitated by qPCR, and sequenced on one lane for 76 cycles from each end of the fragments on a HiSeq 4000 using a HiSeq 4000 sequencing kit version 1 at University of Illinois' Roy J. Carver Biotechnology Center. Resulting Fastq files were generated and demultiplexed with bcl2fastq v2.17.1.14 Conversion Software (Illumina). Reads were 75 base pairs in length and sequenced to a depth of approximately 12 million reads per sample.

Transcriptome assembly – To assemble a trancriptome for *H. cinerea,* we concatenated both forward and reverse direction reads into two single reads. We randomly subsampled 50 million reads from each of these concatenated read datasets using seqtk (https://github.com/lh3/seqtk). The subsampled reads comprised 7% of our overall dataset and were used to assemble the transcriptome using Oyster River Protocol (ORP) version 1.1.1. The ORP is a pipeline that constructs multiple transcriptomes using different, well-vetted assemblers and then merges them together to form a single higher quality transcriptome (MacManes 2017). By taking advantage of the strengths and weaknesses of different approaches, the ORP improves the likelihood of building a high quality, de novo transcriptome (MacManes 2017). The ORP uses the four assemblers; Trinity version 2.4.0 (Grabherr et al. 2011), Shannon version 0.0.2 (Kannan et al. 2016), and SPAdes assembler version 3.11 with a kmer length of 35, and SPAdes assembler with a different kmer length of 55 (Bankevich et al. 2012). The transcriptomes generated from each technique were then merged together using OrthoFuser (MacManes 2017) and corrected for initial errors using RCorrector 1.01 (Song and Florea 2015). Using trimmomatic version 0.36 (Bolger et al. 2014), we removed any adaptors that remained on transcripts using aggressive adaptor removal and performed gentle quality trimming at a Phred score of ≤ 2 (MacManes 2014). The quality of our final transcriptome was evaluated using BUSCO version 3.0.1 (Simão et al. 2015) and TransRate version 1.0.3 (Smith-Unna et al. 2016). BUSCO scores provide a quantitative assessment of the quality of the assembled transcriptome based on the appropriate representation of conserved genes expected to be present across all eukaryotic cells. The Transrate quality metric is based on an analysis of contigs to determine if they are accurate, complete, and non-redundant, and provides an optimal score for "good" well supported contigs.

RNA statistical analyses – We annotated our transcriptome using the peptide databases corresponding to three available frog genomes. We use Diamond version 0.9.10 (Buchfink et al. 2015) to annotate our transcriptome using available information based on *Xenopus tropicalis* (Coordinators 2016), *Nanorana parkeri* (Sun et al. 2015), and *Rana catesbeiana* (Hammond et al. 2017). We also use the UniRef90 database (Consortium 2018) to match sequences that correspond to known peptides in non-amphibian taxa. We quantify transcript abundance based on pseudo-alignments for each sample using Kallisto (version 0.43.0) (Bray et al. 2016) and import these data into the R statistical environment for downstream analyses, version 3.5.0 (Team 2018).

To explore differential expression between location and egg/tadpole salinity, we used the R packages "pcaExplorer" (Marini 2018) and "DESeq2" (Love et al. 2014). For these analyses, location and egg/tadpole salinity were considered fixed effects with alpha values set at 0.05. We used Panther classification system version 13.1 to perform gene ontology (GO) enrichment analyses (Huaiyu et al. 2016) and use the results of the GO-enrichment to identify proteins within certain groups to identify functionally important genes across locations and salinities (Huaiyu et al. 2013).

Results

Tadpole Survival – Tadpole survival on the final day of acclimations during which tadpoles were gradually transitioned into their respective tadpole salinity was driven by the three-way interaction between egg salinity, tadpole salinity, and location ($\chi^2_9 = 26.998$; $p < 0.001$) (Fig. 1). We also found significant differences in survivorship through time ($\chi^2_{19} = 44.8$; $p < 0.001$) with

high survivorship in all treatments except the 12ppt treatment (Fig. 2). Within the 12ppt treatment, the coastal individuals hatched from the freshwater egg environment had the highest survival through day 6, with lower survival among coastal tadpoles hatched from 4ppt, inland tadpoles hatched from 4ppt, and inland tadpoles hatched in freshwater.

Tadpole Measurements -- The mass of tadpoles that survived to the final day of the 6-day acclimation period was best described by an additive model. Mass was only impacted by the salinity of the tadpole environment ($\chi^2_7 = 70.38$; $p < 0.0001$) and was not significantly impacted by egg salinity or location (egg environment: $\chi^2_7 = 0.72$; $p = 0.39$; location: $\chi^2_7 = 0.56$; $p = 0.45$) (Fig. 3). In the freshwater treatments, individuals weighed approximately 0.20g (95% Confidence Interval (C.I.) 0.015-0.023g), while tadpoles in the 12ppt treatments weighed approximately 0.02g (C.I. 0.0069 – 0.011). An additive model also best described tadpole length. Specifically, there were no effects of the egg environment ($\chi^2_7 = 0.60$; $p = 0.43$), and only a weak effect of location ($\chi^2_7 = 3.43$; $p = 0.063$) as well as a difference in length due to tadpole salinity ($\chi^2_7 = 57.26$; $p < 0.0001$) (Fig. 4). In freshwater tadpole treatments, inland tadpoles were slightly longer than coastal tadpoles with an approximate length of 11.2mm (C.I. 10.5-12.1mm) compared to the coastal length of 10.3mm (C.I. 9.7-10.2mm). In the 12ppt treatments, inland tadpoles were approximately 8.7mm (C.I. 8.2-9.4mm) in length, compared to an average length for coastal individuals of 8.0mm (C.I. 7.4-8.6mm).

Consistent with these patterns, plasma osmolality was also affected by the additive effects of location ($\chi^2_7 = 5.92$; $p = 0.015$) and tadpole salinity ($\chi^2_7 = 58.13$; $p < 0.0001$) (Fig. 5). In general, coastal tadpoles had higher plasma solute concentrations than inland tadpoles across all

salinities. In freshwater, coastal tadpoles had internal solute concentrations of approximately 275 milliOsmoles per liter (mOsm/L) (C.I. 260-294 mOsm/L) compared to 255 mOsm/L (C.I. 239-272 mOsm/L) within inland tadpoles in freshwater. In the 12ppt treatments, coastal tadpoles had an approximate plasma osmolality of 465 mOsm/L (C.I. 435-497 mOsm/L) while inland tadpoles held internal concentrations of approximately 430 mOsm/L (C.I. 401-462 mOsm/L).

Transcriptome Assembly – After filtering our original transcriptome produced by the Oyster River Protocol for "good" contigs based on the transrate metric, the assembled transcriptome consisted of 126,042 total transcripts and 55,631 aligned reads. The updated transrate score on the filtered dataset was 0.49, improved from the original transrate score of 0.30. Our BUSCO score was 96.4%, indicating that our filtered dataset contains the majority of conserved genes expected in eukaryotic cells. We annotated 24,533 transcripts (44.1%) within the aligned transcriptome.

Gene Expression – From the Deseq2 analysis, we found 1,924 genes that were differentially expressed between coastal and inland tadpoles after accounting for the effects of the salinity treatments (Fig. 6 & 7). We contrast the freshwater egg/freshwater tadpole treatment against the freshwater egg/6ppt tadpole treatment to determine how tadpole salinity affects gene expression and found 108 genes that are differentially expressed (Fig. 8). We contrast the freshwater egg/ 6ppt tadpole treatment against the 4ppt egg/6ppt tadpole treatment to determine how saltwater exposure during the egg stage affects gene expression in tadpoles (Fig. 8) and found differential expression of 79 genes as a result of the egg environment. Finally, we compare the freshwater egg/freshwater tadpole treatment against the 4ppt egg/4ppt tadpole environment to determine

overall differences in gene expression due to salinity and find 80 differentially expressed genes (Fig. 8).

Location Gene Ontology – Panther's Gene Ontology classified 458 of the annotated genes that emerged as differentially expressed between coastal and inland populations into known molecular functional groups) (Fig. 9). Of these, 174 genes (37.6%) were classified as binding genes (GO:0005488), which refers to genes that regulate interactions of molecules between cells. There were 187 genes (40.4%) identified that regulate catalytic activity (GO:0003824), which regulate biochemical reactions and often encode enzymes. Thirty-one differentially expressed genes (6.7%) were identified that encode transporter activity (GO:0005215), which allow for movement of substances in and out of cells. Fifteen genes (3.2%) encode signal transducer activity (GO:0004871), which convey signals across cells to trigger cellular signals. Twenty genes (4.3%) encode receptor activity (GO:0004872), which combined with signal transducers to initiate changes in cell activity, and 28 genes (3.9%) contribute to structural molecule activity (GO:0005198) which help maintain cellular structure. Finally, 3 genes (0.6%) contributed to antioxidant activity (GO:0016209), which typically are components that limit oxidation reactions that could lead to cellular damage.

When classified more broadly into biological functions, 992 differentially expressed genes between coastal and inland populations could be matched to biological processes. Three hundred genes (30.2%) encoded cellular processes (GO:0009987) which is a general description of a variety of processes carried out at a cellular level and can include cell communication, cell cycle, gene silencing, intercellular transport, or protein folding among others. Two hundred and fifty-

six genes (25.8%) regulate metabolic processes (GO:0008152) which encapsulates cell-growth related processes like DNA repair and replication, protein synthesis, etc. Eighty-six genes (8.7%) contribute to biological regulation (GO:0065007) which is another broad category that encapsulates proteins that regulate cellular activity. One hundred and four genes (10.5%) contribute to cellular component organization (GO:0065007) which concerns the assembly or disassembly of cellular components. Sixty-six genes (6.7%) contribute to cellular response to stimuli (GO:0050896), and 61 genes (6.1%) contribute to localization processes within cells (GO:0051179). Forty-five genes (4.5%) contribute to developmental processes (GO:0032502), with another 45 genes (4.5%) regulating multicellular organismal processes (GO:0032501) which encapsulates a variety of processes including larval behavior, keratinization, circadian regulation of gene expression and translation, organ growth, etc. Nine genes (0.9%) are related to immune system processes (GO:0002376), 7 genes (0.7%) contribute to reproduction (GO:0000003), 8 genes (0.8%) toward biological adhesion (GO:0022610), and 3 genes (0.3%) contribute to locomotion (GO:0040011) (Fig. 9).

Salinity Gene Ontology – For the freshwater egg/freshwater tadpole vs. 4ppt egg/4ppt tadpole salinity contrast, there were only 53 differentially expressed genes with known molecular functions (Fig. 10). Twenty-two of those genes (41.5%) encoded binding activity, while 19 genes (35.8%) encoded catalytic activity. Two genes (3.8 %) encoded translation regulation activity (GO:0045182), which are involved in protein synthesis and modification, and 6 genes (11.3%) contribute to structural molecule activity, 2 additional genes (3.8%) encode transporters, and 2 genes (3.8%) encode signal transducer activity. When classified into biological processes, we found 96 annotated genes with known biological processes. Twenty-nine genes (30.2%)

contribute to cellular processes, 28 genes (29.2%) denote metabolic processes, 10 genes (10.4%) contribute to cellular component organization, 3 genes (3.1%) denote developmental processes, 3 genes (3.1%) encode multicellular organismal processes, 9 genes (9.4%) contribute to biological regulation, 8 genes (8.3%) encode responses to stimuli, and 6 genes (6.3%) denotes localization processes (Fig. 10).

Of the annotated genes that were differentially expressed due to the tadpole environment (e.g., freshwater egg/freshwater tadpole vs. freshwater egg/6ppt tadpole contrast), 39 could be categorized to a molecular function (Fig. 11). Sixteen genes (41%) contribute to binding, 15 genes (38.5%) contribute to catalytic activity, 2 genes (5.1%) contributing structural molecular activity, and 6 genes (10.5%) the encode transporters. Within this same contrast, 90 genes could be classified into a biological process (Fig. 11). Of those, 29 genes (32.2%) contribute to cellular processes, 25 genes (27.8%) toward metabolic processes, 6 genes (6.7%) encode responses to stimulus, 2 genes (2.2%) denote developmental processes, 8 genes (8.9%) encode cellular component organization, 11 genes (12.2%) contribute to biological regulation, 2 genes (2.2%) denoting multicellular organismal processes, and 7 genes (7.8%) contributing to localization.

For the genes that emerged as different according to egg environment (e.g., freshwater egg/6ppt tadpole vs. 4ppt egg/6ppt tadpole contrast), only 17 annotated genes could be classified to a molecular function (Fig. 12). Eight genes (47.1%) encoded different binding activity, and seven genes (41.2%) denote different catalytic activity. One gene (5.9%) were expressed in the structural molecule activity and transporter categories each. However, 38 annotated genes could be classified into a biological process (Fig. 12). Thirteen genes (34.2%) contribute to cellular

processes, 12 genes (31.6%) denote metabolic processes, three genes (7.9%) each denote cellular component organization, localization, and response to stimulus. Two genes (5.3%) contribute to biological regulation, while one gene (2.6%) encodes multicellular organismal processes and developmental processes each.

Discussion

Adaptive evolution occurs when natural selection causes a genetic change within a population. To firmly demonstrate that adaptive evolution has occurred in a population, studies must establish a genetic basis for a phenotypic change, contest alternative mechanisms that can produce phenotypic differences across populations (e.g., plasticity), and link responses to a causal agent (Gienapp et al. 2008, Merilä and Hendry 2014). In this study, we show that there is differential gene expression following saltwater exposure across coastal and inland frog populations. And finally, we provide a mechanistic link between multiple genetic and phenotypic responses in response to a specific environmental driver – salinity. Thus, our study provides strong evidence that observed differences in the distribution, habitat selection, and life history traits (e.g. chapters 1 and 2) between coastal and inland populations of American green tree frogs are the result of adaptive evolution to tolerate saltwater.

Differences in Gene Expression Across Locations: After accounting for salinity effects, there were 1,924 differentially expressed transcripts between coastal and inland frog populations (Fig. 7), which accounts for 3.5% of the aligned genes in our transcriptome. The differentially expressed genes classified according to molecular function show that the majority of differentially expressed genes are involved in catalytic activity and cellular binding (Fig. 9).

Broadly, catalytic genes encode enzymes and proteins that contribute to molecular functioning by facilitating biochemical reactions, while genes that contribute to binding are important for interactions between molecules or cells (Huaiyu et al. 2013). The binding gene with the greatest increase in expression in coastal populations compared to inland populations is fgg (Fig. 13), which encodes a component of fibrinogen. Fibrinogen is a glycoprotein that circulates within the body of vertebrates and is often converted into fibrin to create blood clots following injury. Fibrinogen can also contribute to tissue revascularization and repair by mediating capillary tube formation and angiogenesis (Mosesson 2005). The upregulation of these components in coastal individuals may contribute to higher blood plasma osmolality in coastal tadpoles (Fig. 5) or possibly contribute to the maintenance and development of vasculature given the higher osmotic pressure in coastal tadpoles due to greater plasma osmolality. The two transporters that were the most upregulated in coastal populations were mfsd14a (1663.5-fold increase) and slc20a1(250.7-fold increase) (Fig. 13). Mfsd14a is a member of the large major facilitator superfamily (MFS) which broadly transport small solutes across membranes in response to chemiosmotic gradients (Pao et al. 1998), but the specific solute transported by this particular protein is unknown. Slc20a1, on the other hand, is a sodium-dependent phosphate co-transporter. The primary function of this house-keeping protein is to maintain internal balance of inorganic phosphate, a key electrolyte, so that signal transduction, cell membrane production, and energy exchange can function properly. Phosphate can become imbalanced given malnutrition or if internal plasma concentrations become thin via over-hydration or over-dialysis, and upregulation of this protein suggests that coastal frogs experience an imbalance of internal phosphate concentrations, possibly due to altered internal water balance (Beck et al. 2010, Miyamoto et al. 2011).

We also observed differences in gene expression in genes that constitute physiological pathways that are upregulated in response to stress. Oxidative stress and inflammation can be produced by increases in internal toxins, pathogens, or solute concentrations, and up-regulation of stress-related pathways are good diagnostic features of inflammation. In tadpoles, we observed that components of the chemokine and cytokine signaling inflammation pathway, the oxidative stress response pathway, the p53 pathway, and vasopressin synthesis pathway are differentially expressed across coastal and inland populations. Chemokines and cytokines interact in a complex network to mediate internal inflammation (Turner et al. 2014), and we observed 9 genes within this pathway with differential expression (Fig. 14). We observed a similar pattern of expression in the more general oxidative stress pathway in which coastal frogs up-regulate two genes, but down regulate a third (Fig. 14). The p53 pathway is well known for its role in human cancers, but it is a conserved (but complex) pathway across vertebrates that functions broadly to ensure proper DNA replication and cell division in cells disrupted by internal stressors (Harris and Levine 2005). In frogs in this study, we observed the upregulation of 4 genes from the p53 pathway in coastal populations (Fig. 14). Finally, the vasopressin pathway constructs the anti-diuretic hormone vasopressin, which regulates internal water balance by adjusting blood serum osmolality via cellular cascades (Lu et al. 2007) and we observed an upregulation of two genes within this pathway in coastal frogs (Fig. 14).

Contribution of Phenotypic Plasticity -- It is possible that early life exposure to salt stress could alter genetic and phenotypic pathways to generate differences across coastal and inland populations that mimic adaptive evolution. Indeed, plasticity is increasingly implicated as the driver of differences across populations in response to environmental change rather than genetic

divergence (Urban et al. 2014, Hendry 2015, Cattau et al. 2018). To understand how much environment influences outcomes, we exposed eggs to either freshwater or 4ppt water from the moment of fertilization and after hatching gradually transferred them into either freshwater, 4ppt, 6ppt, 8ppt, or 12ppt saltwater. If the egg environment largely impacted development, we expected to see large differences due to salinity treatment in both gene expression as well as with the physical condition of tadpoles following the 6-day acclimation. However, we see little impact of egg environment in the physical condition assays including mass (Fig. 3), length (Fig. 4), or osmolality (Fig. 5). Importantly, location significantly affected the survival (Fig. 1 & 2), length (Fig. 4), and osmolality (Fig. 5) of tadpoles, which supports the hypothesis that genetic differences rather than environmentally induced plasticity drive differences between coastal and inland frogs.

For each of the contrasts on gene expression between the different treatments, we found 108 differentially expressed genes between the freshwater egg/freshwater tadpole and freshwater egg/6ppt tadpole treatments, 79 differentially expressed genes in the freshwater egg/ 6ppt tadpole and 4ppt egg/6ppt tadpole treatment, and 80 differentially expressed genes in the freshwater egg/freshwater tadpole treatment against the 4ppt egg/4ppt tadpole environment (Fig. 8). Across the four different salinity treatments, the amount of differential expression is much lower than the differences in gene expression that emerged between coastal and inland populations, and only accounts for 0.02%, 0.01%, and 0.01% of the aligned transcripts, respectively. These results strongly suggest that divergence among locations is the key driver of observed differences in responses to salinity rather than environmentally induced plasticity.

Despite the limited influence of environment on gene expression, there were some notable differences in gene expression according to salinity. Interestingly, in the treatments that compared eggs laid in freshwater vs 4ppt (e.g., fw egg/6ppt vs. 4ppt egg/6ppt treatments), the only transporter protein that was differentially expressed is a chloride transporter (clic5) that was downregulated in the individuals hatched from 4ppt water (Fig. 8). This finding went against our expectation that early exposure to saltwater would increase transcripts that code for chloride or sodium transporters. In the freshwater egg/freshwater tadpole treatment vs. 4ppt egg/4ppt tadpole contrast, adrm1-b emerged as the most up-regulated gene (362-fold increase) in the 4ppt/4ppt treatment. This gene is a component of proteasomes that break down proteins tagged for destruction by ubiquitin (Stone and Morris 2014). Within that same salinity contrast, AQP5 is the second most highly upregulated gene (41-fold increase) (Fig. 8) and is involved in water reabsorption for cells and tissues experiencing water loss (Suzuki et al. 2015). This water channel protein has been immuno-located in the apical membrane of epithelial cells in the bladder of adult *Xenopus* frogs (Suzuki et al. 2015), and is upregulated in frogs in response to dehydration (Shibata et al. 2014). Indeed, this family of proteins (AQPs) was *a-priori* expected to be differentially expressed because it is a key protein in the maintenance of water balance among vertebrates (Takata et al. 2004, Uchiyama and Konno 2006, Suzuki et al. 2007, Suzuki and Tanaka 2009, Ogushi et al. 2010, Pandey et al. 2010a, Saitoh et al. 2014, Shibata et al. 2014). Additionally, we also observed an 8.5-fold increase in AQP5 expression in the individuals from the freshwater egg/6ppt tadpole treatment compared to the freshwater egg/freshwater tadpole treatment (Fig. 8).

Missing Candidate Genes – We hypothesized that other AQP isoforms, vcan, sos1, crhbp, mapkap1, nkain, and hsp isoforms would all be differentially expressed across locations/salinities because they have been shown to contribute to either stress responses or maintaining osmotic balance in other taxa. Interestingly, none of these emerged as differentially expressed among treatments or locations, although they were all present in the transcriptome.

A recent paper investigated how gene expression differs between a salt-specialist frog species, *Fejervarya cancrivora*, and a closely related, salt intolerant species *Fejervarya limnocharis* (Shao et al. 2015). Although the Shao et al. study demonstrates transcriptomic differences that encode mechanisms that regulate ionic balance, we did not see any overlap with the genes expressed in *Hyla cinerea* and the genes reported within that paper. There are three possible reasons why: First, the Shao et al. paper compares genes expressed in the kidneys and skin of adult frogs. In addition to the fact that each tissue is expected to respond differently to osmotic stress, it is likely that responses to salt exposure differ between tadpoles and adults, as tadpoles typically osmoregulate similar to teleosts and also lack a well-developed kidney (Hillyard et al. 2009b). Second, *Fejervarya cancrivora* is perhaps the most salt-tolerant frog species known and have been found in marine habitats with salinities exceeding seawater (Gordon et al. 1961, Gordon and Tucker 1965, Wygoda et al. 2011, Wu et al. 2014). *F. cancrivora* persist in seawater by increasing (and tolerating) blood plasma solute concentrations to such a degree that the internal solute concentrations remain hypertonic to the environment (Gordon et al. 1961, Gordon and Tucker 1965, Gordon and Tucker 1968, Balinsky 1981, Uchiyama and Yoshizawa 1992). Although we do see increases in plasma osmolality in coastal *H. cinerea* (Fig. 5), plasma osmolality in coastal tadpoles does not exceed the solute concentrations in the high salinity

treatments (e.g., 8 or 12ppt) which suggests that key differences in saltwater tolerance strategies exist across the anuran phylogeny. Third, the Shao et al. study highlights only a few up-regulated genes that contribute to known osmotic functions. It is possible there is some overlap in gene expression between species, but these genes were simply not reported in the paper.

Three important considerations that we do not include in our study are transgenerational epigenetics, maternal effects, and post-transcriptional regulation, each of which may influence the ability of populations to adapt to novel environments. Epigenetic modification produces heritable changes to gene expression through methylation, histone modification, and non-coding RNA (e.g., small interfering RNA) rather than through allelic changes within the genome (Jaenisch and Bird 2003). To be clear, differences in gene expression across populations certainly confirm a genetic basis for trait differences among populations, but do not confirm that trait differences are due to changes in the frequency of adaptive alleles across populations. If epigenetic modification explains the adaptive responses, we should expect the genomes between coastal and inland frogs to remain highly conserved, and instead see greater differences in the transcriptome. Frog genomes are notoriously difficult to sequence, but future studies should use transcriptomes to understand the role of epigenetics in producing differences in gene expression that may facilitate adaptive responses to environmental changes. Secondly, maternal effects are a blend of plasticity and genetics whereby the phenotype of an organism is not only determined by its own genotype and environment, but also by the genotype and environment experienced by its mother (Kirkpatrick and Lande 1989, Marshall and Uller 2007, Räsänen and Kruuk 2007). Maternally affected traits are expected to increase in prevalence when the environment experienced by the mother and offspring matches (Kirkpatrick and Lande 1989). By passing

necessary mRNA and proteins to offspring through the egg, maternal effects may explain up to 96% of the variation in improved offspring fitness in stressful environments (Chirgwin et al. 2016). Finally, post-transcriptional processes including microRNA or other RNA-binding proteins can divest or amplify expressed mRNA thus distorting our ability to make appropriate conclusions about actual cellular function (Evans 2015). Further, it is possible that several candidate genes that contribute to osmotic balance were not differentially expressed in our dataset simply because the response occurred previously in development and the mRNA is no longer being expressed. An upcoming study on tadpole proteomes will provide clues about the impact of post-transcriptional regulation by quantifying protein abundance and correlating protein abundance to gene expression.

Conclusions -- Adult anurans are sensitive to water quality due to permeable skin used for osmoregulation (Bentley and Yorio 1979), and a limited capacity to concentrate solutes and tolerate hyperosmotic body fluids (Balinsky 1981, Hillyard et al. 2009b). However, as shown in this study, some species such as *Hyla cinerea*, have evolved modest levels of salt-tolerance and can inhabit brackish, coastal habitats. Although there is evidence suggesting that coastal, chronically salt-exposed populations of *H. cinerea* are diverging from inland, salt-naïve *H. cinerea* populations, we had yet to characterize whether phenotypic differences across coastal and inland populations have a genetic basis and what genes underpin salt tolerance in coastal frog populations. In this study, we show that despite exposing frogs to different salinities during the environmentally sensitive egg stage, the largest differences in gene expression emerge between coastal and inland *H. cinerea* populations. Several of these differentially expressed genes encode proteins that are likely contributing to enhanced salt tolerance in coastal

populations. These findings strongly support the hypothesis that coastal populations of *H. cinerea* along the North Carolina coast have locally adapted to rising salinity.

Literature Cited

Albecker, M. A., and M. W. McCoy. 2017. Adaptive responses to salinity stress across multiple life stages in anuran amphibians. Frontiers in Zoology **14**:40.

Alexander, H. K., G. Martin, O. Y. Martin, and S. Bonhoeffer. 2014. Evolutionary rescue: linking theory for conservation and medicine. Evolutionary Applications **7**:1161-1179.

Alvarado, R. H., and A. Moody. 1970. Sodium and chloride transport in tadpoles of the bullfrog Rana catesbeiana. Am J Physiol **218**:1510-1516.

Balinsky, J. B. 1981. Adaptation of Nitrogen metabolism to hyperosmotic environment in amphibia. Journal of Experimental Zoology **215**:335-350.

Bankevich, A., S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, and A. V. Pyshkin. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of computational biology **19**:455-477.

Barrett, R. D. H., and D. Schluter. 2008. Adaptation from standing genetic variation. Trends in Ecology & Evolution **23**:38-44.

Bates, D., M. Martin, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models using lme4. Journal of Statistical Software **2015**:1-48.

Beck, L., C. Leroy, S. Beck-Cormier, A. Forand, C. Salaün, N. Paris, A. Bernier, P. Ureña-Torres, D. Prié, M. Ollero, and L. Coulombel. 2010. The phosphate transporter PiT1 (Slc20a1) revealed as a new essential gene for mouse liver development. . PLOS one **5**:p.e9148.

Bell, G. 2013. Evolutionary rescue and the limits of adaptation. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**:20120080.

Bell, G. 2017. Evolutionary Rescue. Annual Review of Ecology, Evolution and Systematics **48**:605-627.

Bell, G., and A. Gonzalez. 2009. Evolutionary rescue can prevent extinction following environmental change. Ecology Letters **12**:942-948.

Bentley, A. J., and T. Yorio. 1979. Do Frogs Drink?. Journal of Experimental Biology **79**:41-46.

Berdahl, A., C. J. Torney, E. Schertzer, and S. A. Levin. 2015. On the evolutionary interplay between dispersal and local adaptation in heterogeneous environments. Evolution.

Bernabò, I., A. Bonacci, F. Coscarelli, M. Tripepi, and E. Brunelli. 2013. Effects of salinity stress on Bufo balearicus and Bufo bufo tadpoles: Tolerance, morphological gill alterations and Na(+)/K(+)-ATPase localization. Aquat Toxicol **132-133**:119-133.

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. . Bioinformatics **30**:2114-2120.

Bourne, E. C., G. Bocedi, J. M. Travis, R. J. Pakeman, R. W. Brooker, and K. Schiffers. 2014. Between migration load and evolutionary rescue: dispersal, adaptation and the response of spatially structured populations to environmental change. Proceedings of the Royal Society of London B: Biological Sciences **281**:20132795.

Brady, S. P. 2012. Road to evolution? Local adaptation to road adjacency in an amphibian (Ambystoma maculatum). Scientific reports **2**.

Bray, N. L., H. Pimentel, P. Melsted, and L. Pachter. 2016. Near-optimal probabilistic RNA-seq quantification. Nature biotechnology **34**:525.

Buchfink, B., C. Xie, and D. H. Huson. 2015. Fast and sensitive protein alignment using DIAMOND. Nature Methods **12**:59-60.

Burnham, K. P., and D. R. Anderson. 2003. Model selection and multimodel inference: a practical information-theoretic approach. Springer Science & Business Media.

Carlson, S. M., C. J. Cunningham, and P. A. H. Westley. 2014. Evolutionary rescue in a changing world. Trends in Ecology & Evolution **29**:521-530.

Castillo, A. M., D. M. T. Sharpe, C. K. Ghalambor, and L. F. De León. 2018. Exploring the effects of salinization on trophic diversity in freshwater ecosystems: A quantitative review. Hydrobiologia **807**:1-17.

Cattau, C. E., R. J. Fletcher Jr, R. T. Kimball, C. W. Miller, and W. M. Kitchens. 2018. Rapid morphological change of a top predator with the invasion of a novel prey. Nature Ecology and Evolution **2**:108.

Chevin, L. M., R. Lande, and G. M. Mace. 2010. Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. PLoS Biol **8**:e1000357.

Chirgwin, E., D. J. Marshall, C. M. Sgrò, and K. Monro. 2016. The other 96%: Can neglected sources of fitness variation offer new insights into adaptation to global change? Evolutionary Applications **10**:267-275.

Church, J. A., P. U. Clark, A. Cazenave, J. M. Gregory, S. Jevrejeva, A. Levermann, M. A. Merrifield, G. A. Milne, R. S. Nerem, P. D. Nunn, A. J. Payne, W. T. Pfeffer, D. Stammer, and A. S. Unnikrishnan. 2013. IPCC Fifth Assessment Report (AR5), Climate Change 2013: The Physical Science Basis.1-124.

Consortium, U. 2018. UniProt: the universal protein knowledgebase. . Nucleic acids research **46**:2699.

Coordinators, N. R. 2016. Database resources of the national center for biotechnology information. Nucleic acids research.

Craft, C., J. Clough, J. Ehman, S. Joye, R. Park, S. Pennings, H. Guo, and M. Machmuller. 2009. Forecasting the effects of accellerated sea-level rise on tidal marsh ecosystem services Frontiers in Ecology and the Environment **7**:73-78.

Cunningham, H. R., L. J. Rissler, L. B. Buckley, and M. C. Urban. 2016. Abiotic and biotic constraints across reptile and amphibian ranges. Ecography **39**:1-8.

Darwin, C. 1859. On The Origin of Species by Means of Natural Selection, or Preservation of Favoured Races in the Struggle for Life. John Murray, London.

Dawson, D. C., and X. Liu. 2009. Osmoregulation: Some principals of water and solute transport.*in* D. H. Evans, editor. Osmotic and Ionic Regulation. CRC Press, Boca Raton, FL.

Day, J. W., R. R. Christian, D. M. Boesch, A. Yáñez-Arancibia, J. Morris, R. R. Twilley, L. Naylor, L. Schaffner, and C. Stevenson. 2008. Consequences of Climate Change on the Ecogeomorphology of Coastal Wetlands. Estuaries and Coasts **31**:477-491.

DeFaveri, J., and J. Merila. 2013. Local Adaptation to Salinity in the three-spined stickleback. Journal of Evolutionary Biology **27**:290-302.

Evans, T. G. 2015. Considerations for the use of transcriptomics in identifying the 'genes that matter' for environmental adaptation. Journal of Experimental Biology **218**:1925-1935.

Evans, T. G., and G. E. Hofmann. 2012. Defining the limits of physiological plasticity: how gene expression can assess and predict the consequences of ocean change. Philosophical Transactions of the Royal Society B: Biological Sciences **367**:1733-1745.

Evans, T. G., and G. N. Somero. 2008. A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish *Gillichthys mirabilis*: osmosensors to effectors. Journal of Experimental Biology **211**:3636-3649.

Forsman, A. 2015. Rethinking phenotypic plasticity and its consequences for individuals, populations and species. Heredity **115**:276-284.

Gienapp, P., C. Teplitsky, J. S. Alho, J. A. Mills, and J. Merilä. 2008. Climate change and evolution: disentangling environmental and genetic responses. Molecular ecology **17**:167-178.

Gomez-Mestre, I., and M. Tejado. 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. Evolution **57**:1889-1899.

Gomulkiewicz, R., and R. D. Holt. 1995. When does evolution by natural selection prevent extinction? . Evolution **49**:201-207.

Gomulkiewicz, R., R. D. Holt, and M. Barfield. 1999. The effects of density dependence and immigration on local adaptation and niche evolution in a black-hole sink environment. Theoretical population biology **55**:283-296.

Gonzalez, A., O. Ronce, R. Ferriere, and M. E. Hochberg. 2013. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**:20120404.

Gordon, M. S., K. Schmidt-Nielsen, and H. M. Kelly. 1961. Osmotic regulation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **38**:659-678.

Gordon, M. S., and V. A. Tucker. 1965. Osmotic regulation in the tadpoles of the crab-eating frog (*Rana cancrivora)* Journal of Experimental Biology **42**:437-445.

Gordon, M. S., and V. A. Tucker. 1968. Further observations on the physiology of salinity adaptation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **49**:185-193.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica:183-190.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, and Z. Chen. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. . Nature biotechnology **29**:644.

Hammond, S. A., R. L. Warren, B. P. Vandervalk, E. Kucuk, H. Khan, E. A. Gibb, P. Pandoh, H. Kirk, Y. Zhao, M. Jones, and A. J. Mungall. 2017. The North American bullfrog draft genome provides insight into hormonal regulation of long noncoding RNA. Nature Communications **8**:1433.

Harris, S. L., and A. J. Levine. 2005. The p53 pathway: positive and negative feedback loops. Oncogene **24**:2899.

Hart, B. T., P. Bailey, R. Edwards, K. Hortle, K. James, A. McMahon, C. Meredith, and S. K. 1991. A review of the salt sensitivity of the Australian fresh-water biota. . Hydrobiologia **210**.

Havird, J. C., R. P. Henry, and A. E. Wilson. 2013. Altered expression of Na(+)/K(+)-ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: a meta-analysis of 59 quantitative PCR studies over 10 years. Comp Biochem Physiol Part D Genomics Proteomics **8**:131-140.

Hendry, A. P. 2015. Key Questions on the Role of Phenotypic Plasticity in Eco-Evolutionary Dynamics. Journal of Heredity:esv060.

Herbert, E. R., P. Boon, A. J. Burgin, S. C. Neubauer, R. B. Franklin, M. Ardón, K. N. Hopfensperger, L. P. Lamers, and P. Gell. 2015. A global perspective on wetland salinization: ecological consequences of a growing threat to freshwater wetlands. Ecosphere **6**:1-43.

Hildebrandt, J. 1997. Changes in Na+/K+-ATPase expression during adaptive cell differentiation in avian nasal salt gland. Journal of Experimental Biology **200**:1895-1904.

Hillyard, S., N. Møbjerg, S. Tanaka, and E. Larsen. 2009a. Osmotic and ion regulation in amphibians. Osmotic and ionic regulation: Cells and animals:367-441.

Hillyard, S. D., N. Møbjerg, S. Tanaka, and E. H. Larsen. 2009b. Osmotic Regulation in Amphibians.*in* D. H. Evans, editor. Osmotic and ionic Regulation. CRC Press, Boca Raton, FL.

Holt, R., and R. Gomulkiewicz. 2004. Conservation implications of niche conservatism and evolution in heterogeneous environments. Pages 244-264 Evolutionary Conservation Biology. Cambridge University Press.

Holt, R. D., M. Barfield, and R. Gomulkiewicz. 2004a. Temporal variation can facilitate niche evolution in harsh sink environments. The American Naturalist **164**:187-200.

Holt, R. D., T. M. Knight, and M. Barfield. 2004b. Allee effects, immigration, and the evolution of species' niches. The American Naturalist **163**:253-262.

Hopkins, G. R., and J. E. D. Brodie. 2015. Occurrence of Amphibians in Saline Habitats: A Review and Evolutionary Perspective. Herpetological Monographs **29**:1-27.

Hsu, W.-T., C.-S. Wu, J.-C. Lai, Y.-K. Chiao, C.-H. Hsu, and Y.-C. Kam. 2012. Salinity acclimation affects survival and metamorphosis of crab-eating frog tadpoles. Herpetologica **68**:14-21.

Huaiyu, M., X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, and P. D. Thomas. 2016. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res.

Huaiyu, M., A. Muruganujan, J. T. Casagrande, and P. D. Thomas. 2013. Large-scale gene function analysis with the PANTHER classification system. Nature Protocols **8**:1551 - 1566.

Jaenisch, R., and A. Bird. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature genetics **33**:245.

James, K. R., B. Cant, and T. Ryan. 2003. Responses of freshwater biota to rising salinity levels and implications for saline water management: a review. Australian Journal of Botany **51**:703-713.

Kannan, S., J. Hui, K. Mazooji, L. Pachter, and D. Tse. 2016. Shannon: An information-optimal de novo RNA-Seq assembler. . bioRxiv:p.039230.

Kassahn, K. S., R. H. Crozier, H. O. Pörtner, and M. J. Caley. 2009. Animal performance and stress: responses and tolerance limits at different levels of biological organisation. . Biological Reviews **84**:277-292.

Kassambara, A., and M. Kosinski. 2018. survminer: Drawing survival curves using 'ggplot2'. R, R statistical environment.

Kaushal, S. S., G. E. Likens, M. L. Pace, R. M. Utz, S. Haq, J. Gorman, and M. Grese. 2018. Freshwater salinization syndrome on a continental scale. PNAS:p.201711234.

Kefford, B. J., G. L. Hickey, A. Gasith, E. Ben-David, J. E. Dunlop, C. G. Palmer, K. Allan, S. C. Choy, and C. Piscart. 2012. Global scale variation in the salinity sensitivity of riverine macroinvertebrates: Eastern Australia, France, Israel and South Africa. PLOS one **7**: p.e35224.

Kemp, A. C., B. P. Horton, S. J. Culver, D. R. Corbett, O. v. d. Plassche, W. R. Gehrels, B. C. Douglas, and A. C. Parnell. 2009. Timing and magnitude of recent accelerated sea-level rise Geology **37**:1035-1038.

Kirkpatrick, M., and R. Lande. 1989. The evolution of maternal characters. Evolution **1989**:485-503.

Kopp, R. E., B. P. Horton, A. C. Kemp, and C. Tebaldi. 2015. Past and future sea-level rise along the coast of North Carolina, USA. Climatic Change **132**:693-707.
Kültz, D. 2005. Molecular and evolutionary basis of the cellular stress response. Annual Review of Physiology **67**:225-257.
Kusakabe, M., A. Ishikawa, M. Ravinet, K. Yoshida, T. Makino, A. Toyoda, A. Fujiyama, and J. Kitano. 2017. Genetic basis for variation in salinity tolerance between stickleback ecotypes. Molecular ecology **26**:304-319.
Lamichhaney, S., A. M. Barrio, N. Rafati, G. Sundström, C. J. Rubin, E. R. Gilbert, J. Berglund, A. Wetterbom, L. Laikre, M. T. Webster, and M. Grabherr. 2012. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. Proceedings of the National Academy of Sciences **109**:19345-19350.
Lande, R. 2009. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. Journal of Evolutionary Biology **22**:1435-1446.
Lee, C. E., M. Kiergaard, G. W. Gelembiuk, B. D. Eads, and M. Posavi. 2011. Pumping ions: rapid parallel evolution of ionic regulation following habitat invasions. Evolution **65**:2229-2244.
Lindsey, H. A., J. Gallie, S. Taylor, and B. Kerr. 2013. Evolutionary rescue from extinction is contingent on a lower rate of environmental change. Nature **494**:463-467.
Lorenz, J. J. 2014. A review of the effects of altered hydrology and salinity on vertebrate fauna and their habitats in northeastern Florida Bay. . Wetlands **34**.
Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. **15**:550.
Lu, H. A., T. X. Sun, T. Matsuzaki, X. H. Yi, J. Eswara, R. Bouley, M. McKee, and D. Brown. 2007. Heat shock protein 70 interacts with aquaporin-2 and regulates its trafficking. Journal of Biological Chemistry **282**:28721-28732.
Lynch, M., and R. Lande. 1993. Evolution and Extinction in response to environmental change. Pages 234-250. *in* P. M. Kareiva, editor. Biotic interactions and global change. Sinauer Associates.
Lynch, M., and R. Lande. 1998. The critical effective size for a genetically secure population. . Animal Conservation **1**:70-72.
Maclean, I. M., and R. J. Wilson. 2011. Recent ecological responses to climate change support predictions of high extinction risk. Proc Natl Acad Sci U S A **108**:12337-12342.
MacManes, M. D. 2014. On the optimal trimming of high-throughput mRNA sequence data. . Frontiers in Genetics **5**:1-7.
MacManes, M. D. 2017. The Oyster River Protocol: A Multi Assembler and Kmer Approach For de novo Transcriptome Assembly. .
Manda, A. K., A. S. Giuliano, and T. R. Allen. 2014. Influence of artificial channels on the source and extent of saline water intrusion in the wind tide dominated wetlands of southern Albemarle estuarine system (USA). Environmental Earth Science **71**:4409-4419.
Marini, F. 2018. pcaExplorer: Interactive Visualization of RNA-seq Data Using a Principal Components Approach.
Marshall, D. J., and T. Uller. 2007. When is a maternal effect adaptive? . Oikos **116**:1957-1963.
Martin, G., R. Aguile, J. Ramsayer, O. Kaltz, and O. Ronce. 2013. The probability of evolutionary rescue: towards a quantitative comparison between theory and evolution experiments. Phil. Trans. R. Soc. B **368**:20120088.
McCormick, S. D., and D. Bradshaw. 2006. Hormonal control of salt and water balance in Vertebrates. Comparative Endocrinology **147**:3-8.

McNab, B. K. 2002. The physiological ecology of vertebrates: a view from energetics. Cornell University Press.
Merilä, J., and A. P. Hendry. 2014. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. Evolutionary Applications **7**:1-14.
Michener, W. K., E. R. Blood, K. L. Bildstein, M. M. Brinson, and L. R. Gardner. 1997. Climate change, hurricanes and tropical storms, and rising sea level in coastal wetlands. Ecological Applications **7**:770-801.
Miyamoto, K. I., S. Haito-Sugino, S. Kuwahara, A. Ohi, K. Nomura, M. Ito, M. Kuwahata, S. Kido, S. Tatsumi, I. Kaneko, and H. Segawa. 2011. Sodium-dependent phosphate cotransporters: lessons from gene knockout and mutation studies. Journal of pharmaceutical sciences **100**:3719-3730.
Montagna, P. A., M. Alber, P. Doering, and M. S. F. Connor. 2002. Freshwater inflow: science, policy, and management. Estuaries:1243-1245.
Moritz, C., and R. Agudo. 2013. The future of species under climate change: resilience or decline? Science **341**:504-508.
Morris, J. T., P. V. Sundarshwar, C. T. Nietch, B. Kjerfve, and D. R. Cahoon. 2002. Responses of coastal wetlands to rising sea level. Ecology **83**:2869-2877.
Mosesson, M. W. 2005. Fibrinogen and fibrin structure and functions. Journal of Thrombosis and Haemostasis **3**:1894–1904.
Mulligan, R. P., J. P. Walsh, and H. M. Wadman. 2012. Storm surge and surface waves in a large and shallow estuarine system during the passage of a hurricane Journal of Waterway, Port, Coastal, and Ocean Engineering.
Nonaka, E., R. Svanbäck, X. Thibert-Plante, G. Englund, and Å. Brännström. 2015. Mechanisms by Which Phenotypic Plasticity Affects Adaptive Divergence and Ecological Speciation. The American Naturalist **186**:E126-143.
Nunney, L. 2015. Adapting to a Changing Environment: Modeling the Interaction of Directional Selection and Plasticity. Journal of Heredity.
Ogushi, Y., G. Akabane, T. Hasegawa, H. Mochida, M. Matsuda, M. Suzuki, and S. Tanaka. 2010. Water adaptation strategy in anuran amphibians: molecular diversity of aquaporin. Endocrinology **151**:165-173.
Ogushi, Y. A., G.; Hasegawa, T.; Mochida, H.; Matsuda, M.; Suzuki, M.; Tanaka, S. 2010. Water Adaptation Strategy in Anuran Amphibians: Molecular Diversity of Aquaporin. Endocrinology **151**:165-173.
Pandey, R. N., S. Yaganti, S. Coffey, J. Frisbie, K. Alnajjar, and D. Goldstein. 2010a. Expression and immunolocalization of aquaporins HC-1, -2, and -3 in Cope's gray treefrog, Hyla chrysoscelis. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **157**:86-94.
Pandey, R. N., S. Yaganti, S. Coffey, J. Frisbie, K. Alnajjar, and D. Goldstein. 2010b. Expression and immunolocalization of aquaporins HC-1,-2, and-3 in Cope's gray treefrog,< i> Hyla chrysoscelis</i>. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **157**:86-94.
Pao, S. S., I. T. Paulsen, and M. H. Saier. 1998. Major facilitator superfamily. Microbiology and Molecular Biology Reviews **62**:1-34.
Parkinson, R. W. 1994. Sea-level rise and the fate of tidal wetlands Journal of Coastal Research **10**:987-989.

Pfennig, D. W., M. A. Wund, E. C. Snell-Rood, T. Cruickshank, C. D. Schlichting, and A. P. Moczek. 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends in Ecology & Evolution **25**:459-467.

Pinder, A. M., S. A. Halse, J. M. McRae, and R. J. Shiel. 2004. Aquatic invertebrate assemblages of wetlands and rivers in the weatbelt region of Western Australia. . Records of the Western Australian Museum Supplement **67**.

Piscart, C., J. C. Moreteau, and J. N. Beisel. 2005. Biodiversity and structure of macroinvertebrate communities along a small permanent salinity gradient (Meurthe River, France). Hydrobiologia **551**.

Räsänen, K., and L. Kruuk. 2007. Maternal effects and evolution at ecological timescales. Functional Ecology **21**:408-421.

Riginos, C., and C. W. Cunningham. 2005. Invited review: local adaptation and species segregation in two mussel (*Mytilus edulis*× *Mytilus trossulus*) hybrid zones. Molecular ecology **14**:381-400.

Rios-López, N. 2008. Effects of increased salinity on tadpoles of two anurans from a Caribbean coastal wetland in relation to their natural abundance. Amphibia-Reptilia **29**:7-18.

Ruiz-Jarabo, I., C. A. Gonzalez-Wevar, R. Oyarz, J. Fuentes, E. Poulin, C. Bertran, and L. Vargas-Chacoff. 2016. Isolation Driven Divergence in Osmoregulation in *Galaxias maculatus(*Jenyns, 1848) (Actinopterygii: Osmeriformes). PLOS one **11**:e0154766.

Saitoh, Y., Y. Ogushi, Y. Shibata, R. Okada, S. Tanaka, and M. Suzuki. 2014. Novel Vasotocin-Regulated Aquaporins Expressed in the Ventral Skin of Semiaquatic Anuran Amphibians: Evolution of Cutaneous Water-Absorbing Mechanisms. Endocrinology **155**:2166-2177.

Scheiner, S. M., M. Barfield, and R. D. Holt. 2017. The genetics of phenotypic plasticity: Genetic assimilation, the Baldwin effect, and evolutionary rescue. Ecology and evolution **7**:8788-8803.

Schuler, M. S., and R. A. Relyea. 2018. A Review of the Combined Threats of Road Salts and Heavy Metals to Freshwater Systems. BioScience **68**:327-335.

Shao, Y., L. J. Wang, L. Zhong, M. L. Hong, H. M. Chen, R. W. Murphy, D. D. Wu, Y. P. Zhang, and J. Che. 2015. Transcriptomes reveal the genetic mechanisms underlying ionic regulatory adaptations to salt in the crab-eating frog. Scientific reports **5**:17551.

Shibata, Y., T. Sano, N. Tsuchiya, R. Okada, H. Mochida, S. Tanaka, and M. Suzuki. 2014. Gene expression and localization of two types of AQP5 in Xenopus tropicalis under hydration and dehydration. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, **307**:R44-R56.

Simão, F. A., R. M. Waterhouse, P. Ioannidis, E. V. Kriventseva, and E. M. Zdobnov. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics **31**:3210-3212.

Smith-Unna, R., C. Boursnell, R. Patro, J. M. Hibberd, and S. Kelly. 2016. TransRate: reference-free quality assessment of de novo transcriptome assemblies. . Genome research **26**:1134 - 1144.

Song, L., and L. Florea. 2015. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. GigaScience **4**:48.

Stone, H. R., and J. R. Morris. 2014. DNA damage emergency: cellular garbage disposal to the rescue? Oncogene **33**:805.

Sun, Y. B., Z. J. Xiong, X. Y. Xiang, S. P. Liu, W. W. Zhou, X. L. Tu, L. Zhong, L. Wang, D. D. Wu, B. L. Zhang, and C. L. Zhu. 2015. Whole-genome sequence of the Tibetan frog

Nanorana parkeri and the comparative evolution of tetrapod genomes. . Proceedings of the National Academy of Sciences **112**:pp.E1257-E1262.

Suzuki, M., T. Hasegawa, Y. Ogushi, and S. Tanaka. 2007. Amphibian aquaporins and adaptation to terrestrial environments: a review. Comp Biochem Physiol A Mol Integr Physiol **148**:72-81.

Suzuki, M., Y. Shibata, Y. Ogushi, and R. Okada. 2015. Molecular machinery for vasotocin-dependent transepithelial water movement in amphibians: aquaporins and evolution. The Biological Bulletin **229**:109-119.

Suzuki, M., and S. Tanaka. 2009. Molecular and cellular regulation of water homeostasis in anuran amphibians by aquaporins. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **153**:231-241.

Takata, K., T. Matsuzaki, and Y. Tajika. 2004. Aquaporins: water channel proteins of the cell membrane. Progress in Histochemistry and Cytochemistry **39**:1-83.

Team, R. C. 2018. R: A Language and Environment for Statistical Computing.

Therneau, T. 2015. survival: A package for survival analysis in S. R statistical environmnet.

Titus, J. G., and C. Richman. 2001. Maps of lands vulnerable to sea level rise: modeled elevations along the US Atlantic and Gulf coasts. Climate Research **18**:205-228.

Travis, J. 1994. Evaluating the adaptive role of morphological plasticity. Ecological Morphology:99-122.

Turner, M. D., B. Nedjai, T. Hurst, and D. J. Pennington. 2014. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research **1843**:2563-2582.

Uchiyama, M., and N. Konno. 2006. Hormonal regulation of ion and water transport in anuran amphibians. Gen Comp Endocrinol **147**:54-61.

Uchiyama, M., and H. Yoshizawa. 1992. Salinity tolerance and structure of external and internal gills in tadpoles of the crab-eating frog, Rana cancrivora. Cell and Tissue Research **267**:35-44.

Urban, M. C., G. Bocedi, A. P. Hendry, J.-B. Mihoub, G. Pe'er, A. Singer, J. R. Bridle, L. G. Crozier, L. De Meester, W. Godsoe, A. Gonzalez, J. J. Hellmann, R. D. Holt, A. Huth, K. Johst, C. B. Krug, P. W. Leadley, S. C. F. Palmer, J. H. Pantel, A. Schmitz, P. A. Zollner, and J. M. J. Travis. 2016. Improving the forecast for biodiversity under climate change. Science **353**.

Urban, M. C., J. L. Richardson, and N. A. Reidenfelds. 2014. Plasticity and genetic adaptation mediate amphibian and reptile responses to climate change. Evolutionary Applications **7**:88-103.

Vander Wal, E., D. Garant, M. Festa-Bianchet, and F. Pelletier. 2013. Evolutionary rescue in vertebrates: evidence, applications and uncertainty. . Phil. Trans. R. Soc. B **368**: p.20120090.

Via, S., and R. Lande. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution:505-522.

Wallace, A. R. 1859. On the Tendency of Varieties to depart indefinitely from the Original Type. John Murray, London.

West-Eberhard, M. J. 2003. Developmental plasticity and evolution. Oxford University Press, Oxford, England.

Whitehead, A., F. Galvez, S. Zhang, L. M. Williams, and M. F. Oleksiak. 2011a. Functional Genomics of Physiological Plasticity and Local Adaptation in Killifish. Journal of Heredity **102**:499-511.

Whitehead, A., J. L. Roach, S. Zhang, and F. Galvez. 2011b. Genomic mechanisms of evoloved physiological plasticity in killifish distributed along an environmental salinity gradient. Proceedings of the National Academy of Science **108**:6193-6198.

Whitehead, A., J. L. Roach, S. Zhang, and F. Galvez. 2012. Salinity- and population-dependent genome regulatory response during osmotic acclimation in the killifish (*Fundulus heteroclitus*) gill. Journal of Experimental Biology **215**:1293-1305.

Williams, K., K. C. Ewel, R. P. Stumpf, F. E. Putz, and T. W. Workman. 1999. Sea-level rise and coastal forest retreat on the west coast of florida, USA Ecology **80**:2045-2063.

Williams, K., M. MacDonald, and L. d. S. L. Sternberg. 2003. Interactions of storm, drought, and sea-level rise on coastal forest- A case study. Journal of Coastal Research **19**:1116-1121.

Williams, S. J. 2013. Sea Level Rise Implications for Coastal Regions Journal of Coastal Research **63**:184-196.

Willmer, P., G. Stone, and I. Johnston. 2005. Environmental Physiology of Animals. 2nd edition. Blackwell Publishing, Oxford, England.

Wu, C. S., W. K. Yang, T. H. Lee, I. Gomez-Mestre, and Y. C. Kam. 2014. Salinity acclimation enhances salinity tolerance in tadpoles living in brackish water through increased Na(+) , K(+) -ATPase expression. J Exp Zool A Ecol Genet Physiol **321**:57-64.

Wygoda, M. L., T. F. Dabruzzi, and W. A. Bennett. 2011. Cutaneous resistance to evaporative water loss in the Crab-eating frog (*Fejervarya cancrivora*) Journal of Herpetology **45**:417-420.

Figures and tables

Figure 4-1. Tadpole survival on the final day of acclimations according to final salinity. The light blue line refers to coastal tadpoles that were laid in freshwater, the dark blue line refers to coastal tadpoles that were laid in 4ppt water, the light green line denotes inland tadpoles that were laid in freshwater, and the dark green line indicates inland tadpoles that were laid in 4ppt water. The vertical line occurs at 8ppt to highlight differences in survival. At 8ppt, 91% (C.I. 86-94%) of coastal tadpoles from FW egg environment survived, compared to 73% (C.I. 66-78%) of coastal tadpoles hatched in 4ppt water. If laid in freshwater, 80% (C.I. 75-85%) of inland tadpoles survived while 77% (C.I. 70-83%) of inland tadpoles survived if laid in 4ppt water.

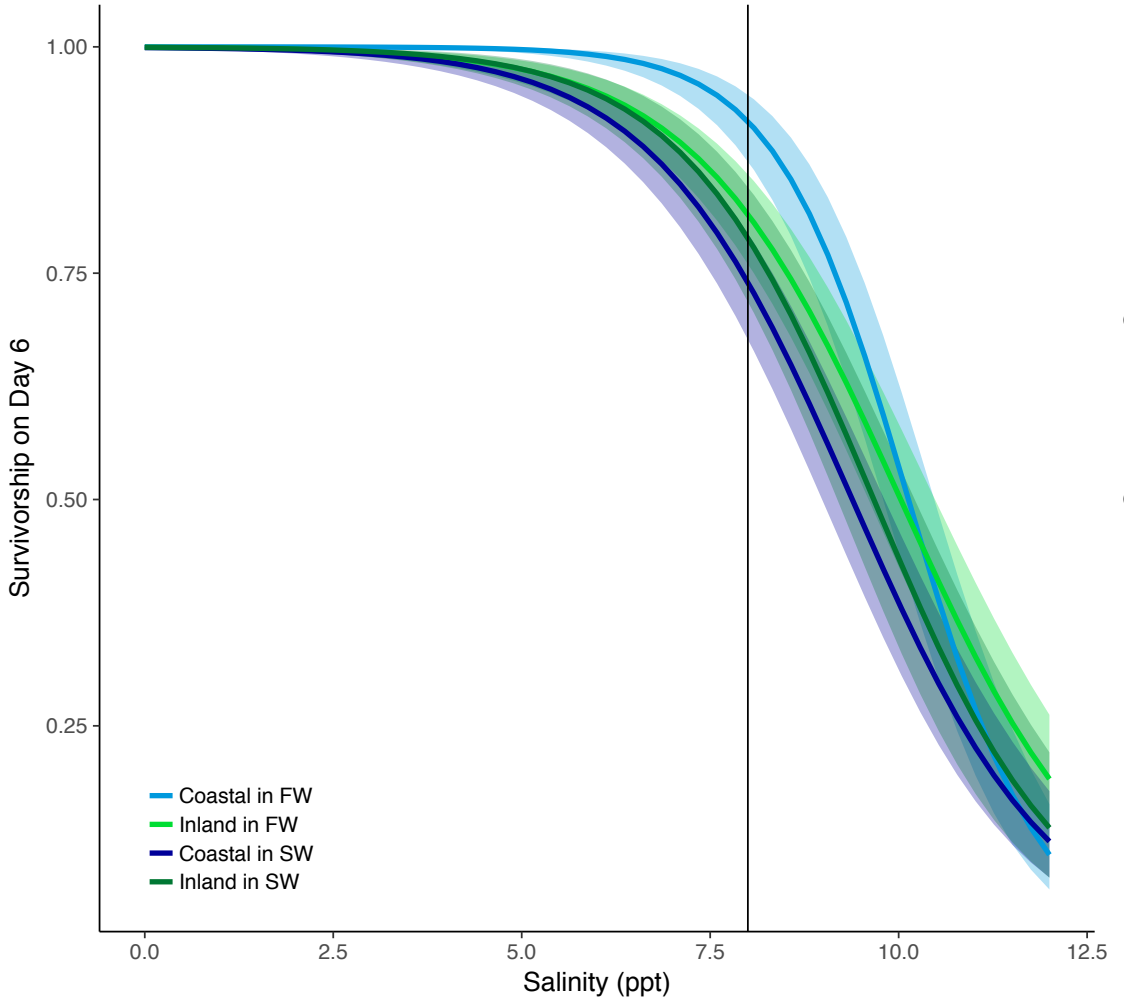


Figure 4-2. Survival through time according to the target salinity on the final day of the acclimations. Panels indicate the target salinity. All four treatments are shown on each panel through time in days. The light blue line refers to coastal tadpoles that were laid in freshwater, the dark blue line refers to coastal tadpoles that were laid in 4ppt water, the light green line denotes inland tadpoles that were laid in freshwater, and the dark green line indicates inland tadpoles that were laid in 4ppt water.

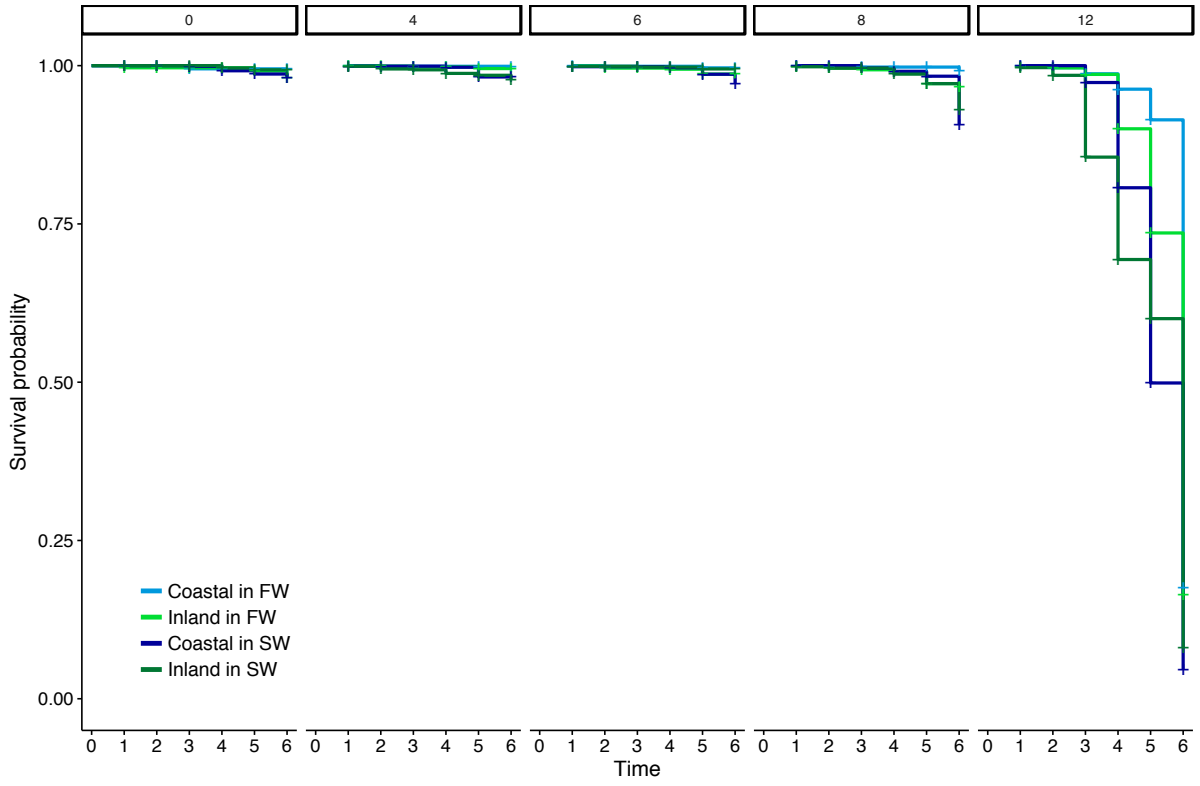


Figure 4-3. Wet mass of tadpoles according to environmental salinity on the final day of the acclimations. Lines are fitted means with dots depicting offset raw data points. The light blue line refers to coastal tadpoles that were laid in freshwater, the dark blue line refers to coastal tadpoles that were laid in 4ppt water, the light green line denotes inland tadpoles that were laid in freshwater, and the dark green line indicates inland tadpoles that were laid in 4ppt water.

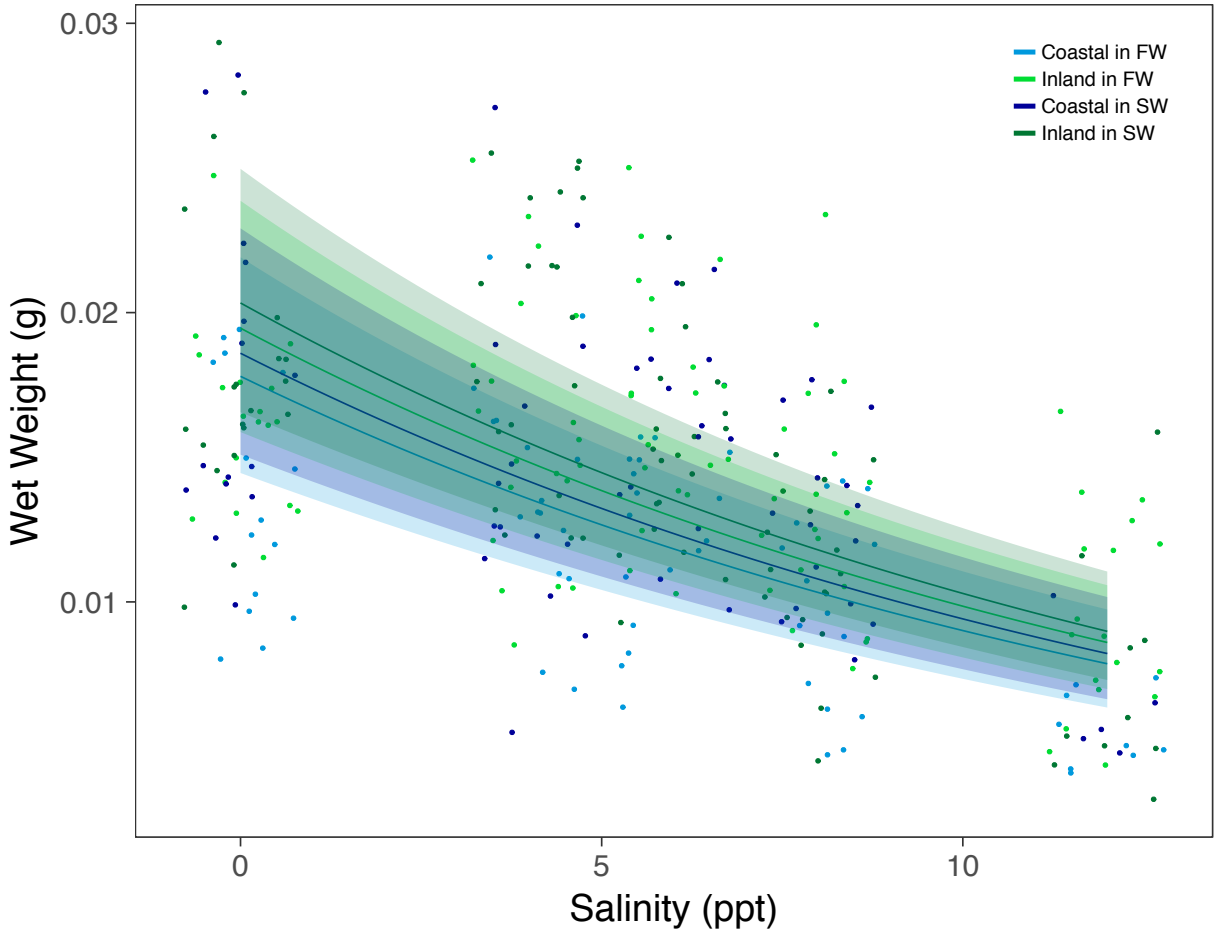


Figure 4-4. Length of tadpoles according to environmental salinity on the final day of the acclimations. Length is the tip of the snout to the tip of the tail in millimeters. Lines are fitted means with dots depicting offset raw data points. The light blue line refers to coastal tadpoles that were laid in freshwater, the dark blue line refers to coastal tadpoles that were laid in 4ppt water, the light green line denotes inland tadpoles that were laid in freshwater, and the dark green line indicates inland tadpoles that were laid in 4ppt water.

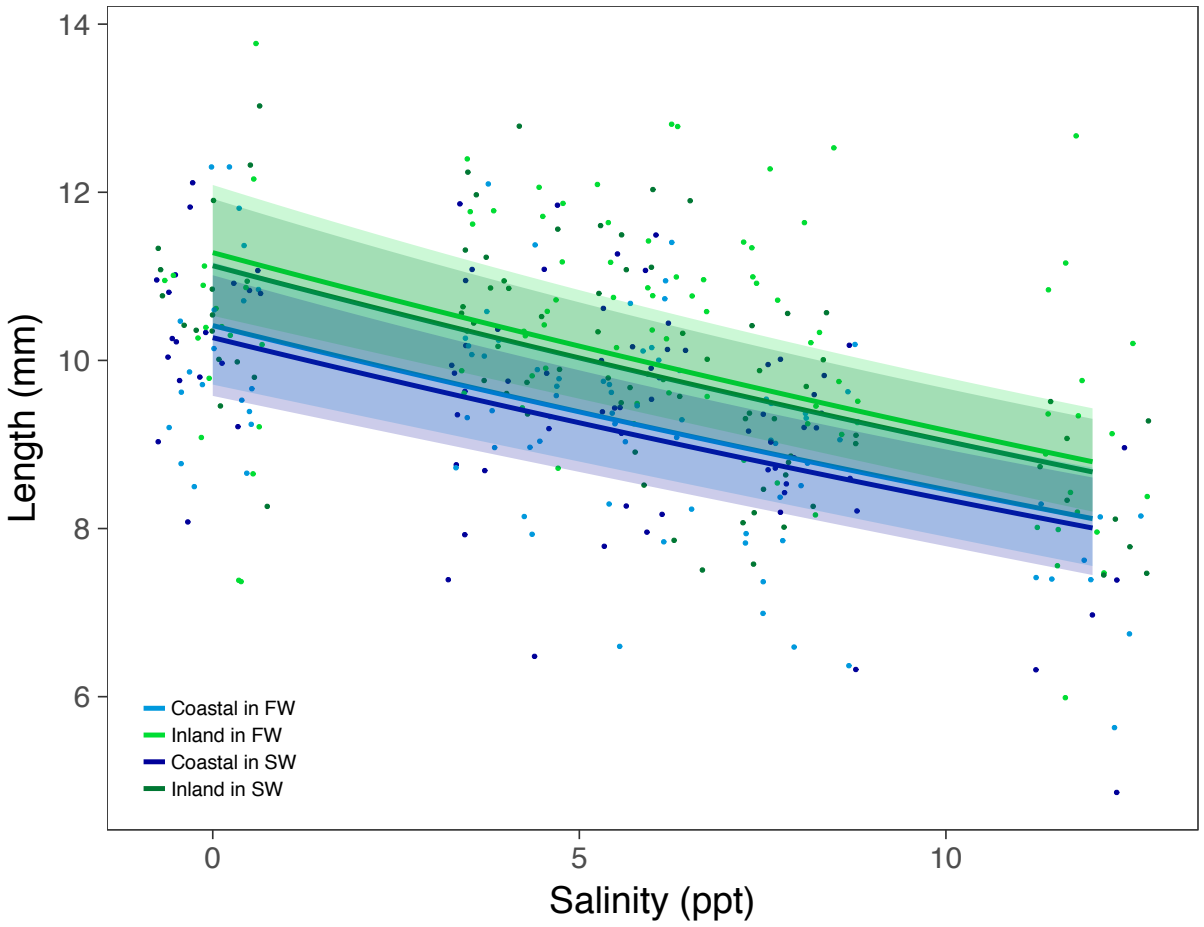


Figure 4-5. Plasma osmolality (mOsm/L) of tadpoles from different environmental salinities on the final day of the acclimations. Lines are fitted means with dots depicting raw offset data points. The light blue line refers to coastal tadpoles that were laid in freshwater, the dark blue line refers to coastal tadpoles that were laid in 4ppt water, the light green line denotes inland tadpoles that were laid in freshwater, and the dark green line indicates inland tadpoles that were laid in 4ppt water.

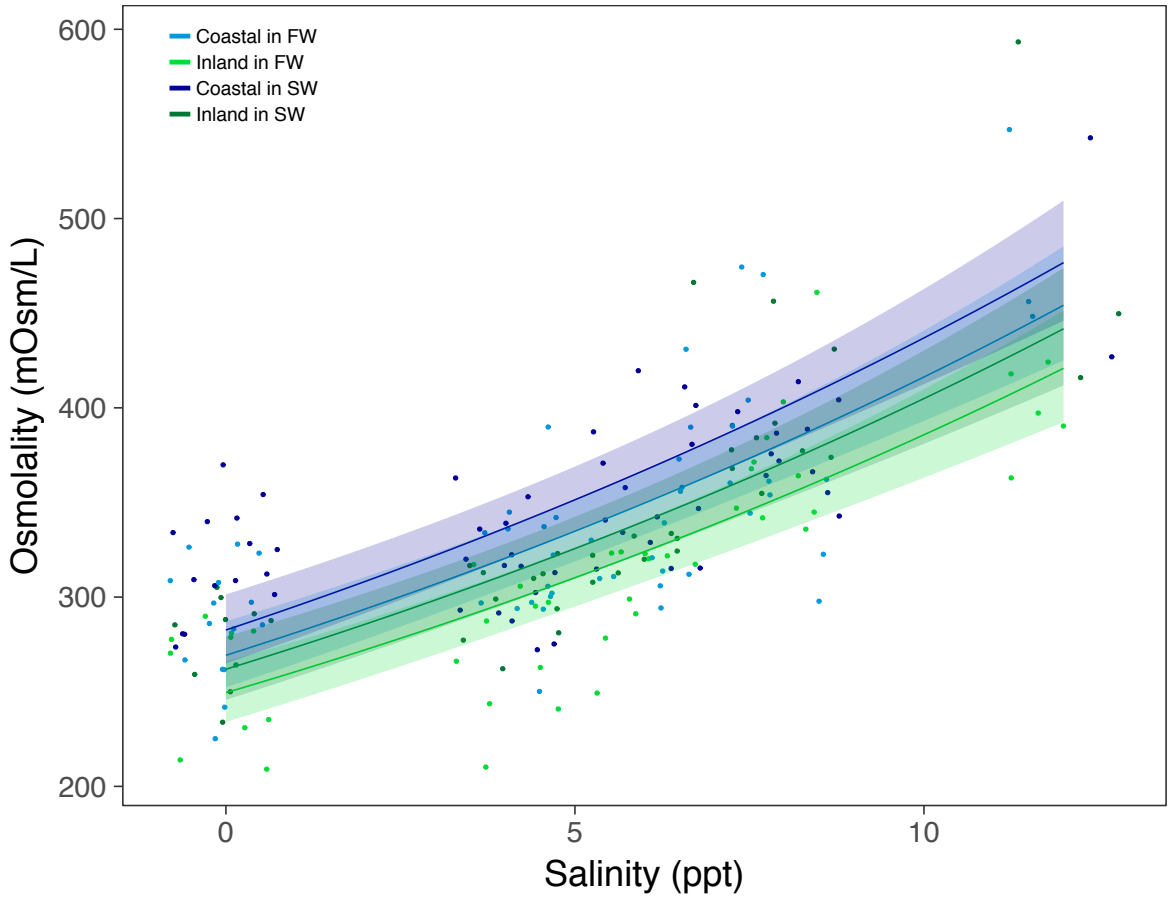


Figure 4-6. Principal Components Analysis showing differential expression patterns between coastal and inland populations. Green denotes coastal populations, while blue denotes inland. PC1 explains 10.25% of the variance, while PC2 explains 8.12%. Ellipses represent 95% confidence ellipse.

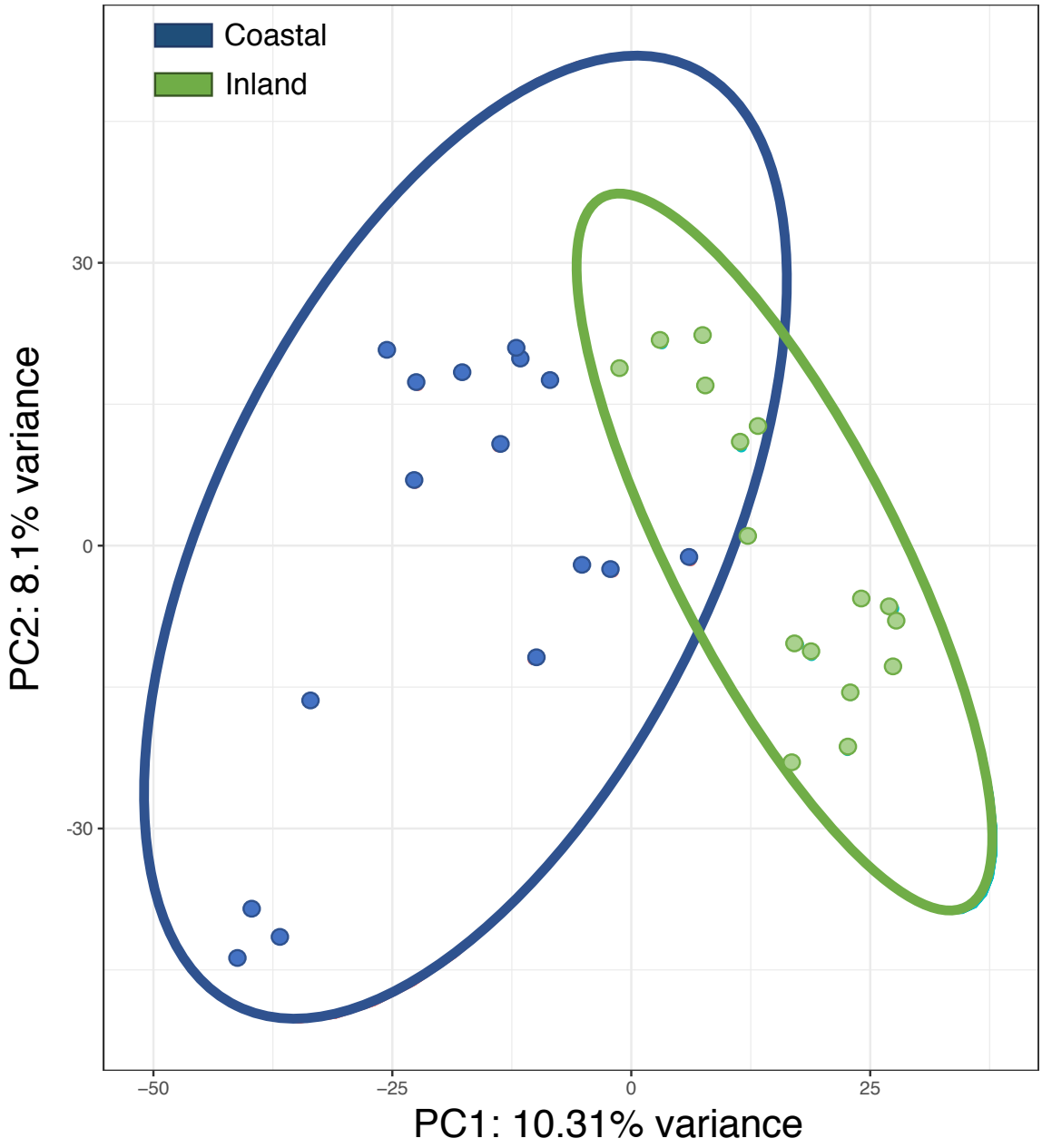


Figure 4-7. Heatmap showing differentially expressed genes between coastal and inland populations. The left panel shows genes that were the most upregulated in coastal populations relative to inland populations. The right panel shows genes that were the most downregulated in coastal populations compared to inland populations. The gene names are available in the supplementary material.

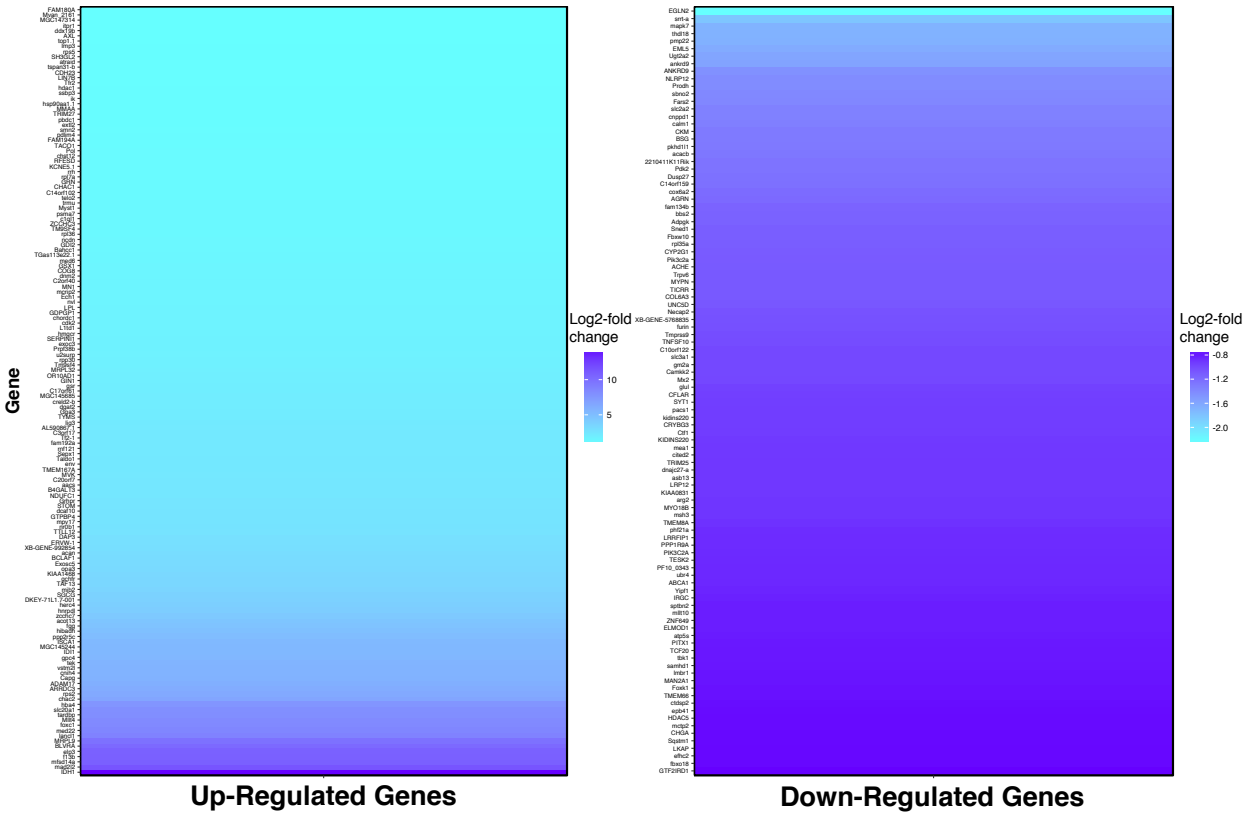


Figure 4-8. Heatmap showing differentially expressed genes between the three salinity contrasts. Gray space indicates that gene was not differentially expressed in that treatment. The first panel shows log2-fold change in gene expression in the 4ppt egg/4ppt tadpole vs. FW egg/FW tadpole treatment, the second panel shows log2-fold change in gene expression in the FW egg/FW tadpole vs. FW egg/6ppt tadpole treatment, and the third panel shows log2-fold change in gene expression in the 4ppt egg/6ppt tadpole vs. FW egg/6ppt tadpole treatment. In this plot, the log2-fold change is relative to the second treatment. For example, the gene adrm1-b is purple, which indicates that it is upregulated in the 4ppt egg/4ppt tadpole compared to the FW egg/FW tadpole treatment.

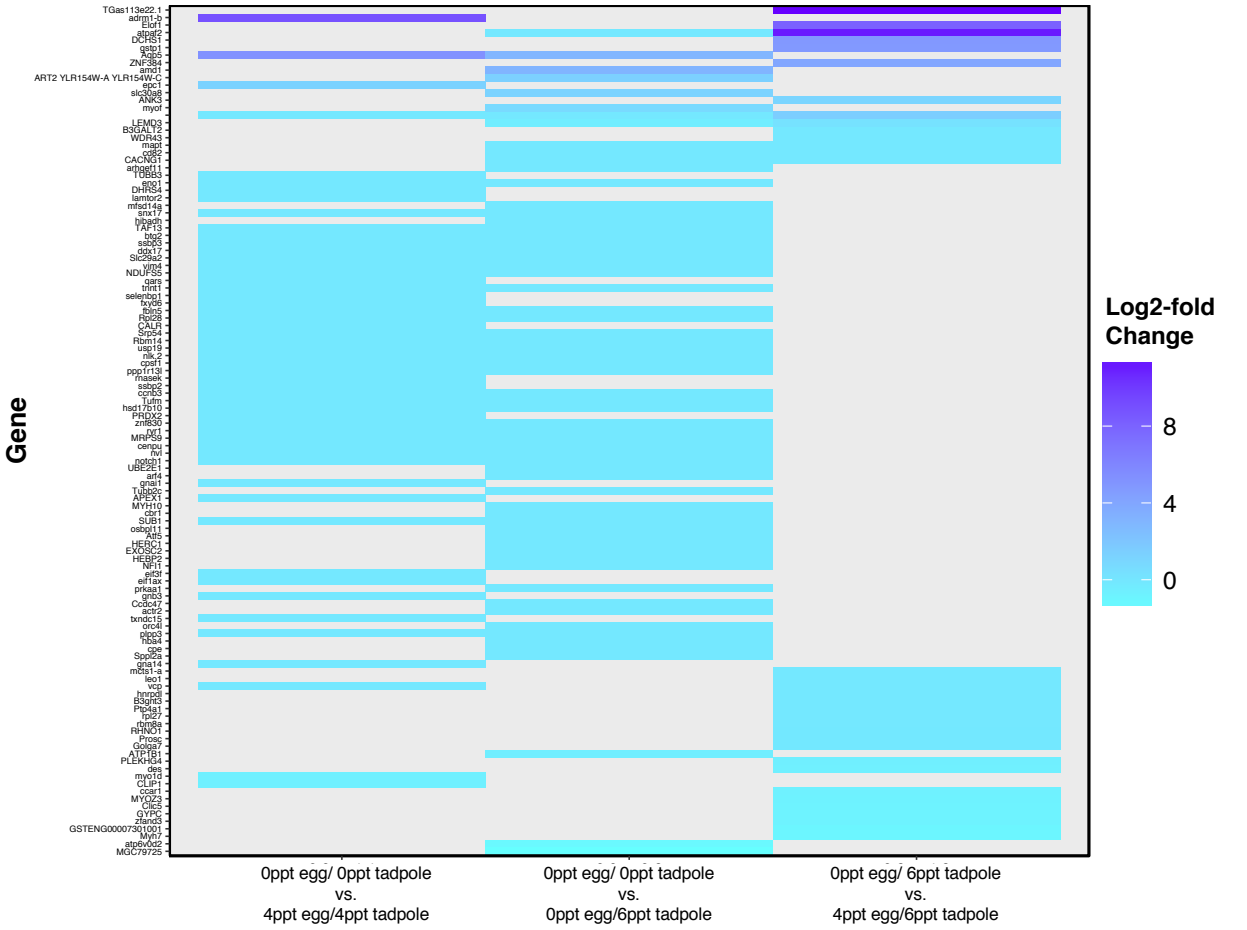


Figure 4-9. Gene Ontology of differentially expressed annotated genes between coastal and inland populations. The top panel shows the number of genes classified into different molecular functions, while the bottom panel shows the genes that were classified into biological processes. Sometimes the same genes could be categorized into both molecular functions and biological processes.

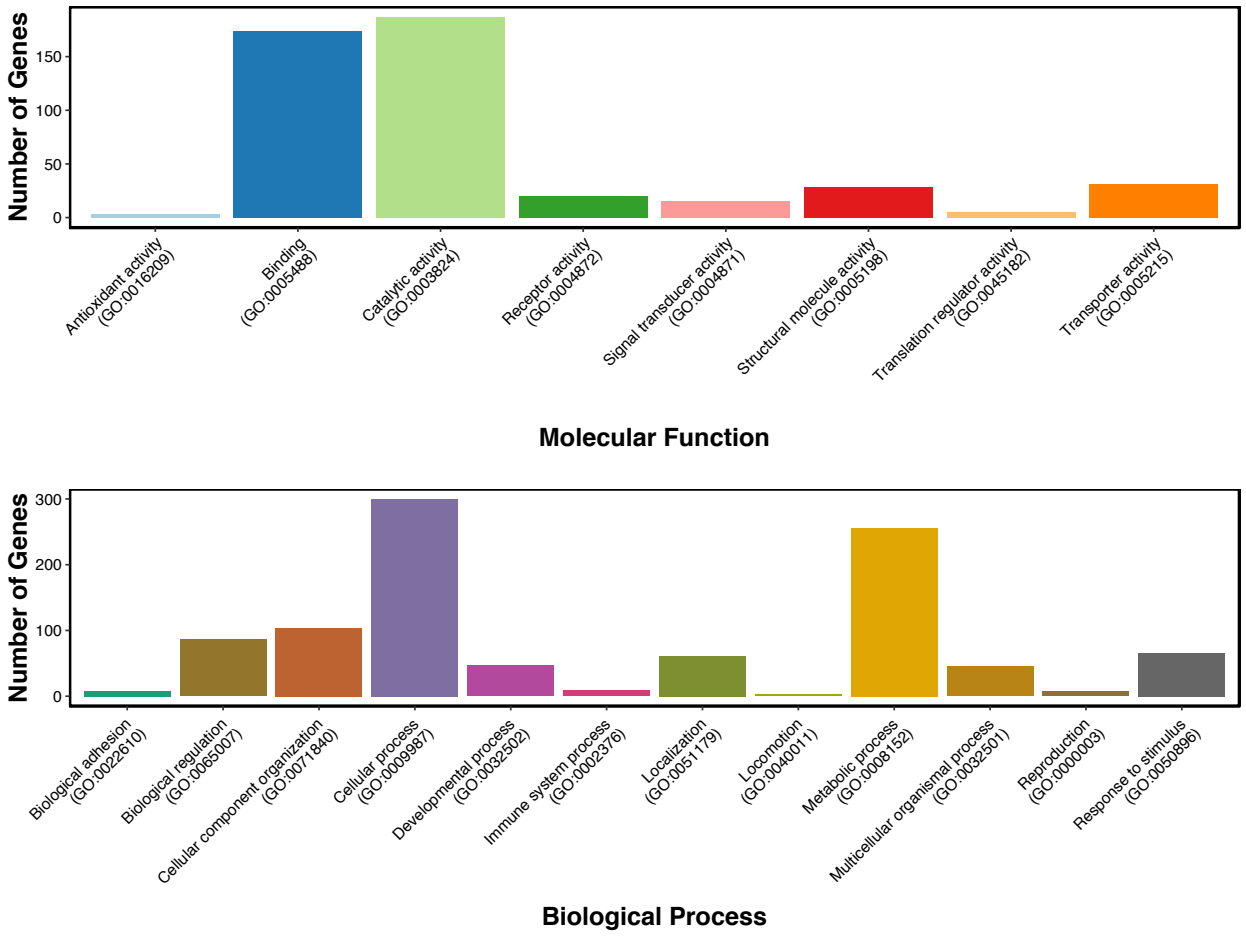


Figure 4-10. Gene Ontology of differentially expressed annotated genes between FW egg/FW tadpole vs. 4ppt egg/4ppt tadpole contrast. The top panel shows the number of genes classified into different molecular functions, while the bottom panel shows the genes that were classified into biological processes. Sometimes the same genes could be categorized into both molecular functions and biological processes.

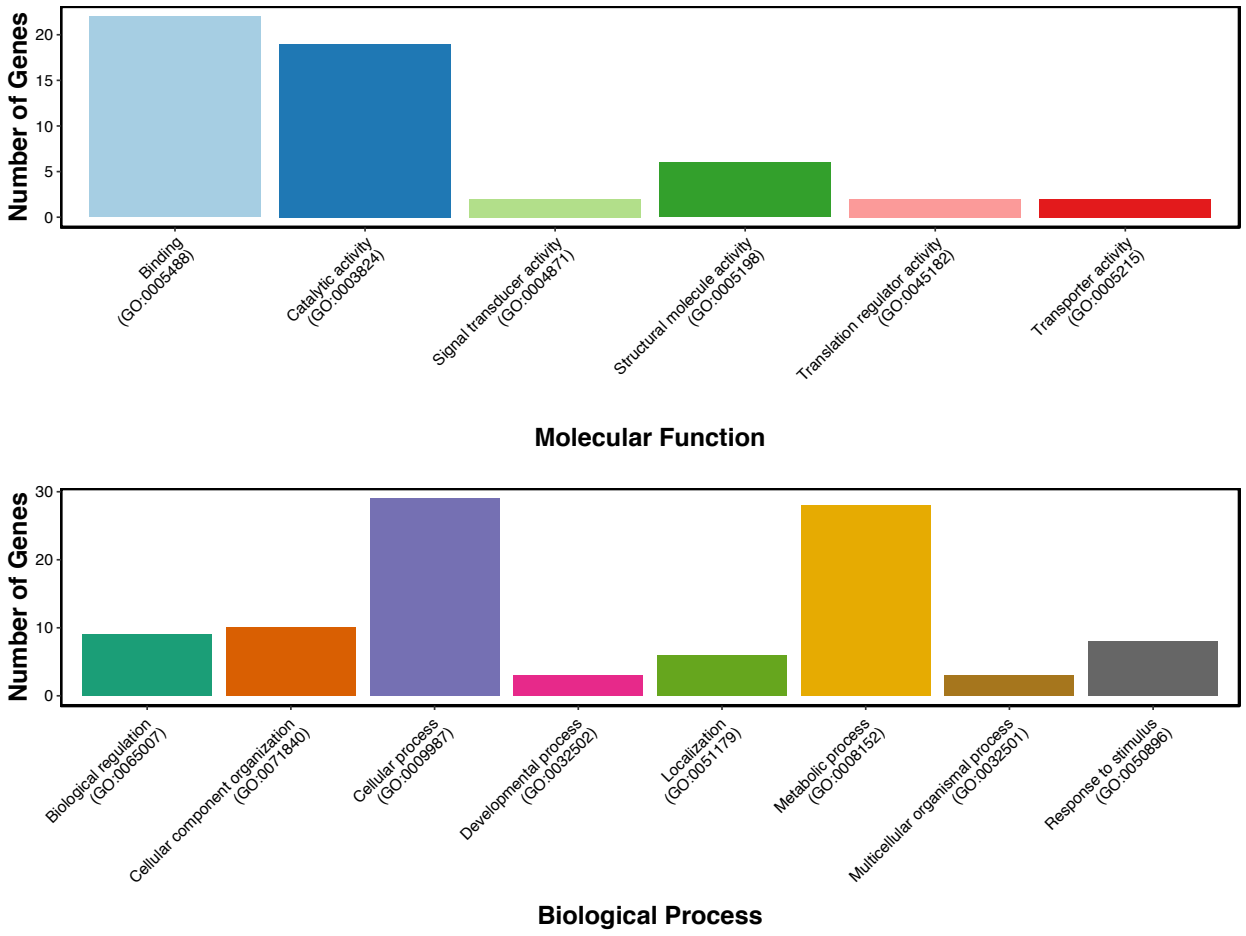


Figure 4-11. Gene Ontology of differentially expressed annotated genes between FW egg/FW tadpole vs. FW egg/6ppt tadpole contrast. The top panel shows the number of genes classified into different molecular functions, while the bottom panel shows the genes that were classified into biological processes. Sometimes the same genes could be categorized into both molecular functions and biological processes.

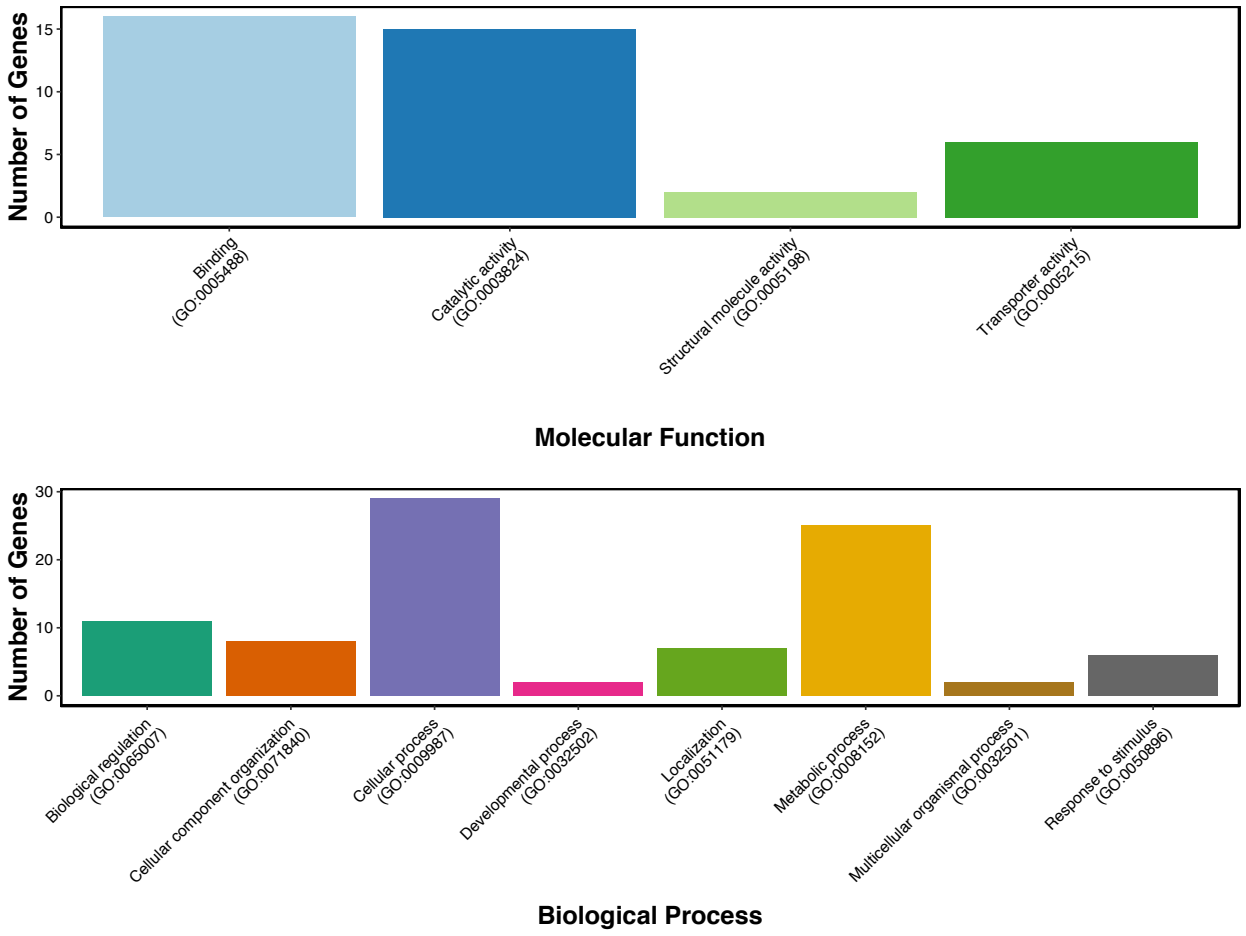


Figure 4-12. Gene Ontology of differentially expressed annotated genes between FW egg/6ppt tadpole vs. 4ppt egg/6ppt tadpole contrast. The top panel shows the number of genes classified into different molecular functions, while the bottom panel shows the genes that were classified into biological processes. Sometimes the same genes could be categorized into both molecular functions and biological processes.

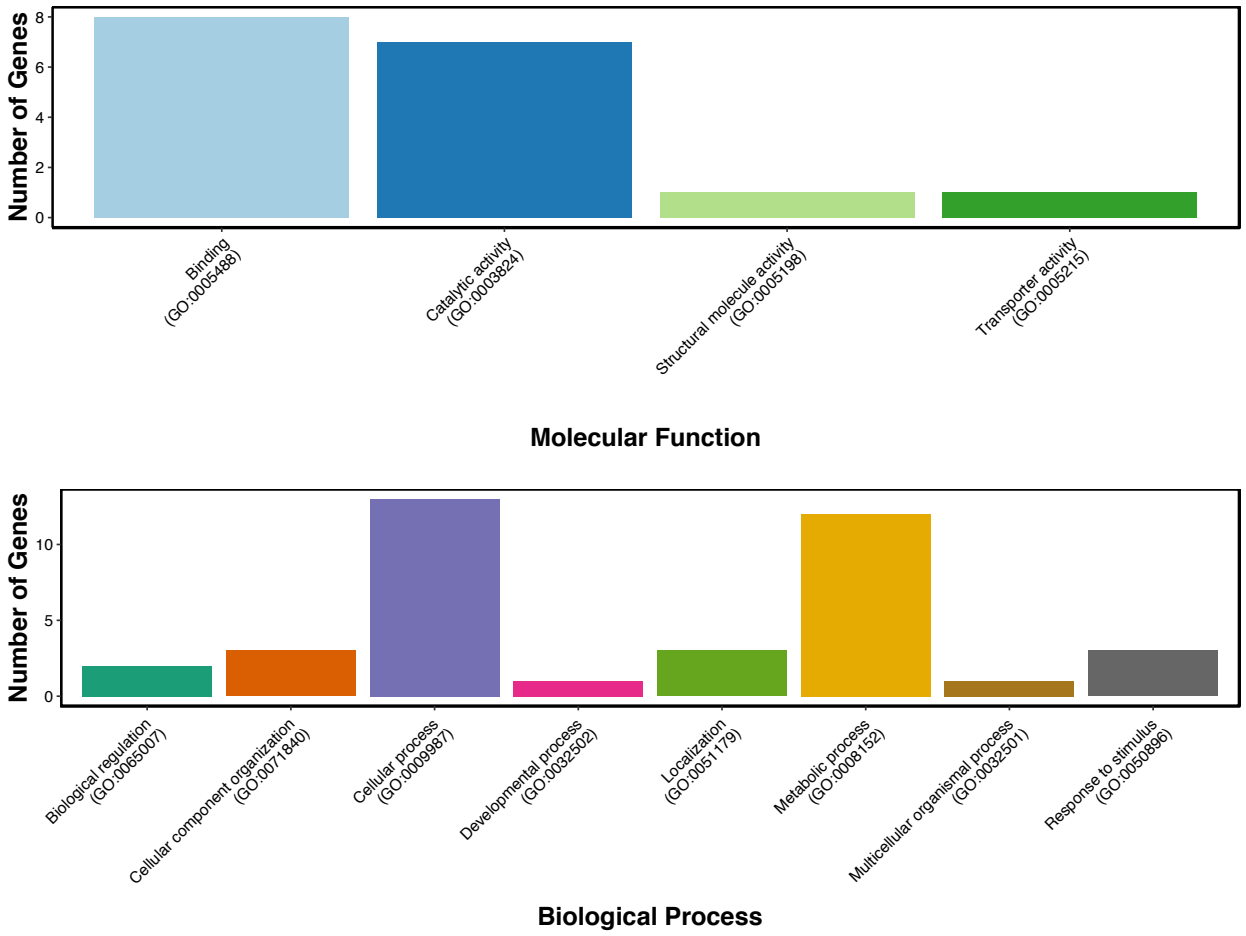


Figure 4-13. Differentially expressed genes that contribute to binding and transporter activity between coastal and inland populations. The top panel shows the log2-fold change in genes classified into binding functions, while the bottom panel shows the log2-fold change in genes classified as transporter genes. Log2-fold changes reflect expression in coastal populations compared to inland populations.

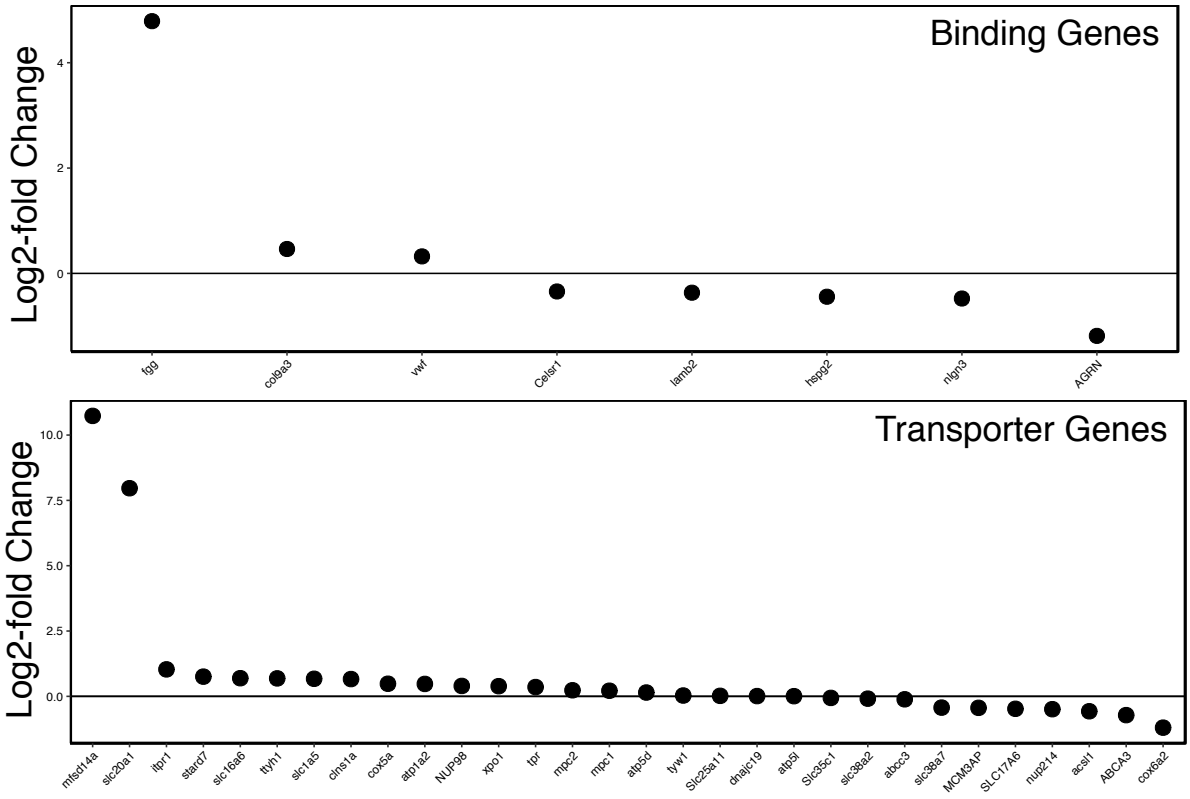
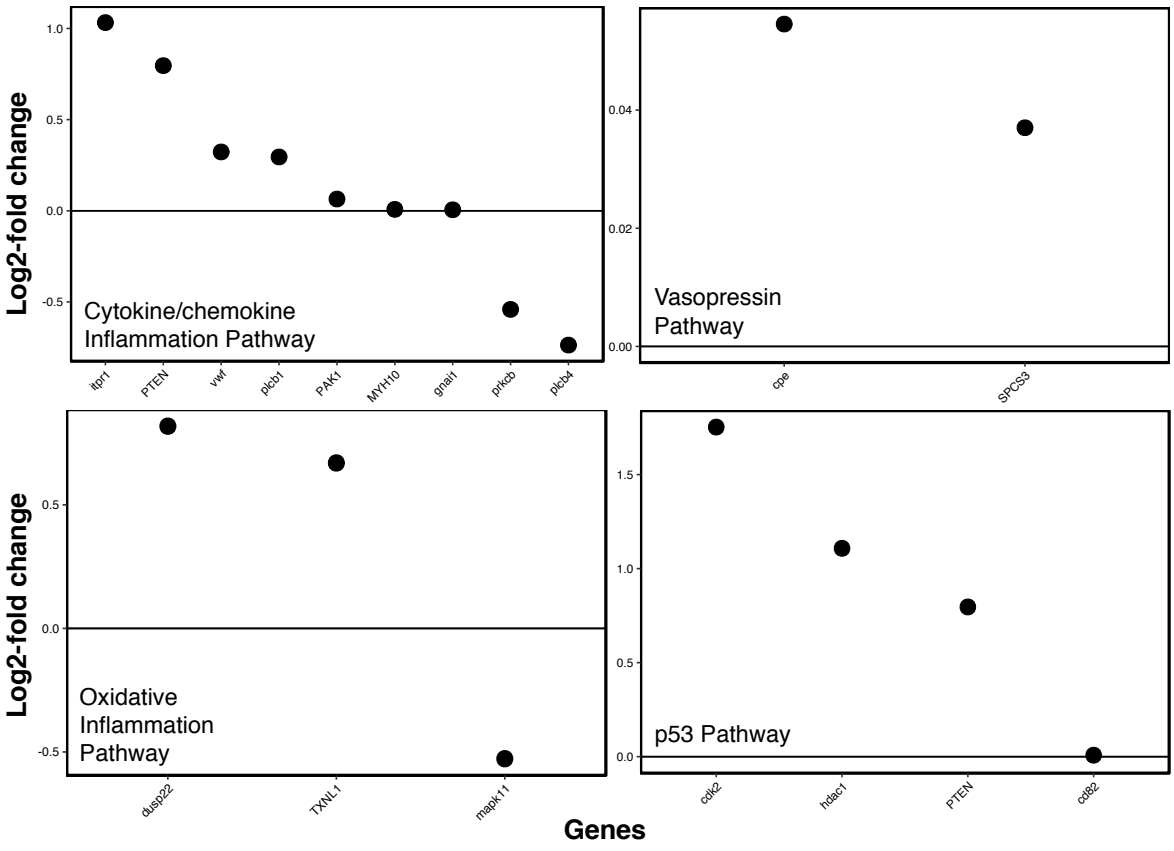


Figure 4-14. Differentially expressed genes that contribute to stress pathways between coastal and inland populations. The top panel shows the log2-fold change in genes classified into binding functions, while the bottom panel shows the log2-fold change in genes classified as transporter genes. The top left plot shows the genes involved in the cytokine/chemokine inflammation pathway. The top right plot shows genes involved in constructing vasopressin hormone. Bottom left shows genes involved in the oxidative inflammation pathway, and the bottom right shows the genes involved in the p53 pathway. In each of these plots, a number above zero (horizontal line) indicates these genes were upregulated in coastal populations relative to inland.



Supplementary Material:

4S-1**: List of most upregulated genes in coastal populations.** This data is meant to assist in the interpretation of the left-hand panel in Figure 7, in which the gene names and values may be too small to interpret.

Gene	Log2- Fold Change
aacs	2.51612961
acan	3.14156541
acot13	4.64053824
ADAM17	5.99707333
AL590867.1	2.34053523
ARRDC3	6.22478525
atraid	1.05259307
AXL	1.03680187
B4GALT3	2.5199182
Bahcc1	1.47303159
BCLAF1	3.21555883
BLVRA	10.2101753
C14orf102	1.31391481
C17orf61	2.06462859
c1ql1	1.38737028
C20orf7	2.5028449
C2orf40	1.60687376
C3orf17	2.35892487
Capg	5.83948463
CDH23	1.08147831
cdk2	1.75260823
CHAC1	1.29645433
chac2	6.65805672
chordc1	1.74177628
chst12	1.23735167
cnih4	5.75980783
COG8	1.55225685
creld2-b	2.11311301
DAP3	2.93387416
dcaf10	2.69384576
ddx19b	1.03526976

dgat2	2.12144593
DKEY-71L1.7-001	3.91460264
dnm2	1.58845931
Ech1	1.63850649
elp3	10.6605819
env	2.43216722
ERVW-1	2.96345885
exoc3	1.8309241
Exosc5	3.26779939
extl2	1.13011251
f13b	10.7000484
FAM180A	1.0061252
fam192a	2.38895734
FAM194A	1.21535986
fgg	4.78913012
foxc1	8.37939084
gchfr	3.49099932
GDI2	1.43613093
GDPGP1	1.74002556
Gga3	2.18638075
GIN1	2.02235764
gpc4	5.48679327
Grhpr	2.6536008
GRN	1.29222933
gsr	2.03922516
GSX1	1.53938395
GTPBP4	2.69612064
hba4	7.62764638
hdac1	1.10794736
herc4	4.01815133
hibadh	4.93738592
hmgcr	1.78336237
hnrpdl	4.15458082
hsp90aa1.1	1.12142111
IDH1	13.9944005
IDI1	5.46243756
ik	1.11575375
Imp3	1.03754903
ISCA1	5.36948315
itpr1	1.0322663

KCNE5.1	1.24582137
KIAA1468	3.447632
L1td1	1.76461867
lancl1	8.76907201
lig3	2.32335137
LIN7B	1.08616626
LPL	1.69187195
mad2l2	11.329642
mcrip2	1.63431377
med22	8.55062546
med6	1.51565764
mfsd14a	10.7335217
MGC145244	5.43206982
MGC145685	2.10561458
MGC147314	1.0159904
mib2	3.65978475
Mllt4	8.31368925
MMAA	1.12404296
MN1	1.61145389
mpv17	2.70967735
MRPL32	1.95378706
MRPL9	9.79497179
Mvan_2161	1.0102758
MVK	2.47556243
Myst1	1.36635836
ncdn	1.42409162
NDUFC1	2.52495495
nr0b1	2.7472029
nvl	1.65115374
opa3	3.29704098
OR10AD1	1.97973856
pbdc1	1.12812664
pdlim4	1.19435613
Pol	1.22816742
ppp2r5c	5.11345765
Prpf38b	1.83230536
psma7	1.36728532
RFESD	1.24111549
rnf121	2.39746408
rpl36	1.40117505

rpl7a	1.28240121
rpp30	1.87758291
rps2	6.38018722
rps5	1.03952157
rrh	1.26967115
Sepx1	2.40231475
SERPINI1	1.83080398
SGCG	3.82160696
SH3GL2	1.04006792
slc20a1	7.96506141
smn2	1.14572415
ssbp3	1.11069088
STOM	2.65458747
TACO1	1.21621389
TAF13	3.53234636
Taldo1	2.42590059
tardbp	8.0355647
tek	5.69103387
telo2	1.31789698
Tf2-1	2.36937456
Tfr2	1.08996481
TGas113e22.1	1.50643664
Tm9sf4	1.91012545
TM9SF4	1.3958438
TMEM167A	2.44008415
top1.1	1.03715353
TRIM27	1.12724259
trmu	1.36215114
tspan31-b	1.07092677
TTLL12	2.78823321
TYMS	2.19026618
u2surp	1.8496272
vstm2l	5.7454443
XB-GENE-992854	3.01605048
ZCCHC3	1.39495897
zcchc7	4.3766589

4S-2**: List of most downregulated genes in coastal populations.** This data is meant to assist in the interpretation of the right-hand panel in Figure 7, in which the gene names and values may be too small to interpret.

Gene	Log2-Fold Change
2210411K11Rik	-1.2510059
ABCA1	-0.8429979
acacb	-1.2775975
ACHE	-1.0904211
Adpgk	-1.1413101
AGRN	-1.1863814
ankrd9	-1.5793013
ANKRD9	-1.4516239
arg2	-0.8993904
asb13	-0.9069456
atp5s	-0.806364
bbs2	-1.1419002
BSG	-1.2973577
C10orf122	-0.9905566
C14orf159	-1.2276128
calm1	-1.3297101
Camkk2	-0.9882067
CFLAR	-0.9445586
CHGA	-0.7695686
cited2	-0.9098547
CKM	-1.2983423
cnppd1	-1.3431869
COL6A3	-1.0843009
cox6a2	-1.1979806
CRYBG3	-0.9334174
ctdsp2	-0.7765466
Ctf1	-0.9280479
CYP2G1	-1.1015109

dnajc27-a	-0.9069536
Dusp27	-1.2352115
efhc2	-0.7680114
EGLN2	-2.2285547
ELMOD1	-0.8096248
EML5	-1.6366439
epb41	-0.772942
fam134b	-1.147164
Fars2	-1.3813702
fbxo18	-0.7675386
Fbxw10	-1.1152266
Foxk1	-0.7832894
furin	-1.035493
glul	-0.9515384
gm2a	-0.988407
GTF2IRD1	-0.7584103
HDAC5	-0.7719614
IRGC	-0.8221606
KIAA0831	-0.9064932
kidins220	-0.9341514
KIDINS220	-0.9151966
LKAP	-0.7680964
lmbr1	-0.789469
LRP12	-0.9065498
LRRFIP1	-0.8566742
MAN2A1	-0.7878125
mapk7	-1.6864217
mctp2	-0.7713278
mea1	-0.9108787
mllt10	-0.8105211
msh3	-0.8961391
Mx2	-0.9831257
MYO18B	-0.8973417
MYPN	-1.0847344
Necap2	-1.0481207

NLRP12	-1.4206728
pacs1	-0.936577
Pdk2	-1.2490639
PF10_0343	-0.8457786
phf21a	-0.8608986
Pik3c2a	-1.0927038
PIK3C2A	-0.8540177
PITX1	-0.7977413
pkhd1l1	-1.2871075
pmp22	-1.6723768
PPP1R9A	-0.854675
Prodh	-1.4144988
rpl35a	-1.1133154
samhd1	-0.7938661
sbno2	-1.3930407
slc2a2	-1.3450419
slc3a1	-0.9893694
Sned1	-1.1152505
sptbn2	-0.8106318
Sqstm1	-0.7688743
srrt-a	-1.815642
SYT1	-0.9379786
tbk1	-0.7948916
TCF20	-0.7953846
TESK2	-0.8490179
thdl18	-1.6837015
TICRR	-1.0846027
TMEM66	-0.7819218
TMEM8A	-0.8794118
Tmprss9	-1.0174708
TNFSF10	-1.0142705
TRIM25	-0.908048
Trpv6	-1.0889467
ubr4	-0.8455087
Ugt2a2	-1.5888467

UNC5D	-1.073869
XB-GENE-5768835	-1.0415724
Yipf1	-0.8317193
ZNF649	-0.8099827

CONCLUSION

This dissertation integrated theoretical, empirical, computational, and next generation genomic techniques to address fundamental questions in evolutionary ecology about the ability of species to evolve and occupy a new habitat type. We show that chronically salt-exposed, coastal frog populations are diverging from salt-naïve, inland frog populations in response to saltwater exposure across life stages. We find differences between locations in adults during reproduction and oviposition site choice behavior (Chapter 1), egg hatching (Chapters 1 & 4), early-stage tadpole survivorship (Chapters 1 & 4), as well as survivorship and growth rates throughout larval development (Chapter 3), and size at and time to metamorphosis (Chapter 3). We also identify a suite of genetic mechanisms (Chapter 4) that likely contribute to the enhanced ability of coastal populations of this species to persist in saline habitats. Finally, we examine the influence of a variety of biological processes on a species' adaptive potential in a changed environment including life history strategy (Chapters 2 & 3), density dependence (Chapter 3), phenotypic plasticity (Chapter 2), and genetic variation (Chapter 3). Although this dissertation has answered many questions and accomplished many of the goals established at the outset of this work, this research has also revealed new and additional questions. After five years of thinking about evolution, environmental change, and frogs, I have three main lingering questions that remain unanswered:

1) Determining the rate of evolution that allowed these frogs (or any freshwater organism) to occupy and persist in saline marshes. Although we can certainly make general estimations about the rates of evolutionary change based on our model (Chapter 3), at this point such estimates

would be largely conjecture. Indeed, given that a primary motivation for this research has been to understand whether evolution can keep pace with the rates of contemporary environmental change, this is a particularly noticeable gap in this dissertation and in our understanding of adaptive processes in general. Nevertheless, we can glean some insights about the minimum rates of evolutionary change for coastal frogs. The last glacial maximum occurred approximately 21,000 years before present. Phylogeographic analyses suggest that during that ice age, Holoarctic frogs (including *Hyla cinerea*) occupied refugia in Florida and Texas, and following the recession of glaciers, expanded northward along the coast to their current range (Barrow et al. 2017). Around the same time, Pamlico Creek was being progressively inundated, destined to become the drowned river Albemarle/Pamlico estuary system (Riggs and Ames 2003, Pre et al. 2011). Thus, it may be that the earliest possible time that frogs could have occupied coastal, saline habitats was approximately 20,000 years before present. Future work may use different genomic techniques than those discussed in Chapter 4 to plumb additional information hidden within our transcriptomic data to better predict rates of evolutionary change.

2.) Will *Hyla cinerea*, or another amphibian species, ever become fully marine? More specifically, are there limits to saltwater adaptation for frogs or any freshwater animal, or will frogs continue to evolve to tolerate increased osmotic stress until they are capable of tolerating and inhabiting full-strength seawater? It may be possible, since there is at least one species of frog (*Fejervarya cancrivora*) that has evolved to live and breed in marine mangrove forests that fringe the Java Sea (Gordon et al. 1961, Gordon and Tucker 1968, Shao et al. 2015).

3.) What is happening with other frog species regarding salt-tolerance? Will all frogs be able to respond adaptively to increased exposure to saltwater? We use *H. cinerea* because this species has successfully hurdled evolutionary barriers that prevent saltwater tolerance, and as a result, much can be learned about evolutionary transitions. Although not included in this dissertation, I conducted a project in which I duplicated the experiments conducted in Chapter 1 on two additional species in the same genus as *H. cinerea*; *Hyla chrysoscelis* and *Hyla squirrella,* with the purpose of exploring whether other frog species show similar responses to saltwater as *Hyla cinerea*. Both frogs' ranges overlap with *H. cinerea* with two important differences. First, *H. chrysoscelis* has not been observed in a wetland within 20 miles of the coast, although is sympatric otherwise. Second, although *H. squirrella* is found in coastal and inland wetlands, this species is observed exclusively in freshwater wetlands at the coast. The results from that project show three distinct responses across these three species. Coastal *Hyla squirrella* tadpoles actually appear to be maladapted to saltwater exposure compared to inland populations, possibly due to an ecological trap occurring with oviposition site choice behaviors. *Hyla chrysoscelis* tadpoles have high survivorship that matches coastal *Hyla cinerea* survivorship, but none of the eggs hatch if salinities exceed 2ppt. Thus, it could be high embryonic sensitivity that excludes *Hyla chrysoscelis* from inhabiting coastal wetlands.

In fact, it appears that *Hyla cinerea* is unique in its ability to demonstrate adaptive differences among even closely related species, suggesting that adaptation to saltwater exposure very well may be the exception rather than the norm (which is corroborated by work by Brady (2013), Brady (2017)). Therefore, to understand likelihoods of evolution, we also need to better understand the different factors that slow or constrain evolution in species that do not appear to

be able to adapt, such as those occurring in *Hyla squirrella* or *Hyla chrysoscelis*. Questions of this nature are often unanswered because the species that could not evolve have gone extinct and are no longer available for study. But if we can identify locations/populations facing intensified selective pressures prior to major losses, we may be able to identify traits associated with adaptation or maladaptation and examine how these traits interact with contemporary selective pressure from environmental change to affect adaptive responses (Urban et al. 2016).

Literature Cited:

2017. R: A language and environment for statistical computing. . R Core Development Team. Vienna, Austria, R Foundation for Statistical Computing.

Abrams, P. A. 1995. Implications of dynamically variable traits for identifying, classifying, and measuring direct and indirect effects in ecological communities. American Naturalist:112-134.

Aitken, S. N., S. Yeaman, J. A. Holliday, T. Wang, and S. Curtis-McLane. 2008. Adaptation, migration or extirpation: climate change outcomes for tree populations. Evolutionary Applications **1**:95-111.

Albecker, M. A., W. B. Brantley Jr, and M. W. McCoy. 2018. Diet and Nematode Infection Differ Between Coastal and Inland Populations of Green Treefrogs (*Hyla cinerea*). Southeastern Naturalist **17**:155-165.

Albecker, M. A., and M. W. McCoy. 2017. Adaptive responses to salinity stress across multiple life stages in anuran amphibians. Frontiers in Zoology **14**:40.

Alexander, H. K., G. Martin, O. Y. Martin, and S. Bonhoeffer. 2014. Evolutionary rescue: linking theory for conservation and medicine. Evolutionary Applications **7**:1161-1179.

Alford, R. A., and R. N. Harris. 1988. Effects of larval growth history on anuran metamorphosis. The American Naturalist **131**:91-106.

Allran, J. W., and W. H. Karasov. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environmental Toxicology and Chemistry, **20**:769-775.

Alvarado, R. H., and A. Moody. 1970. Sodium and chloride transport in tadpoles of the bullfrog Rana catesbeiana. Am J Physiol **218**:1510-1516.

Anderson, J. T., N. Perera, B. Chowdhury, and T. Mitchell-Olds. 2015. Microgeographic patterns of genetic divergence and adaptation across environmental gradients in Boechera stricta (Brassicaceae). American Naturalist **186**:S60-S73.

Araujo, M. B., and C. Rahbek. 2006. Ecology. How does climate change affect biodiversity? Science **313**:1396-1397.

Baldwin, A. H., and I. A. Mendelssohn. 1998. Effects of salinity and water level on coastal marshes: an experimental test of disturbance as a catalyst for vegetation change. Aquatic Botany **61**:255-268.

Baldwin, J. M. 1896. A new factor in evolution. American Naturalist **30**:441-451.

Balinsky, J. B. 1981. Adaptation of Nitrogen metabolism to hyperosmotic environment in amphibia. Journal of Experimental Zoology **215**:335-350.

Bankevich, A., S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, and A. V. Pyshkin. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of computational biology **19**:455-477.

Barfield, M., R. D. Holt, and R. Gomulkiewicz. 2011. Evolution in Stage-structured Populations. The American Naturalist **177**:397-409.

Barrett, R. D. H., and D. Schluter. 2008. Adaptation from standing genetic variation. Trends in Ecology & Evolution **23**:38-44.

Barrow, L. N., J. A. Soto-Centeno, A. R. Warwick, A. R. Lemmon, and E. Moriarty Lemmon. 2017. Evaluating hypotheses of expansion from refugia through comparative phylogeography of south-eastern Coastal Plain amphibians. Journal of Biogeography, **44**:2692-2705.

Bates, D., M. Martin, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models using lme4. Journal of Statistical Software **2015**:1-48.

Beck, L., C. Leroy, S. Beck-Cormier, A. Forand, C. Salaün, N. Paris, A. Bernier, P. Ureña-Torres, D. Prié, M. Ollero, and L. Coulombel. 2010. The phosphate transporter PiT1 (Slc20a1) revealed as a new essential gene for mouse liver development. . PLOS one **5**:p.e9148.

Bell, G. 2013. Evolutionary rescue and the limits of adaptation. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**:20120080.

Bell, G. 2017. Evolutionary Rescue. Annual Review of Ecology, Evolution and Systematics **48**:605-627.

Bell, G., and A. Gonzalez. 2009. Evolutionary rescue can prevent extinction following environmental change. Ecology Letters **12**:942-948.

Bell, G., and A. Gonzalez. 2011. Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration. Science **332**:1327-1330.

Bellard, C., C. Bertelsmeier, P. Leadley, W. Thuiller, and F. Courchamp. 2012. Impacts of climate change on the future of biodiversity. Ecology Letters **15**:365-377.

Bennett, J. E., A. Racine-Poon, and J. C. Wakefield. 1996. MCMC for nonlinear hierarchical models. London, UK: Chapman and Hall.

Bentley, A. J., and T. Yorio. 1979. Do Frogs Drink?. Journal of Experimental Biology **79**:41-46.

Berdahl, A., C. J. Torney, E. Schertzer, and S. A. Levin. 2015. On the evolutionary interplay between dispersal and local adaptation in heterogeneous environments. Evolution.

Bernabò, I., A. Bonacci, F. Coscarelli, M. Tripepi, and E. Brunelli. 2013. Effects of salinity stress on Bufo balearicus and Bufo bufo tadpoles: Tolerance, morphological gill alterations and Na(+)/K(+)-ATPase localization. Aquat Toxicol **132-133**:119-133.

Berven, K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology **71**:1599-1608.

Berven, K. A., and D. E. Gill. 1983. Interpreting geographic variation in life-history traits. American Zoologist **23**:85-97.

Beverton, R. J. H., and S. J. Holt. 1957. On the dynamics of exploited fish populations. Fishery Investigations Series II, Vol. XIX, Ministry of Agriculture. Fisheries and Food **1**:957.

Boeye, J., J. M. Travis, R. Stoks, and D. Bonte. 2013. More rapid climate change promotes evolutionary rescue through selection for increased dispersal distance. Evolutionary Applications **6**:353-364.

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. . Bioinformatics **30**:2114-2120.

Bolker, B., and C. D. Team. 2017. bbme: Tools for general maximum likelihood estimation. R

Bolker, B. M., M. E. Brooks, C. J. Clark, S. W. Geange, J. R. Poulsen, M. H. H. Stevens, and J. S. S. White. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends in Ecology & Evolution **24**:127-135.

Bolnick, D. I., and P. Nosil. 2007. Natural selection in populations subject to migration load. Evolution **61**:2229-2243.

Bourne, E. C., G. Bocedi, J. M. Travis, R. J. Pakeman, R. W. Brooker, and K. Schiffers. 2014. Between migration load and evolutionary rescue: dispersal, adaptation and the response of spatially structured populations to environmental change. Proceedings of the Royal Society of London B: Biological Sciences **281**:20132795.

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity in plants. Advances in Genetics **13**:115-155.

Bradshaw, W. E., and C. M. Holzapfel. 2006. Climate change. Evolutionary response to rapid climate change. Science **312**:1477-1478.
Brady, S. P. 2012. Road to evolution? Local adaptation to road adjacency in an amphibian (Ambystoma maculatum). Scientific reports **2**.
Brady, S. P. 2013. Microgeographic maladaptive performance and deme depression in response to roads and runoff. PeerJ **1**:e163.
Brady, S. P. 2017. Environmental exposure does not explain putative maladaptation in road-adjacent populations. Oecologia **184**:931-942.
Brand, A. B., J. W. Snodgrass, M. T. Gallagher, R. E. Casey, and R. Van Meter. 2010. Lethal and sublethal effects of embryonic and larval exposure of Hyla versicolor to stormwater pond sediments. Archives of Environmental Contamination and Toxicology **58**:325-331.
Bray, N. L., H. Pimentel, P. Melsted, and L. Pachter. 2016. Near-optimal probabilistic RNA-seq quantification. Nature biotechnology **34**:525.
Brook, B. W., and C. J. Bradshaw. 2006. Strength of Evidence for Density Dependence in Abundance Time Series of 1198 Species. Ecology **87**:1445-1451.
Brown, M. E., and S. C. Walls. 2013. Variation in salinity tolerance among larval anurans: Implications for community composition and the spread of an invasive, non-native species. Copeia **2013**:543-551.
Brysse, K., N. Oreskes, J. O'Reilly, and M. Oppenheimer. 2013. Climate change prediction- Erring on the side of least drama? Global Environmental Change **23**:327-337.
Buchfink, B., C. Xie, and D. H. Huson. 2015. Fast and sensitive protein alignment using DIAMOND. Nature Methods **12**:59-60.
Bürger, R. 1999. Evolution of genetic variability and the advantage of sex and recombination in changing environments. Genetics **153**:1055-1069.
Burger, R., and M. Lynch. 1995. Evolution and extinction in a changing environment: a quantitative-genetic analysis. Evolution:151-163.
Burkhart, J. G., J. C. Helgen, D. J. Fort, K. Gallagher, D. Bowers, T. L. Propst, M. Gernes, J. Magner, M. D. Shelby, and G. Lucier. 1998. Induction of mortality and malformation in *Xenopus laevis* embryos by water sources associated with field frog deformities. Environmental Health Perspectives **106**:841.
Burnham, K. P., and D. R. Anderson. 2003. Model selection and multimodel inference: a practical information-theoretic approach. Springer Science & Business Media.
Buskirk, J. V., and G. Saxer. 2001. Delayed costs of an induced defense in tadpoles? Morphology, hopping, and development rate at metamorphosis. Evolution **55**:821-829.
Carignan, V., and M.-A. Villard. 2002. Selecting Indicator Species to Monitor Ecological Integrity: A Review. Environmental Monitoring and Assessment **78**:45-61.
Carlson, S. M., C. J. Cunningham, and P. A. H. Westley. 2014. Evolutionary rescue in a changing world. Trends in Ecology & Evolution **29**:521-530.
Castillo, A. M., D. M. T. Sharpe, C. K. Ghalambor, and L. F. De León. 2018. Exploring the effects of salinization on trophic diversity in freshwater ecosystems: A quantitative review. Hydrobiologia **807**:1-17.
Cattau, C. E., R. J. Fletcher Jr, R. T. Kimball, C. W. Miller, and W. M. Kitchens. 2018. Rapid morphological change of a top predator with the invasion of a novel prey. Nature Ecology and Evolution **2**:108.
Chen, I. C., J. K. Hill, R. Ohlemuller, D. B. Roy, and C. D. Thomas. 2011. Rapid range shifts of species associated with high levels of climate warming. Science **333**:1024-1026.

Chevin, L. M., R. Gallet, R. Gomulkiewicz, R. D. Holt, and S. Fellous. 2013. Phenotypic plasticity in evolutionary rescue experiments. . Phil. Trans. R. Soc. B **368**:p.20120089.
Chevin, L. M., and R. Lande. 2010. When do adaptive plasticity and genetic evolution prevent extinction of a density-regulated population? . Evolution **64**:1143-1150.
Chevin, L. M., R. Lande, and G. M. Mace. 2010. Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. PLoS Biol **8**:e1000357.
Chinathamby, K., R. D. Reina, P. C. Bailey, and B. K. Lees. 2006. Effects of salinity on the survival, growth and development of tadpoles of the brown tree frog, Litoria ewingii. Australian Journal of Zoology **54**:97-105.
Chirgwin, E., D. J. Marshall, C. M. Sgrò, and K. Monro. 2016. The other 96%: Can neglected sources of fitness variation offer new insights into adaptation to global change? Evolutionary Applications **10**:267-275.
Chown, S. L. 2012. Trait-based approaches to conservation physiology: forecasting environmental change risks from the bottom up. Philos Trans R Soc Lond B Biol Sci **367**:1615-1627.
Christensen, H., A. C. J. Burgers, and C. H. Li. 1961. Demonstration of Aldosterone and Corticosteron as the principal steroids formed in incubates of adrenals of American Bullfrog (*Rana catesbeiana*) and stimulation of their production by mammalian adrenocorticotropin. Comparative Endocrinology **1**:37-50.
Christman, S. P. 1974. Geographic variation for salt water tolerance in the frog *Rana sphenocephala* Copeia **1974**:773-778.
Christy, M. T., and C. R. Dickman. 2002. Effects of salinity on tadpoles of the green and golden bell frog (Litoria aurea). Amphibia-Reptilia **23**:1-11.
Church, J. A., P. U. Clark, A. Cazenave, J. M. Gregory, S. Jevrejeva, A. Levermann, M. A. Merrifield, G. A. Milne, R. S. Nerem, P. D. Nunn, A. J. Payne, W. T. Pfeffer, D. Stammer, and A. S. Unnikrishnan. 2013. IPCC Fifth Assessment Report (AR5), Climate Change 2013: The Physical Science Basis.1-124.
Consortium, U. 2018. UniProt: the universal protein knowledgebase. . Nucleic acids research **46**:2699.
Coordinators, N. R. 2016. Database resources of the national center for biotechnology information. Nucleic acids research.
Cope, W. G., R. B. Bringolf, D. B. Buchwalter, T. J. Newton, C. G. Ingersoll, N. Wang, T. Augspurger, F. J. Dwyer, M. C. Barnhart, R. J. Neves, and E. Hammer. 2008. Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. Journal of the North American Benthological Society **27**:451-462.
Craft, C., J. Clough, J. Ehman, S. Joye, R. Park, S. Pennings, H. Guo, and M. Machmuller. 2009. Forecasting the effects of accellerated sea-level rise on tidal marsh ecosystem services Frontiers in Ecology and the Environment **7**:73-78.
Crispo, E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. Evolution **61**:2469-2479.
Crother, B. I., and C. L. Fontenot. Amphibian and Reptile Monitoring in the Ponchartrain-Maurepas Region. Lake Pontchartrain Basin Research Program (PBRP):35.
Crother, B. I., and C. L. Fontenot. 2006. Amphibian and Reptile Monitoring in the Ponchartrain-Maurepas Region. Lake Pontchartrain Basin Research Program (PBRP):35.
Crouse, D. T., L. B. Crowder, and H. Caswell. 1987. A stage-based population model for Loggerhead Sea Turtles and implications for conservation. Ecology **68**:1412-1423.
Crutzen, P. J. 2002. Geology of mankind. Nature **415**:23.

Cunningham, H. R., L. J. Rissler, L. B. Buckley, and M. C. Urban. 2016. Abiotic and biotic constraints across reptile and amphibian ranges. Ecography **39**:1-8.
DaLaune, R. D., and S. R. Pezeshki. 1994. The influence of subsidence and saltwater intrustion on coastal marsh stability: Louisiana gulf coast Journal of Coastal Research **12**:77-89.
DaLaune, R. D., S. R. Pezeshki, and W. H. P. Jr. 1987. Response of Coastal plants increase in submergence and salinity. Journal of Coastal Research **3**:535-546.
Darwin, C. 1859. On The Origin of Species by Means of Natural Selection, or Preservation of Favoured Races in the Struggle for Life. John Murray, London.
Davis, M. B., and R. G. Shaw. 2001. Range shifts and adaptive responses to Quaternary climate change. Science **292**:673-679.
Davis, M. B., R. G. Shaw, and J. R. Etterson. 2005. Evolutionary responses to changing climate. Ecology **86**:1704-1714.
Dawson, D. C., and X. Liu. 2009. Osmoregulation: Some principals of water and solute transport.*in* D. H. Evans, editor. Osmotic and Ionic Regulation. CRC Press, Boca Raton, FL.
Dawson, T. P., S. T. Jackson, J. I. House, I. C. Prentice, and G. M. Mace. 2011. Beyond predictions: biodiversity conservation in a changing climate. Science **332**:53-58.
Day, J. W., R. R. Christian, D. M. Boesch, A. Yáñez-Arancibia, J. Morris, R. R. Twilley, L. Naylor, L. Schaffner, and C. Stevenson. 2008. Consequences of Climate Change on the Ecogeomorphology of Coastal Wetlands. Estuaries and Coasts **31**:477-491.
DeFaveri, J., and J. Merila. 2013. Local Adaptation to Salinity in the three-spined stickleback. Journal of Evolutionary Biology **27**:290-302.
Denver, R. J. 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis American Zoologist **37**:172-184.
DeWitt, T. J., A. Sih, and D. S. Wilson. 1998. Costs and limits of phenotypic plasticity. Trends in Ecology & Evolution **12**:77-81.
Dietz, T. H., and R. H. Alvarado. 1974. Na and Cl transport across gill chamber epithelium of Rana catesbeiana tadpoles. Am J Physiol **226**:764-770.
Domingues, C. M., J. A. Church, N. J. White, P. J. Gleckler, S. E. Wijffels, P. M. Barker, and J. R. Dunn. 2008. Improved estimates of upper-ocean warming and multi-decadal sea-level rise. Nature **453**:1090-1093.
Dougherty, C. K., and G. R. Smith. 2006. Acute effects of road de-icers on the tadpoles of three anurans. Applied Herpetology **3**:87-93.
Emerson, S. B. 1986. Heterochrony and frogs: the relationship of a life history trait to morphological form. The American Naturalist **127**:167-183.
Evans, T. G. 2015. Considerations for the use of transcriptomics in identifying the 'genes that matter' for environmental adaptation. Journal of Experimental Biology **218**:1925-1935.
Evans, T. G., and G. E. Hofmann. 2012. Defining the limits of physiological plasticity: how gene expression can assess and predict the consequences of ocean change. Philosophical Transactions of the Royal Society B: Biological Sciences **367**:1733-1745.
Evans, T. G., and G. N. Somero. 2008. A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish *Gillichthys mirabilis*: osmosensors to effectors. Journal of Experimental Biology **211**:3636-3649.
Ferrari, M. C., and D. P. Chivers. 2009. Sophisticated early life lessons: threat-sensitive generalization of predator recognition by embryonic amphibians. Behavioral Ecology **20**:1295-1298.

Ficetola, G. F., and F. De Bernardi. 2006. Trade-off between larval development rate and post-metamorphic traits in the frog Rana latastei. Evolutionary Ecology **20**:143-158.

Foden, W. B., S. H. Butchart, S. N. Stuart, J. C. Vié, H. R. Akçakaya, A. Angulo, L. M. DeVantier, A. Gutsche, E. Turak, L. Cao, and S. D. Donner. 2013. Identifying the world's most climate change vulnerable species: a systematic trait-based assessment of all birds, amphibians and corals. PLOS one **8**:p.e65427.

Foden, W. B., G. M. Mace, J.-C. Vié, A. Angulo, S. H. M. Butchart, L. DeVantier, H. T. Dublin, A. Gutsche, S. N. Stuart, and E. Turak. 2009. Species susceptibility to climate change impacts. Pages 77-88 *in* J.-C. Vie, C. Hilton-Taylor, and S. N. Stuart, editors. Wildlife in a Changing World: An Analysis of the 2008 IUCN Red List of Threatened Species, Barcelona, Spain.

Forsman, A. 2015. Rethinking phenotypic plasticity and its consequences for individuals, populations and species. Heredity **115**:276-284.

Fraser, D. J., L. K. Weir, L. Bernatchez, M. M. Hansen, and E. B. Taylor. 2011. Extent and scale of local adaptation in salmonid fishes: review and meta-analysis. Heredity **106**:404-420.

Futuyma, D. J. 2010. Evolutionary constraint and ecological consequences. Evolution **64**:1865-1884.

Geddes, N. A., and S. Mopper. 2006. Effects of environmental salinity on vertebrate florivory and wetland communities. Natural Areas Journal **26**:31-37.

Gelman, A., J. B. Carlin, H. S. Stern, and D. B. Rubin. 2004. Bayesian data analysis. Texts in statistical science series. Chapman & Hall/CRC, Boca Raton, FL.

Ghalambor, C. K., J. K. McKay, S. P. Carroll, and D. N. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Functional Ecology **21**:394-407.

Gibbons, J. W., and J. W. Coker. 1978. Herpetofaunal colonization patterns of atlantic coast barrier islands American Midland Naturalist **99**:219-233.

Gibbons, J. W., C. T. Winne, D. E. Scott, J. D. Willson, X. Glaudas, K. M. Andrews, B. D. Todd, L. A. Fedewa, L. Wilkinson, R. N. Tsaliagos, S. J. Harper, J. L. Greene, T. D. Tuberville, B. S. Metts, M. E. Dorcas, J. P. Nestor, C. A. Young, T. Akre, R. N. Reed, K. A. Buhlmann, J. Norman, D. A. Croshaw, C. Hagen, and B. B. Rothermel. 2006. Remarkable amphibian biomass and abundance in an isolated wetland: implications for wetland conservation. Conserv Biol **20**:1457-1465.

Gienapp, P., C. Teplitsky, J. S. Alho, J. A. Mills, and J. Merilä. 2008. Climate change and evolution: disentangling environmental and genetic responses. Molecular ecology **17**:167-178.

Glennemeier, K. A., and R. J. Denver. 2002. Developmental changes in interrenal responsiveness in anuran amphibians. Integr Comp Biol **42**:565-573.

Gomez-Mestre, I., and M. Tejado. 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. Evolution **57**:1889-1899.

Gomez-Mestre, I., and M. Tejado. 2005. Adaptation or Exaptation? An experimental test of hypotheses on the origin of salinity tolerance in Bufo calamita. Journal of Evolutionary Biology **18**:847-855.

Gomez-Mestre, I., M. Tejedo, and J. Merilä. 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. Evolution **57**:1889-1899.

Gomulkiewicz, R., and R. D. Holt. 1995. When does evolution by natural selection prevent extinction? . Evolution **49**:201-207.

Gomulkiewicz, R., R. D. Holt, and M. Barfield. 1999. The effects of density dependence and immigration on local adaptation and niche evolution in a black-hole sink environment. Theoretical population biology **55**:283-296.

Gonzalez, A., O. Ronce, R. Ferriere, and M. E. Hochberg. 2013. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**:20120404.

Gordon, M. S. 1962. Osmotic regulation in the Green Toad (*Bufo viridis*) Journal of Experimental Biology **39**:261-270.

Gordon, M. S. 1965. Intracellular osmoregulation in skeletal muscle during salinity adaptation in two species of toads Biological Bulletin **128**:218-229.

Gordon, M. S., K. Schmidt-Nielsen, and H. M. Kelly. 1961. Osmotic regulation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **38**:659-678.

Gordon, M. S., and V. A. Tucker. 1965. Osmotic regulation in the tadpoles of the crab-eating frog (*Rana cancrivora)* Journal of Experimental Biology **42**:437-445.

Gordon, M. S., and V. A. Tucker. 1968. Further observations on the physiology of salinity adaptation in the crab-eating frog (Rana cancrivora). Journal of Experimental Biology **49**:185-193.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica:183-190.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, and Z. Chen. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. . Nature biotechnology **29**:644.

Grant, K. P., and L. R. Licht. 1995. Effects of ultraviolet radiation on life-history stages of anurans from Ontario, Canada. Canadian Journal of Zoology **73**:2292-2301.

Gunzburger, M. S. 2006. Reproductive ecology of the green treefrog (Hyla cinerea) in Northwestern Florida. The American midland naturalist **155**:321-328.

Hamer, A. J., and M. J. McDonnell. 2008. Amphibian ecology and conservation in the urbanising world- a review Biological Conservation **141**:2432-2449.

Hammond, S. A., R. L. Warren, B. P. Vandervalk, E. Kucuk, H. Khan, E. A. Gibb, P. Pandoh, H. Kirk, Y. Zhao, M. Jones, and A. J. Mungall. 2017. The North American bullfrog draft genome provides insight into hormonal regulation of long noncoding RNA. Nature Communications **8**:1433.

Haramura, T. 2007. Salinity tolerance of eggs of Buergeria japonica (Amphibia, Anura) inhabiting coastal areas. Zoolog Sci **24**:820-823.

Haramura, T. 2011. Use of oviposition sites by a Rhacophorid frog inhabiting a coastal area in Japan Journal of Herpetology **45**:432-437.

Hardy, J. D. 1953. Notes on the distribution of *Mycrohyla carolinensis* in southern Maryland. Herpetologica **8**:162-166.

Hardy, J. D. J. 1972. Amphibians of the Chesapeake Bay Region Chesapeake Science **13**:S123-S128.

Harley, C. D. 2011. Climate change, keystone predation, and biodiversity loss. Science **334**:1124-1127.

Harris, R. N. 1999. The Anuran Tadpole: Evolution and maintenance. Pages 279-294 *in* R. Mcdiarmid and R. Altig, editors. Tadpole. University of Chicago Press, Chicago, IL.

Harris, S. L., and A. J. Levine. 2005. The p53 pathway: positive and negative feedback loops. Oncogene **24**:2899.

Hart, B. T., P. Bailey, R. Edwards, K. Hortle, K. James, A. McMahon, C. Meredith, and S. K. 1991. A review of the salt sensitivity of the Australian fresh-water biota. . Hydrobiologia **210**.

Havird, J. C., R. P. Henry, and A. E. Wilson. 2013. Altered expression of Na(+)/K(+)-ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: a meta-analysis of 59 quantitative PCR studies over 10 years. Comp Biochem Physiol Part D Genomics Proteomics **8**:131-140.

Hendry, A. P. 2015. Key Questions on the Role of Phenotypic Plasticity in Eco-Evolutionary Dynamics. Journal of Heredity:esv060.

Herbert, E. R., P. Boon, A. J. Burgin, S. C. Neubauer, R. B. Franklin, M. Ardón, K. N. Hopfensperger, L. P. Lamers, and P. Gell. 2015. A global perspective on wetland salinization: ecological consequences of a growing threat to freshwater wetlands. Ecosphere **6**:1-43.

Heyer, R., M. A. Donnelly, M. Foster, and R. Mcdiarmid. 2014. Measuring and monitoring biological diversity: standard methods for amphibians. Smithsonian Institution.

Hildebrandt, J. 1997. Changes in Na+/K+-ATPase expression during adaptive cell differentiation in avian nasal salt gland. Journal of Experimental Biology **200**:1895-1904.

Hillyard, S., N. Møbjerg, S. Tanaka, and E. Larsen. 2009a. Osmotic and ion regulation in amphibians. Osmotic and ionic regulation: Cells and animals:367-441.

Hillyard, S. D., N. Møbjerg, S. Tanaka, and E. H. Larsen. 2009b. Osmotic Regulation in Amphibians.*in* D. H. Evans, editor. Osmotic and ionic Regulation. CRC Press, Boca Raton, FL.

Hintz, W. D., and R. A. Relyea. 2017. Impacts of road deicing salts on the early-life growth and development of a stream salmonid: Salt type matters. Environ Pollut.

Hoffmann, A. A., and C. M. Sgrò. 2011. Climate change and evolutionary adaptation. Nature **470**:479-485.

Holt, R., and R. Gomulkiewicz. 2004. Conservation implications of niche conservatism and evolution in heterogeneous environments. Pages 244-264 Evolutionary Conservation Biology. Cambridge University Press.

Holt, R. D. 2003. On the evolutionary ecology of species' ranges. Evolutionary ecology research **5**:159-178.

Holt, R. D. 2011. Evolution in source-sink environments: implications for niche conservatism. . Pages 23-57 Sources, Sinks and Sustainability. Cambridge University Press, Cambridge, UK.

Holt, R. D., M. Barfield, and R. Gomulkiewicz. 2004a. Temporal variation can facilitate niche evolution in harsh sink environments. The American Naturalist **164**:187-200.

Holt, R. D., T. M. Knight, and M. Barfield. 2004b. Allee effects, immigration, and the evolution of species' niches. The American Naturalist **163**:253-262.

Hopkins, G. R., and J. E. D. Brodie. 2015. Occurrence of Amphibians in Saline Habitats: A Review and Evolutionary Perspective. Herpetological Monographs **29**:1-27.

Hota, A. K. 1994. Growth in Amphibians. Gerontology **40**:147-160.

Hsu, W.-T., C.-S. Wu, J.-C. Lai, Y.-K. Chiao, C.-H. Hsu, and Y.-C. Kam. 2012. Salinity acclimation affects survival and metamorphosis of crab-eating frog tadpoles. Herpetologica **68**:14-21.

Huaiyu, M., X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, and P. D. Thomas. 2016. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res.

Huaiyu, M., A. Muruganujan, J. T. Casagrande, and P. D. Thomas. 2013. Large-scale gene function analysis with the PANTHER classification system. Nature Protocols **8**:1551 - 1566.

Istock, C. A. 1967. The evolution of complex life cycle phenomena: an ecological perspective. Evolution **21**:592-605.

Jaenisch, R., and A. Bird. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature genetics **33**:245.

James, K. R., B. Cant, and T. Ryan. 2003. Responses of freshwater biota to rising salinity levels and implications for saline water management: a review. Australian Journal of Botany **51**:703-713.

Kannan, S., J. Hui, K. Mazooji, L. Pachter, and D. Tse. 2016. Shannon: An information-optimal de novo RNA-Seq assembler. . bioRxiv:p.039230.

Karraker, N. E. 2007. Are embryonic and larval green frogs (Rana clamitans) insensitive to road deicing salt. Herpetological Conservation and Biology **2**:35-41.

Karraker, N. E., and G. R. Ruthig. 2009. Effect of road deicing salt on the susceptibility of amphibian embryos to infection by water molds. Environmental Research **109**:40-45.

Kassahn, K. S., R. H. Crozier, H. O. Pörtner, and M. J. Caley. 2009. Animal performance and stress: responses and tolerance limits at different levels of biological organisation. . Biological Reviews **84**:277-292.

Kassambara, A., and M. Kosinski. 2018. survminer: Drawing survival curves using 'ggplot2'. R, R statistical environment.

Kaushal, S. S., G. E. Likens, M. L. Pace, R. M. Utz, S. Haq, J. Gorman, and M. Grese. 2018. Freshwater salinization syndrome on a continental scale. PNAS:p.201711234.

Kearney, B. D., P. G. Byrne, and R. D. Reina. 2012. Larval tolerance to salinity in three species of Australian anuran: an indication of saline specialisation in Litoria aurea. PLOS one **7**:e43427.

Kefford, B. J., G. L. Hickey, A. Gasith, E. Ben-David, J. E. Dunlop, C. G. Palmer, K. Allan, S. C. Choy, and C. Piscart. 2012. Global scale variation in the salinity sensitivity of riverine macroinvertebrates: Eastern Australia, France, Israel and South Africa. PLOS one **7**: p.e35224.

Kemp, A. C., B. P. Horton, S. J. Culver, D. R. Corbett, O. v. d. Plassche, W. R. Gehrels, B. C. Douglas, and A. C. Parnell. 2009. Timing and magnitude of recent accelerated sea-level rise Geology **37**:1035-1038.

Kemp, A. C., B. P. Horton, J. P. Donnelly, M. E. Mann, M. Vermeer, and S. Rahmstorf. 2011. Climate related sea level variations over the past two millennia. PNAS **108**:11017-11022.

Kiesecker, J. M., and A. R. Blaustein. 1997. Influences of egg laying behavior on pathogenic infection of amphibian eggs. Conservation BIology **11**:214-220.

Kirkpatrick, M., and R. Lande. 1989. The evolution of maternal characters. Evolution **1989**:485-503.

Knighton, A. D., K. Mills, and C. D. Woodroffe. 1991. Tidal-creek extension and saltwater intrusion in northern Australia. Geology **19**:831-834.

Konno, N., S. Hyodo, K. Matsuda, and M. Uchiyama. 2006. Effect of osmotic stress on expression of a putative facilitative urea transporter in the kidney and urinary bladder of the marine toad, Bufo marinus. Journal of Experimental Biology **209**:1207-1216.

Kopp, R. E., B. P. Horton, A. C. Kemp, and C. Tebaldi. 2015. Past and future sea-level rise along the coast of North Carolina, USA. Climatic Change **132**:693-707.

Kubisch, A., R. D. Holt, H.-J. Poethke, and E. A. Fronhofer. 2014. Where am I and why? Synthesizing range biology and the eco-evolutionary dynamics of dispersal. Oikos **123**:5-22.

Kültz, D. 2005. Molecular and evolutionary basis of the cellular stress response. Annual Review of Physiology **67**:225-257.

Kusakabe, M., A. Ishikawa, M. Ravinet, K. Yoshida, T. Makino, A. Toyoda, A. Fujiyama, and J. Kitano. 2017. Genetic basis for variation in salinity tolerance between stickleback ecotypes. Molecular ecology **26**:304-319.

Lamichhaney, S., A. M. Barrio, N. Rafati, G. Sundström, C.-J. Rubin, E. R. Gilbert, J. Berglund, A. Wetterbom, L. Laikre, and M. T. Webster. 2012a. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. Proceedings of the National Academy of Sciences **109**:19345-19350.

Lamichhaney, S., A. M. Barrio, N. Rafati, G. Sundström, C. J. Rubin, E. R. Gilbert, J. Berglund, A. Wetterbom, L. Laikre, M. T. Webster, and M. Grabherr. 2012b. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. Proceedings of the National Academy of Sciences **109**:19345-19350.

Lamichhaney, S., F. Han, J. Berglund, C. Wang, M. S. Almén, M. T. Webster, B. R. Grant, P. R. Grant, and L. Andersson. 2016. A beak size locus in Darwin's finches facilitated character displacement during a drought. Science **352**:470-474.

Lande, R. 2009. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. Journal of Evolutionary Biology **22**:1435-1446.

Lande, R., and S. Shannon. 1996. The role of genetic variation in adaptation and population persistence in a changing environment. Evolution **50**:434-437.

Langhans, M., B. Peterson, A. Walker, G. R. Smith, and J. E. Rettig. 2009. Effects of salinity on survivorship of wood frog (Rana sylvatica) tadpoles.

Laurila, A., S. Karttunen, and J. Merilä. 2002. Adaptive phenotypic plasticity and genetics of larval life histories in two Rana temporaria populations. Evolution **56**:617-627.

Lawler, J. J., S. L. Shafer, B. A. Bancroft, and A. R. Blaustein. 2010. Projected climate impacts for the amphibians of the Western hemisphere. Conserv Biol **24**:38-50.

Lee, C. E. 1999. Rapid and repeated invasions of fresh water by the saltwater copepod Eurytemora affinis. Evolution **53**:1423-1434.

Lee, C. E., M. Kiergaard, G. W. Gelembiuk, B. D. Eads, and M. Posavi. 2011. Pumping ions: rapid parallel evolution of ionic regulation following habitat invasions. Evolution **65**:2229-2244.

Levis, N. A., A. Serrato-Capuchina, and D. W. Pfennig. 2017. Genetic accommodation in the wild: evolution of gene expression plasticity during character displacement. Journal of evolutionary biology.

Lewis, O. T. 2006. Climate change, species-area curves and the extinction crisis. Philos Trans R Soc Lond B Biol Sci **361**:163-171.

Lillywhite, H. B. 2006. Water relations of tetrapod integument. J Exp Biol **209**:202-226.

Lindsey, H. A., J. Gallie, S. Taylor, and B. Kerr. 2013. Evolutionary rescue from extinction is contingent on a lower rate of environmental change. Nature **494**:463-467.

Loaiciga, H. A. 2003. Climate change and ground water Annals of the Association of American Geographers **93**:30-41.
Loreau, M., N. Mouquet, and A. Gonzalez. 2003. Biodiversity as spatial insurance in heterogeneous landscapes. . Proceedings of the National Academy of Sciences **100**:12765-12770.
Lorenz, J. J. 2014. A review of the effects of altered hydrology and salinity on vertebrate fauna and their habitats in northeastern Florida Bay. . Wetlands **34**.
Losos, J. B. 2009. Lizards in an Evolutionary Tree: Ecology and
Adaptive Radiation of Anoles. University of California Press, Berkeley, CA.
Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. **15**:550.
Lu, H. A., T. X. Sun, T. Matsuzaki, X. H. Yi, J. Eswara, R. Bouley, M. McKee, and D. Brown. 2007. Heat shock protein 70 interacts with aquaporin-2 and regulates its trafficking. Journal of Biological Chemistry **282**:28721-28732.
Lynch, M., and R. Lande. 1993. Evolution and Extinction in response to environmental change. Pages 234-250. *in* P. M. Kareiva, editor. Biotic interactions and global change. Sinauer Associates.
Lynch, M., and R. Lande. 1998. The critical effective size for a genetically secure population. . Animal Conservation **1**:70-72.
Maclean, I. M., and R. J. Wilson. 2011. Recent ecological responses to climate change support predictions of high extinction risk. Proc Natl Acad Sci U S A **108**:12337-12342.
MacManes, M. D. 2014. On the optimal trimming of high-throughput mRNA sequence data. . Frontiers in Genetics **5**:1-7.
MacManes, M. D. 2017. The Oyster River Protocol: A Multi Assembler and Kmer Approach For de novo Transcriptome Assembly. .
Malcolm, J. R., C. Liu, R. P. Neilson, L. Hansen, and L. E. E. Hannah. 2006. Global warming and extinctions of endemic species from biodiversity hotspots. Conservation BIology **20**:538-548.
Manda, A. K., A. S. Giuliano, and T. R. Allen. 2014. Influence of artificial channels on the source and extent of saline water intrusion in the wind tide dominated wetlands of southern Albemarle estuarine system (USA). Environmental Earth Science **71**:4409-4419.
Marini, F. 2018. pcaExplorer: Interactive Visualization of RNA-seq Data Using a Principal Components Approach.
Marshall, D. J., S. C. Burgess, and T. Connallon. 2016. Global change, life-history complexity and the potential for evolutionary rescue. Evolutionary Applications **9**:1189-1201.
Marshall, D. J., and T. Uller. 2007. When is a maternal effect adaptive? . Oikos **116**:1957-1963.
Martin, G., R. Aguile, J. Ramsayer, O. Kaltz, and O. Ronce. 2013. The probability of evolutionary rescue: towards a quantitative comparison between theory and evolution experiments. Phil. Trans. R. Soc. B **368**:20120088.
McClanahan, L., Jr. 1964. Osmotic tolerance of the muscles of two desert-inhabiting toads, *Bufo cognatus* and *Scaphiopus couchi*. Comp Biochem Physiol **12**:501-508.
McClanahan, L., Jr., J. N. Stinner, and V. H. Shoemaker. 1978. Skin lipids, water loss, and energy metabolism in a South American tree frog (*Phyllomedusa sauvagei*). Physiological Zoology **51**:179-187.
McCormick, S. D., and D. Bradshaw. 2006. Hormonal control of salt and water balance in Vertebrates. Comparative Endocrinology **147**:3-8.

McCoy, M. W., M. Barfield, and R. D. Holt. 2009. Predator shadows: complex life histories as generators of spatially patterned indirect interactions across ecosystems. Oikos **118**:87-100.

McNab, B. K. 2002. The physiological ecology of vertebrates: a view from energetics. Cornell University Press.

Meehl, G. A., W. M. Washington, W. D. Collins, J. M. Arblaster, A. Hu, L. E. Buja, W. G. Strand, and H. Teng. 2005. How much more climate change and sea level rise? Science **307**:1769-1772.

Merilä, J., and A. P. Hendry. 2014. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. Evolutionary Applications **7**:1-14.

Meteyer, C. U., R. A. Cole, K. A. Converse, D. E. Docherty, M. Wolcott, J. C. Helgen, R. Levey, L. Eaton-Poole, and J. G. Burkhart. 2000. Defining anuran malformations in the context of a developmental problem. The Journal of the Iowa Academy of Science **107**:72-78.

Meyssignac, B., and A. Cazenave. 2012. Sea Level- A review of present day and recent past changes and variability Journal of Geodynamics **58**:96-109.

Michener, W. K., E. R. Blood, K. L. Bildstein, M. M. Brinson, and L. R. Gardner. 1997. Climate change, hurricanes and tropical storms, and rising sea level in coastal wetlands. Ecological Applications **7**:770-801.

Miyamoto, K. I., S. Haito-Sugino, S. Kuwahara, A. Ohi, K. Nomura, M. Ito, M. Kuwahata, S. Kido, S. Tatsumi, I. Kaneko, and H. Segawa. 2011. Sodium-dependent phosphate cotransporters: lessons from gene knockout and mutation studies. Journal of pharmaceutical sciences **100**:3719-3730.

Montagna, P. A., M. Alber, P. Doering, and M. S. F. Connor. 2002. Freshwater inflow: science, policy, and management. Estuaries:1243-1245.

Moorhead, K. K., and M. M. Brinson. 1995. Responses of wetlands to rising sea level in the lower coastal plain of North Carolina. Ecological Applications **5**:261-271.

Mopper, S., and S. Y. Strauss. 2013. Genetic structure and local adaptation in natural insect populations: effects of ecology, life history, and behavior. Springer Science & Business Media.

Moran, N. A. 1994. Adaptation and constraint in the complex life cycles of animals. Annual Review of Ecology and Systematics **25**:573-600.

Morgan, T. H., and C. R. Stockard. 1907. The effects of salts and sugar solutions on the development of the frog's egg Biological Bulletin **13**:272-279.

Moritz, C., and R. Agudo. 2013. The future of species under climate change: resilience or decline? Science **341**:504-508.

Morris, J. T., P. V. Sundarshwar, C. T. Nietch, B. Kjerfve, and D. R. Cahoon. 2002. Responses of coastal wetlands to rising sea level. Ecology **83**:2869-2877.

Mosesson, M. W. 2005. Fibrinogen and fibrin structure and functions. Journal of Thrombosis and Haemostasis **3**:1894–1904.

Mulligan, R. P., J. P. Walsh, and H. M. Wadman. 2012. Storm surge and surface waves in a large and shallow estuarine system during the passage of a hurricane Journal of Waterway, Port, Coastal, and Ocean Engineering.

Munsey, L. D. 1972. Salinity tolerance of the african Pipid Frog. *Xenopus laevis* Copeia **1972**:584-586.

Murren, C. J., J. R. Auld, H. Callahan, C. K. Ghalambor, C. A. Handelsman, M. A. Heskel, J. Kingsolver, H. J. Maclean, J. Masel, and H. Maughan. 2015a. Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. Heredity.

Murren, C. J., J. R. Auld, H. Callahan, C. K. Ghalambor, C. A. Handelsman, M. A. Heskel, J. Kingsolver, H. J. Maclean, J. Masel, and H. Maughan. 2015b. Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. Heredity **115**:293-301.

Nadaeu, C. P., M. C. Urban, and J. R. Bridle. 2017. Climates Past, Present, and Yet-to-come shape climate change vulnerabilities. Trends in Ecology & Evolution **32**:786-800.

Nakkrasae, L. i., S. Phummisutthigoon, and N. Charoenphandhu. 2015. Low salinity increases survival, body weight and development in tadpoles of the Chinese edible frog Hoplobatrachus rugulosus. Aquaculture Research.

Neill, W. T. 1958. The occurrence of amphibians and reptiles in saltwater areas, and a bibliography. Bulletin of Marine Science **8**:1-97.

Nicholls, R. J., and A. Cazenave. 2010. Sea-level rise and its impact on coastal zones. Science **328**:1517-1520.

Nicholls, R. J., and R. S. Tol. 2006. Impacts and responses to sea-level rise: a global analysis of the SRES scenarios over the twenty-first century. Philos Trans A Math Phys Eng Sci **364**:1073-1095.

Nonaka, E., R. Svanbäck, X. Thibert-Plante, G. Englund, and Å. Brännström. 2015. Mechanisms by Which Phenotypic Plasticity Affects Adaptive Divergence and Ecological Speciation. The American Naturalist **186**:E126-143.

Nunney, L. 2015. Adapting to a Changing Environment: Modeling the Interaction of Directional Selection and Plasticity. Journal of Heredity.

Ogushi, Y., G. Akabane, T. Hasegawa, H. Mochida, M. Matsuda, M. Suzuki, and S. Tanaka. 2010. Water adaptation strategy in anuran amphibians: molecular diversity of aquaporin. Endocrinology **151**:165-173.

Ogushi, Y. A., G.; Hasegawa, T.; Mochida, H.; Matsuda, M.; Suzuki, M.; Tanaka, S. 2010. Water Adaptation Strategy in Anuran Amphibians: Molecular Diversity of Aquaporin. Endocrinology **151**:165-173.

Pandey, R. N., S. Yaganti, S. Coffey, J. Frisbie, K. Alnajjar, and D. Goldstein. 2010a. Expression and immunolocalization of aquaporins HC-1, -2, and -3 in Cope's gray treefrog, Hyla chrysoscelis. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **157**:86-94.

Pandey, R. N., S. Yaganti, S. Coffey, J. Frisbie, K. Alnajjar, and D. Goldstein. 2010b. Expression and immunolocalization of aquaporins HC-1,-2, and-3 in Cope's gray treefrog,< i> Hyla chrysoscelis</i>. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **157**:86-94.

Pao, S. S., I. T. Paulsen, and M. H. Saier. 1998. Major facilitator superfamily. Microbiology and Molecular Biology Reviews **62**:1-34.

Parkinson, R. W. 1994. Sea-level rise and the fate of tidal wetlands Journal of Coastal Research **10**:987-989.

Parmesan, C. 2006. Ecological and evolutionary responses to recent climate change. Annual Review of Ecology, Evolution, and Systematics:637-669.

Parsons, K. J., M. Concannon, D. Navon, J. Wang, I. Ea, K. Groveas, C. Campbell, and R. C. Albertson. 2016. Foraging environment determines the genetic architecture and

evolutionary potential of trophic morphology in cichlid fishes. Molecular ecology **25**:6012-6023.
Pearson, R. G. 2006. Climate change and the migration capacity of species. Trends in Ecology & Evolution **21**:111-113.
Peng, M., L. Xie, and L. J. Pietrafesa. 2004. A numerical study of storm surge and inundation in the croatan-albemarle-pamlico estuary system Estuarine, Coastal, and Shelf Science **59**:121-137.
Petranka, J. W., and E. J. Doyle. 2010. Effects of road salts on the composition of seasonal pond communities: can the use of road salts enhance mosquito recruitment? Aquatic Ecology **44**:155-166.
Pfennig, D. W., M. A. Wund, E. C. Snell-Rood, T. Cruickshank, C. D. Schlichting, and A. P. Moczek. 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends in Ecology & Evolution **25**:459-467.
Pigliucci, M., and C. J. Murren. 2003. Perspective: Genetic assimilation and a possible evolutionary paradox: can macroevolution sometimes be so fast as to pass us by?. Evolution **57**:1455-1464.
Pinder, A. M., S. A. Halse, J. M. McRae, and R. J. Shiel. 2004. Aquatic invertebrate assemblages of wetlands and rivers in the weatbelt region of Western Australia. . Records of the Western Australian Museum Supplement **67**.
Piscart, C., J. C. Moreteau, and J. N. Beisel. 2005. Biodiversity and structure of macroinvertebrate communities along a small permanent salinity gradient (Meurthe River, France). Hydrobiologia **551**.
Plummer, M. 2015. rjags: Bayesian Graphical Models using MCMC. R package version 3-15.
Plummer, M., N. Best, K. Cowles, and K. Vines. 2006. coda: Convergence Diagnosis and Output Analysis for MCMC. R News.
Pough, F. H., and S. Kamel. 1984. Post-metamorphic change in activity metabolism of anurans in relation to life history. Oecologia **65**:138-144.
Pre, C. G., S. J. Culver, D. J. Mallinson, K. M. Farrell, D. R. Corbett, B. P. Horton, C. Hillier, S. R. Riggs, S. W. Snyder, and M. A. Buzas. 2011. Rapid Holocene coastal change revealed by high-resolution micropaleontological analysis, Pamlico Sound, North Carolina, USA. Quaternary Research **76**:319-334.
Rader, R. B., D. P. Batzer, and S. A. Wissinger. 2001. Bioassessment and management of North American freshwater wetlands. John Wiley & Sons.
Rahmstorf, S., M. Perrette, and M. Vermeer. 2012. Testing the robustness of semi-empirical sea level projections Climate Dynamics **39**:861-875.
Räsänen, K., and L. Kruuk. 2007. Maternal effects and evolution at ecological timescales. Functional Ecology **21**:408-421.
Reed, T. E., D. E. Schindler, and R. S. Waples. 2011. Interacting effects of phenotypic plasticity and evolution on population persistence in a changing climate. Conservation BIology **25**:56-63.
Reed , T. E., R. S. Waples, D. E. Schindler, J. J. Hard, and M. T. Kinnison. 2010. Phenotypic plasticity and population viability: the importance of environmental predictability. Proceedings of the Royal Society of London B: Biological Sciences **277**.
Refsnider, J. M., and F. J. Janzen. 2010. Putting eggs in one basket: ecological and evolutionary hypotheses for variation in oviposition-site choice. Annual Review of Ecology, Evolution and Systematics **41**:39-57.

Reid, N. M., D. A. Proestou, B. W. Clark, W. C. Warren, J. K. Colbourne, J. R. Shaw, S. I. Karchner, M. E. Hahn, D. Nacci, M. F. Oleksiak, and D. L. Crawford. 2016. The genomic landscape of rapid repeated evolutionary adaptation to toxic pollution in wild fish. Science **354**:1305-1308.

Relyea, R. A. 2002a. Costs of phenotypic plasticity. The American Naturalist **159**:272-282.

Relyea, R. A. 2002b. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs **72**:77-93.

Renn, S. C. P., and M. E. Schumer. 2013. Genetic accommodation and behavioural evolution: insights from genomic studies. Animal Behavior **85**.

Reznick, D. N., and C. K. Ghalambor. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. Genetica **112**:183-198.

Rieger, J. F., C. A. Binckley, and W. J. Resetarits Jr. 2004. Larval performance and oviposition site preference along a predation gradient. Ecology **85**:2094-2099.

Riggs, S. R., and D. V. Ames. 2003. Drowning the North Carolina Coast: Sea-Level Rise and Estuarine Dynamics. Raleigh, NC, North Carolina Sea Grant Program.

Riginos, C., and C. W. Cunningham. 2005. Invited review: local adaptation and species segregation in two mussel (*Mytilus edulis*× *Mytilus trossulus*) hybrid zones. Molecular ecology **14**:381-400.

Rios-López, N. 2008. Effects of increased salinity on tadpoles of two anurans from a Caribbean coastal wetland in relation to their natural abundance. Amphibia-Reptilia **29**:7-18.

Roberts, J. 1970. Variations in salinity tolerance in the Pacific Treefrog, Hyla regilla. Oregon [dissertation]. Corvallis, OR: Oregon State University.

Rohr, J. R., and K. A. McCoy. 2010. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environmental Health Perspectives **2010**:20-32.

Ruibal, R. 1959. The ecology of a brackish water population of *Rana pipiens* Copeia **1959**:315-322.

Ruiz-Jarabo, I., C. A. Gonzalez-Wevar, R. Oyarz, J. Fuentes, E. Poulin, C. Bertran, and L. Vargas-Chacoff. 2016. Isolation Driven Divergence in Osmoregulation in *Galaxias maculatus(*Jenyns, 1848) (Actinopterygii: Osmeriformes). PLOS one **11**:e0154766.

Saitoh, Y., Y. Ogushi, Y. Shibata, R. Okada, S. Tanaka, and M. Suzuki. 2014. Novel Vasotocin-Regulated Aquaporins Expressed in the Ventral Skin of Semiaquatic Anuran Amphibians: Evolution of Cutaneous Water-Absorbing Mechanisms. Endocrinology **155**:2166-2177.

Sanzo, D., and S. J. Hecnar. 2006. Effects of road de-icing salt (NaCl) on larval wood frogs (Rana sylvatica). Environ Pollut **140**:247-256.

Sarkar, S. 2004. From the Reaktionsnorm to the evolution of adaptive plasticity. Phenotypic plasticity. Pages 10-30 *in* T. J. DeWitt and S. M. Scheiner, editors. Functional and conceptual approaches. Oxford University Press, NY., New York.

Savolainen, O., M. Lascoux, and J. Merila. 2013. Ecological genomics of local adaptation. Nat Rev Genet **14**:807-820.

Scavia, D., J. C. Field, D. F. Boesch, R. W. Buddemeier, V. Burkett, D. R. Cayan, M. Fogarty, M. A. Harwell, R. W. Howarth, C. Mason, D. J. Reed, T. C. Royer, A. H. Sallenger, and J. G. Titus. 2002. Climate change impacts on U.S. coastal and marine ecosystems Estuaries **25**:149-164.

Scheiner, S. M. 1993. Genetics and evolution of phenotypic plasticity. Annual Review of Ecology and Systematics **24**:35-68.
Scheiner, S. M. 2014. Bet-hedging as a complex interaction among developmental instability, environmental heterogeneity, dispersal, and life-history strategy. . Ecology and evolution **4**:505-515.
Scheiner, S. M., M. Barfield, and R. D. Holt. 2017. The genetics of phenotypic plasticity: Genetic assimilation, the Baldwin effect, and evolutionary rescue. Ecology and evolution **7**:8788-8803.
Scheiner, S. M., and R. D. Holt. 2012. The genetics of phenotypic plasticity: Variation versus uncertainty. Ecology and evolution **2**.
Schlichting, C. D. 2004. The role of phenotypic plasticity in diversification. Phenotypic plasticity: functional and conceptual approaches:191-200.
Schluter, D., T. D. Price, and L. Rowe. 1991. Conflicting serlection pressures and life history trade-offs. Proceedings of the Royal Society of London B: Biological Sciences **246**:11-17.
Schmidt-Nielsen, B., and R. P. Forster. 1954. The effect of dehydration and low temperature on renal function in the bullfrog. Journal of Cellular and Comparative Physiology **44**:233-246.
Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods **9**:671-675.
Schuler, M. S., and R. A. Relyea. 2018. A Review of the Combined Threats of Road Salts and Heavy Metals to Freshwater Systems. BioScience **68**:327-335.
Schwartz, M. W., L. R. Iverson, A. M. Prasad, S. N. Matthews, and R. J. O'Connor. 2006. Predicting extinctions as a result of climate change. Ecology **87**:1611-1615.
Semlitsch, R. D., D. E. Scott, and J. H. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in Ambystoma talpoideum. Ecology **69**:184-192.
Senior, C. A., R. G. Jones, J. A. Lowe, C. F. Durman, and D. Hudson. 2002. Predictions of extreme precipitation and sea-level rise under climate change Philos Trans A Math Phys Eng Sci **360**:1301-1311.
Shao, Y., L. J. Wang, L. Zhong, M. L. Hong, H. M. Chen, R. W. Murphy, D. D. Wu, Y. P. Zhang, and J. Che. 2015. Transcriptomes reveal the genetic mechanisms underlying ionic regulatory adaptations to salt in the crab-eating frog. Scientific reports **5**:17551.
Shibata, Y., T. Sano, N. Tsuchiya, R. Okada, H. Mochida, S. Tanaka, and M. Suzuki. 2014. Gene expression and localization of two types of AQP5 in Xenopus tropicalis under hydration and dehydration. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, **307**:R44-R56.
Shoemaker, V., S. Hillman, S. Hillyard, D. Jackson, L. McClanahan, P. Withers, and M. Wygoda. 1992. Exchange of water, ions, and respiratory gases in terrestrial amphibians. Environmental physiology of the amphibians:125-150.
Simão, F. A., R. M. Waterhouse, P. Ioannidis, E. V. Kriventseva, and E. M. Zdobnov. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics **31**:3210-3212.
Skelly, D. K. 1992. Field evidence for a cost of behavioral antipredator response in a larval amphibian. Ecology:704-708.
Smith, D. C. 1983. Factors controlling tadpole populations of the chorus frog (Pseudacris triseriata) on Isle Royale, Michigan. Ecology:501-510.
Smith, M. J., E. S. G. Shreiber, M. P. Scroggie, M. Kohout, K. Ough, J. Potts, R. Lennie, D. Turnbull, C. Jin, and T. Clancy. 2006. Associations between anuran tadpoles and salinity

in a landscape mosaic of wetlands impacted by secondary salinisation Freshwater Biology **52**:75-84.
Smith-Unna, R., C. Boursnell, R. Patro, J. M. Hibberd, and S. Kelly. 2016. TransRate: reference-free quality assessment of de novo transcriptome assemblies. . Genome research **26**:1134 - 1144.
Song, L., and L. Florea. 2015. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. GigaScience **4**:48.
Squires, Z. E., P. C. E. Bailey, R. D. Reina, and B. B. M. Wong. 2010. Compensatory growth in tadpoles after transient salinity stress. Marine and Freshwater Research **61**:219-222.
Steffen, W., J. Grinevald, P. Crutzen, and J. McNeill. 2011. The Anthropocene: conceptual and historical perspectives. Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, **369**:842-867.
Stone, H. R., and J. R. Morris. 2014. DNA damage emergency: cellular garbage disposal to the rescue? Oncogene **33**:805.
Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science **306**:1783-1786.
Su, Y.-S., and M. Yajima. 2015. R2jags: Using R to Run 'JAGS'. . R package version 0.5-7.
Sun, Y. B., Z. J. Xiong, X. Y. Xiang, S. P. Liu, W. W. Zhou, X. L. Tu, L. Zhong, L. Wang, D. D. Wu, B. L. Zhang, and C. L. Zhu. 2015. Whole-genome sequence of the Tibetan frog *Nanorana parkeri* and the comparative evolution of tetrapod genomes. . Proceedings of the National Academy of Sciences **112**:pp.E1257-E1262.
Suzuki, M., T. Hasegawa, Y. Ogushi, and S. Tanaka. 2007. Amphibian aquaporins and adaptation to terrestrial environments: a review. Comp Biochem Physiol A Mol Integr Physiol **148**:72-81.
Suzuki, M., Y. Shibata, Y. Ogushi, and R. Okada. 2015. Molecular machinery for vasotocin-dependent transepithelial water movement in amphibians: aquaporins and evolution. The Biological Bulletin **229**:109-119.
Suzuki, M., and S. Tanaka. 2009. Molecular and cellular regulation of water homeostasis in anuran amphibians by aquaporins. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **153**:231-241.
Taigen, T. L., and F. H. Pough. 1985. Metabolic correlates of anuran behavior. American Zoologist **25**:987-997.
Takata, K., T. Matsuzaki, and Y. Tajika. 2004. Aquaporins: water channel proteins of the cell membrane. Progress in Histochemistry and Cytochemistry **39**:1-83.
Team, R. C. 2018. R: A Language and Environment for Statistical Computing.
Therneau, T. 2015. survival: A package for survival analysis in S. R statistical environmnet.
Thirion, J.-M. 2014. salinity of the reproduction habitats of the Western spadefoot Toad Pelobates cultripes (cuvier, 1829), along the atlantic coast of France. Herpetozoa **27**:13-20.
Thomas, C. D., A. Cameron, R. E. Green, M. Bakkenes, L. J. Beaumont, Y. C. Collingham, B. F. Erasmus, M. F. De Siqueira, A. Grainger, and L. Hannah. 2004. Extinction risk from climate change. Nature **427**:145-148.
Thorson, T. B. 1955. The relationship of water economy to terrestrialism in amphibians Ecology **36**:100-116.
Thorson, T. B. 1956. Adjustment of water loss in response to desiccation in amphibians. Copeia **1956**:230-237.

Titus, J. G., and C. Richman. 2001. Maps of lands vulnerable to sea level rise: modeled elevations along the US Atlantic and Gulf coasts. Climate Research **18**:205-228.

Touchon, J. C. 2006. Hatching plasticity in two temperate anurans: responses to pathogen and predation cues. Canadian Journal of Zoology **84**:556-563.

Touchon, J. C., I. Gomez-Mestre, and K. M. Warkentin. 2006. Hatching plasticity in two temperate anurans: responses to a pathogen and predation cues. Canadian Journal of Zoology **84**:556-563.

Touchon, J. C., M. W. McCoy, T. Landberg, J. R. Vonesh, and K. M. Warkentin. 2015. Putting μ/g in a new light: plasticity in life history switch points reflects fine-scale adaptive responses. Ecology **96**:2192-2202.

Touchon, J. C., and K. M. Warkentin. 2008. Reproductive mode plasticity: Aquatic and terrestrial oviposition in a treefrog. PNAS **105**:7495-7499.

Traill, L. W., M. L. Lim, N. S. Sodhi, and C. J. Bradshaw. 2010. Mechanisms driving change: altered species interactions and ecosystem function through global warming. J Anim Ecol **79**:937-947.

Travis, J. 1994. Evaluating the adaptive role of morphological plasticity. Ecological Morphology:99-122.

Tuberville, T. D., J. D. Willson, M. E. Dorcas, and J. W. Gibbons. 2005. Herpetofaunal species richness of southeastern national parks Southeastern Naturalist **4**:537-569.

Turner, M. D., B. Nedjai, T. Hurst, and D. J. Pennington. 2014. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research **1843**:2563-2582.

Uchiyama, M., and N. Konno. 2006. Hormonal regulation of ion and water transport in anuran amphibians. Gen Comp Endocrinol **147**:54-61.

Uchiyama, M., and H. Yoshizawa. 1992. Salinity tolerance and structure of external and internal gills in tadpoles of the crab-eating frog, Rana cancrivora. Cell and Tissue Research **267**:35-44.

Urban, M. C. 2015. Accelerating extinction risk from climate change. Science **348**:571-573.

Urban, M. C., G. Bocedi, A. P. Hendry, J.-B. Mihoub, G. Pe'er, A. Singer, J. R. Bridle, L. G. Crozier, L. De Meester, W. Godsoe, A. Gonzalez, J. J. Hellmann, R. D. Holt, A. Huth, K. Johst, C. B. Krug, P. W. Leadley, S. C. F. Palmer, J. H. Pantel, A. Schmitz, P. A. Zollner, and J. M. J. Travis. 2016. Improving the forecast for biodiversity under climate change. Science **353**.

Urban, M. C., J. L. Richardson, and N. A. Reidenfelds. 2014. Plasticity and genetic adaptation mediate amphibian and reptile responses to climate change. Evolutionary Applications **7**:88-103.

Van Buskirk, J., and S. A. McCollum. 2000. Influence of tail shape on tadpole swimming performance. Journal of Experimental Biology **203**:2149-2158.

Vander Wal, E., D. Garant, M. Festa-Bianchet, and F. Pelletier. 2013. Evolutionary rescue in vertebrates: evidence, applications and uncertainty. . Phil. Trans. R. Soc. B **368**: p.20120090.

Via, S., and R. Lande. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution:505-522.

Vitt, L. J., J. P. Caldwell, H. M. Wilbur, and D. C. Smith. 1990. Amphibians as harbingers of decay. BioScience **40**:418-418.

Vonesh, J. R., and De la Cruz. 2002. Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. Oecologia **133**:325-333.
Vonesh, J. R., and K. M. Warkentin. 2006. Opposite shifts in size at metamorphosis in response to larval and metamorph predators. Ecology **87**:556-562.
Waddington, C. H. 1953. Genetic assimilation of an acquired character. Evolution **7**:118-126.
Wallace, A. R. 1859. On the Tendency of Varieties to depart indefinitely from the Original Type. John Murray, London.
Walther, G.-R., E. Post, P. Convey, A. Menzel, C. Parmesan, T. J. C. Beebee, J.-M. Fromentin, O. Hoegh-Guldberg, and F. Bairlein. 2002. Ecological responses to recent climate change. Nature **416**:389-395.
Walworth, N. G., M. D. Lee, F. X. Fu, D. A. Hutchins, and E. A. Webb. 2016. Molecular and physiological evidence of genetic
assimilation to high CO2 in the marine nitrogen fixer Trichodesmium. Proc. Natl. Acad. Sci. **113**:E7367–E7374.
Wells, K. D. 2007. The Ecology and Behavior of Amphibians. The University of Chicago Press, Chicago, Illinois.
Werner, E. E. 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. The American Naturalist **128**:319-341.
Werner, E. E., and B. R. Anholt. 1996. Predator-induced behavioral indirect effects: consequences to competitive interactions in anuran larvae. Ecology **77**:157-169.
Werner, E. E., and J. F. Gilliam. 1984. The ontogenetic niche and species interactions in size-structured populations. . Annual Review of Ecology and Systematics **15**:393-425.
Werner, E. E., and M. A. McPeek. 1994. Direct and indirect effects of predators on two anuran species along an environmental gradient. Ecology:1368-1382.
Werner, E. E., and S. D. Peacor. 2003. A review of trait-mediated indirect interactions in ecological communities. Ecology **84**:1083-1100.
West-Eberhard, M. J. 2003. Developmental plasticity and evolution. Oxford University Press, Oxford, England.
Whitehead, A., F. Galvez, S. Zhang, L. M. Williams, and M. F. Oleksiak. 2011a. Functional Genomics of Physiological Plasticity and Local Adaptation in Killifish. Journal of Heredity **102**:499-511.
Whitehead, A., J. L. Roach, S. Zhang, and F. Galvez. 2011b. Genomic mechanisms of evoloved physiological plasticity in killifish distributed along an environmental salinity gradient. Proceedings of the National Academy of Science **108**:6193-6198.
Whitehead, A., J. L. Roach, S. Zhang, and F. Galvez. 2012. Salinity- and population-dependent genome regulatory response during osmotic acclimation in the killifish (*Fundulus heteroclitus*) gill. Journal of Experimental Biology **215**:1293-1305.
Whitman, D. W., and A. A. Agrawal. 2009. What is Phenotypic Plasticity and Why is it important? .1-63.
Wijethunga, U., M. Greenlees, and R. Shine. 2016. Living up to its name? The effect of salinity on development, growth, and phenotype of the "marine" toad (Rhinella marina). Journal of Comparative Physiology B **186**:205-213.
Wilbur, H. M. 1980. Complex Life Cycles. Annual Review of Ecology and Systematics **11**:67-93.
Wilbur, H. M., and J. P. Collins. 1973. Ecological aspects of amphibian metamorphosis: nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. Science **182**:1305-1314.

Wilder, A. E., and A. M. Welch. 2014. Effects of Salinity and Pesticide on Sperm Activity and Oviposition Site Selection in Green Treefrogs, Hyla cinerea. Copeia **2014**:659-667.
Williams, K., K. C. Ewel, R. P. Stumpf, F. E. Putz, and T. W. Workman. 1999. Sea-level rise and coastal forest retreat on the west coast of florida, USA Ecology **80**:2045-2063.
Williams, K., M. MacDonald, and L. d. S. L. Sternberg. 2003. Interactions of storm, drought, and sea-level rise on coastal forest- A case study. Journal of Coastal Research **19**:1116-1121.
Williams, S. J. 2013. Sea Level Rise Implications for Coastal Regions Journal of Coastal Research **63**:184-196.
Willmer, P., G. Stone, and I. Johnston. 2005. Environmental Physiology of Animals. 2nd edition. Blackwell Publishing, Oxford, England.
Wood, L., and A. M. Welch. 2015. Assessment of interactive effects of elevated salinity and three pesticides on life history and behavior of southern toad (Anaxyrus terrestris) tadpoles. Environmental Toxicology and Chemistry **34**:667-676.
Wood, S. N. 2011. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. Journal of the Royal Statistical Society **73**:3-36.
Wu, C.-S., and Y.-C. Kam. 2009a. Effects of salinity on the survival, growth, development, and metamorphosis of Fejervarya limnocharis tadpoles living in brackish water. Zoolog Sci **26**:476-482.
Wu, C. S., I. Gomez-Mestre, and Y. C. Kam. 2012. Irreversibility of a bad start: early exposure to osmotic stress limits growth and adaptive developmental plasticity. Oecologia **169**:15-22.
Wu, C. S., and Y. C. Kam. 2009b. Effects of salinity on the survival, growth, development, and metamorphosis of Fejervarya limnocharis tadpoles living in brackish water. Zoolog Sci **26**:476-482.
Wu, C. S., W. K. Yang, T. H. Lee, I. Gomez-Mestre, and Y. C. Kam. 2014. Salinity acclimation enhances salinity tolerance in tadpoles living in brackish water through increased Na(+) , K(+) -ATPase expression. J Exp Zool A Ecol Genet Physiol **321**:57-64.
Wund, M. A. 2012. Assessing the impacts of phenotypic plasticity on evolution. Integr Comp Biol **52**:5-15.
Wund, M. A., J. A. Baker, B. Clancy, J. L. Golub, and S. A. Foster. 2008. A test of the "flexible stem" model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. American Naturalist **172**.
Wygoda, M. L., T. F. Dabruzzi, and W. A. Bennett. 2011. Cutaneous resistance to evaporative water loss in the Crab-eating frog (*Fejervarya cancrivora*) Journal of Herpetology **45**:417-420.

APPENDICES:

Appendix A: IACUC AUP #D302

4/9/14
Administrative Approval by Sue McRae 4/10/14
95

East Carolina University Animal Use Protocol (AUP) Amendment Form
Latest Revision, February 2014

FOR IACUC USE ONLY	
AUP # D302	
Date received: 4/9/14	
Full Review and date:	Designated Reviewer and date:
Approval date: 4/10/14	
Pain Category: E	
Amendments approved: Ø	
Minor Amendment:	
Significant Amendment:	If so, number?

Please fill out completely and email to davenportp@ecu.edu or iacuc@ecu.edu

PROJECT INFORMATION: Please list AUP Number and Title
AUP #D302: The Effects of Saltwater Intrusion on Freshwater Wetland Animals.

Principal Investigator:
Mike McCoy

1. What is the purpose or rationale for the protocol amendment?
Briefly explain in simple, non-technical language the reason(s) for amending the project.
In our original AUP, we did not plan to distinguish between the sexes of collected individuals for the physiological component of the project. However, we have learned that sex-specific differences in the ability to osmoregulate during reproductive events may confound our results unless we control for sex. We wish to amend our AUP by including equal numbers of males and females, which will increase the number of animals needed. Since we plan to collect during reproductive events (i.e. during choruses), this change is necessary to ensure that the results of our study are representative and accurate.

2. Will different people use the animals or are new personnel being added to the AUP?
Will previously approved personnel be assuming new roles or responsibilities? No
Choose an item.
If so, list qualifications and training. N/A
Click here to enter text.

3. Have protocol related hazards (transgenic animals, infectious, chemical or biologic agents) changed with this amendment? No Choose an item.
If so, please describe the hazard and the oversight committee associated with this hazard (see AUP form II.E.1). If any hazardous agents have changed since the original AUP, please fill out the attached Hazardous Agents Form (Appendix 1). Oversight committee approval is required before the amendment can be approved by IACUC.
Click here to enter text.

+60

4. Please indicate changes to the animals or animal numbers.
a. Will the strain or sex of the animals change Yes, the sex will change. Choose an item.
If so, please list changes (use complete strain nomenclature and a brief description of the line/strain when possible). We will include equal numbers of male and female frog individuals (5 of each sex per replicate) individuals from each of the 3 populations.

Appendix B: IACUC AUP Amendment for #D302

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

April 14, 2015

Michael McCoy, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McCoy:

The Amendment to your Animal Use Protocol entitled, "The Effects of Saltwater Intrusion on Freshwater Wetland Animals" (AUP #D302) was reviewed by this institution's Animal Care and Use Committee on April 13, 2015. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

Appendix C: IACUC AUP #D328

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

May 15, 2015

Michael McCoy, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McCoy:

Your Animal Use Protocol entitled, "The Effects of Saltwater on the Reproductive Decisions of Coastal and Inland Frogs" (AUP #D328) was reviewed by this institution's Animal Care and Use Committee on 5/15/15. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

Appendix D: IACUC AUP Amendment #D328

East Carolina University

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

July 19, 2016

Michael McCoy, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McCoy:

The Amendment to your Animal Use Protocol entitled, "The Effects of Saltwater on the Reproductive Decisions of Coastal and Inland Frogs" (AUP #D328) was reviewed by this institution's Animal Care and Use Committee on July 18, 2016. The following action was taken by the Committee:

"Approved as submitted"

Note: An administrative change was made in 4.c. changing 6 groups to 5 groups of 50 hatchlings.

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Eddie Johnson

Eddie Johnson
Vice-Chair, Animal Care and Use Committee

EJ/jd

Enclosure

Appendix E: IACUC AUP #D314

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

January 18, 2017

National Science Foundation
Division of Environmental Biology
4201 Wilson Boulevard
Arlington, VA 22230

Dear Sir or Madam:

The vertebrate animal use described in the following application submitted to the National Science Foundation was reviewed and is congruent with an IACUC-approved animal use protocol:

Title of Application: "Mechanisms of Adaptation to Saltwater Environments by Amphibians"

Name of Principal Investigator: Michael McCoy, Ph.D./Molly Ann Albecker

Name of Institution: East Carolina University

Congruency Approval Date: January 18, 2017

Animal Use Protocol Expiration Date: (D314) June 23, 2017

This institution is fully accredited by AAALAC and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare. The Assurance Number is A3469-01.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

cc: ECU Office of Sponsored Program

Appendix F: IACUC AUP Amendment #D314

East Carolina University

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

August 28, 2015

Michael McCoy, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McCoy:

The Amendment to your Animal Use Protocol entitled, "Interacting Effects of Local Adaptation by Anurans to Salt, Predators and Water Mold", (AUP #D314) was reviewed by this institution's Animal Care and Use Committee on 8/28/15. The following action was taken by the Committee:

"Approved as amended"

****Please contact Dale Aycock prior to any hazard use**

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure