Leaping salt barriers: Mechanisms of salinity tolerance in locally adapted coastal frogs

Conceptual Motivation

Global climate change and other anthropogenic activities are having dramatic and accelerating impacts on habitats and the natural functioning of ecosystems globally[1]. Because environmental change poses a threat to biodiversity in affected landscapes[2], understanding and predicting how species respond to rapid environmental changes is a sorely needed focal point for research[3]. In general, it is assumed that as habitats change and become less suitable, organisms must either acclimate to the novel conditions, move to less affected habitats, adapt *in situ*[4], or be extirpated[5]. Extinction and migration are assumed to be the two most realistic outcomes, given that evolutionary change is classically assumed to occur too slowly to keep pace with contemporary rates of environmental change[6,7]. Thus, adaptation has scarcely been considered when projecting expected impacts of habitat modifications and climate change on biodiversity.

However, recent theoretical and empirical work has demonstrated that rapid evolutionary change and adaptation over short spatiotemporal scales is possible, and there is a growing body of literature demonstrating such responses over ecologically relevant time scales (i.e. over several generations)[8]. Yet few studies have examined the potential for rapid adaptive responses to rescue or buffer losses of species from environmental change. Currently, most models aimed at forecasting how climate change will impact biodiversity do not consider adaptation at all [9], which may exaggerate or bias predicted species losses. One particularly well-cited study (over 4,500 citations) predicts that 15-37% of all currently described species will be "committed to extinction" due to climate change, yet does not consider the potential for adaptation or evolution[10]. Our research is among the first to explicitly examine adaptation to climate change-induced modifications to habitat quality in an extinction-vulnerable species[11,12]. I substitute space for time to investigate patterns of local adaptation in an amphibian species that has increased tolerance to saltwater in coastal wetland habitats that are becoming increasingly saline due to sea level rise[13].
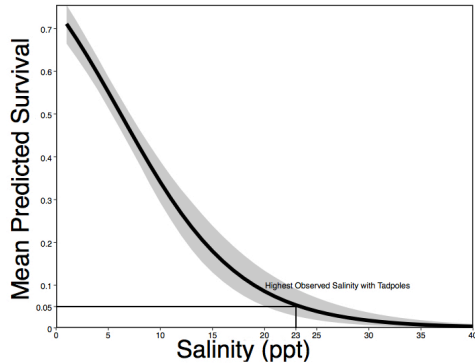
Secondary salinization, or the increase of salt concentrations into freshwater wetlands, is occurring in wetland habitats globally and at unprecedented rates [14]. Both climate change and coastal development are progressively increasing saltwater concentrations in coastal freshwaters via canal dredging, storm surges, saltwater intrusion, and sea level rise (SLR). Because salt is a major abiotic factor affecting species' distributions, the introduction of salt is expected to significantly alter species composition in freshwater habitats, and studies have documented a decline in freshwater species richness as salinity increases. The effects of secondary salinization are likely to be especially severe in regions where the coasts are gently sloped, have low elevation, and are gradually subsiding (sinking), such as North Carolina[15].

As coastal wetlands grow saltier, it is assumed that freshwater species will migrate into more suitable habitats. However habitat fragmentation, urbanization, and poor dispersal capabilities of many organisms tied to freshwater habitats (e.g. fish, turtles, and frogs) often preclude this option. Therefore it is assumed that species inhabiting freshwater coastal wetlands will be locally extirpated as salinities increase. This expectation has greatly influenced management efforts in coastal ecosystems and millions of dollars have been invested to prevent saltwater from contaminating freshwater aquifers and wetlands[16,17]. A better appreciation of adaptive responses by organisms may inform alternative management strategies in the face of habitat changes expected with climate change. For example, facilitated gene flow is a low cost management idea in which "pre-adapted" individuals of a species may be transferred into populations at risk of encountering environmental

challenges, thereby introducing favorable alleles into the population and facilitating the evolutionary processes that permit rapid adaptation[18]. However, before strategies like facilitated gene flow can be implemented, much remains to be understood about how adaptation occurs over short timeframes.

Study System and Objectives

Amphibians are ideal model taxa for studying the effects of increased salt concentrations on freshwater biota. They are important indicator species for wetland communities, comprise a significant proportion of the vertebrate biomass in wetland ecosystems[19], are an under-studied taxa among vertebrates[20], and have been classified by the IUCN as "climate change susceptible" . But most importantly, due to a variety of factors including permeable skin, a life history tied to water, an inability to concentrate and excrete excess salts, and poor dispersal capabilities, frogs are excellent sentinels and ideal research subjects to explore the effects of secondary salinization on salt-sensitive coastal fauna.

Figure 1: The mean predicted survival of all frog species following exposure to saltwater (ppt). This graph includes data from every study that quantified frog mortality (in all life stages) after saltwater exposure. I have highlighted the mean predicted mean survival (0.05) at 23 ppt, which is the highest salinity in which I observed thriving frog populations along the North Carolina coast.

I have found populations of frogs along the North Carolina coast that inhabit wetlands containing salt concentrations (>23ppt) well above levels typically lethal to amphibians (~6ppt) [21], and preliminary studies suggest these coastal populations are more salt tolerant than inland counterparts (Figs. 2-3). *In this study, I will test the hypothesis that chronically salt-exposed coastal frog populations exhibit different (presumably adaptive) physiological responses after experimental exposure to saltwater than freshwater conspecifics from inland populations and inland congeneric species*. I have two major aims for this study. First, I will unravel how particular physiological mechanisms contribute to saltwater tolerance in frogs, which is currently poorly understood. Second, by determining how physiological responses differ according to population, species, and salinity, I will describe reaction norms and patterns of adaptation to elevated salinity levels, which will supply important information about the etiology of adaptive evolution – an understudied necessity required to propel future theoretical, empirical, and managerial work.

Background and Supporting Data

Sea level rise is predicted to significantly impact North Carolina's coastal habitats[22], therefore I conducted field surveys characterizing current amphibian distributions in >20 wetlands along North Carolina's inner- and outer-banks. During these surveys, I documented multiple populations of green treefrogs (*Hyla cinerea*) (both breeding adults and larvae) in North Carolina saltmarsh habitats with salinities above 20ppt, which are approximately 3.8-fold higher than the lethal salinity reported for this species[23]. In light of these findings, I conducted a meta-analysis of all published studies of salt tolerance in frogs and found that these are among the highest salinities in which any North American tree frog species has ever been recorded (Fig.1).

Motivated by these observations, I conducted experiments to test for evidence of local adaptation. First, I ran a common garden experiment to test whether female frogs from coastal populations were less likely to avoid salt water when laying eggs compared to inland populations. I found that not only do coastal frogs show less salt avoidance when choosing where to lay eggs, but also offspring from coastal pairs have a higher probability of hatching in higher salinities (Fig. 2). I also
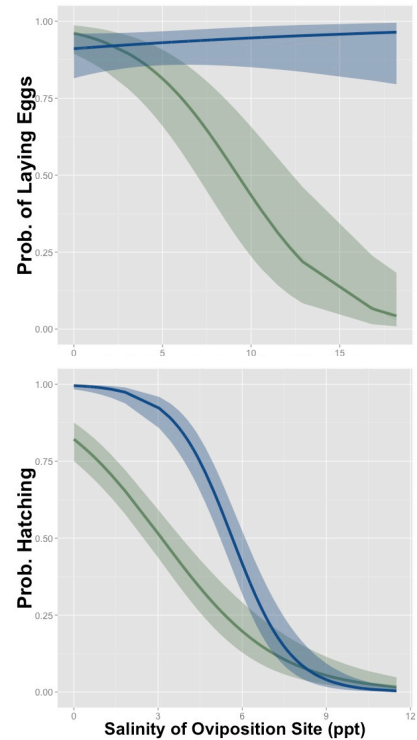
conducted a common garden experiment in which coastal and inland adult green treefrogs (*Hyla cinerea*) and the closely related grey tree frogs (*Hyla chrysoscelis*, which are restricted inland freshwater ponds) were exposed to the same common gradient ("garden") of salinities to examine whether there were differences in blood plasma osmolality following acute salt exposure. I found that inland populations of both species (green and grey treefrogs) showed much lower propensity to regulate solute concentrations (osmolality) in their blood than did coastal green treefrogs (Fig. 3).

Collectively, these findings suggest that we may be documenting local adaption to a significant environmental shift in macrofauna –a remarkable discovery! While these studies are interesting, developing and improving our understanding of the physiological mechanisms that allow this species to persist in salt affected environments is vital for developing baseline understanding of adaptation and for making predictions about which amphibian (and potentially non-amphibian) species might be able to adapt to sea level rise -- and which species will not. Indeed, this knowledge is paramount in the quest to understand mechanisms driving adaptive evolution and also for emerging management strategies.

Proposed Research
The physiological mechanisms that allow frogs to osmoregulate in salty environments are largely unknown -- with even less known about how these mechanisms differ among species and populations. Therefore, I propose to test for differences in osmoregulatory proteins in important osmoregulatory tissues to determine how these proteins and tissues contribute to the observed differences in salinity tolerance. Specifically I will quantify the presence and abundance of three cell surface proteins that are central to maintaining osmotic balance in cells of nearly all vertebrates and in multiple osmoregulatory tissues (kidney, bladder, skin). These proteins include the sodium-potassium pump (NKA), and the sodium-potassium-chloride co-transporter (NKCC). These protein pumps are chief regulators of water, sodium, and chloride ions within cells in vertebrates, and these ionic functions directly correspond to the molecular composition of seawater. Thus, adaptive responses will likely be demonstrated by differential abundances of these proteins within osmoregulatory tissues.
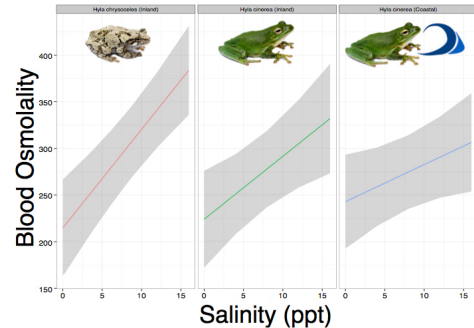

Figure 2: The top panel demonstrates that coastal pairs (blue) have a high probability of laying eggs in a pool as salinity increases, while inland pairs (green) avoid more saline pools. The bottom panel demonstrates that eggs from coastal pairs (blue) were more likely to hatch in higher salinities compared to eggs from inland pairs (green).

I have already collected kidney, bladder, and pelvic skin tissues from adult green treefrogs (*H. cinerea*)(from coastal and inland populations) and grey treefrogs (*H. chrysoscelis*)(never found in salinized coastal wetlands) after acute exposure to one of five salinities (0ppt, 4ppt, 8ppt, 12ppt, and 16ppt) (N=60, 5 salinities x 3 populations [coastal *H. cinerea*, inland *H. cinerea*, inland *H. chrysoscelis*] x 4 replicates) [ECU IACUC approval: D302]. This dose-response, common "gradient" (garden) design allows me to assemble physiological reaction norms to increasing salinity levels, characterize the amount of plasticity among species and populations, and calculate the amount of variation in responses among populations and species—key information in appreciating the mechanistic drivers of adaptation to osmotically stressful environments.

In this proposal I am requesting funds to utilize "Wes", which is a simple western machine that was recently acquired by my institution. I will use this state-of-the-art technology to quantify protein abundance in collected kidney, bladder, and pelvic skin tissues. "Wes" is an ideal alternative to traditional western blotting, because it can quickly conduct immuno-detection assays for large numbers of samples and produce reproducible and accurate estimates of protein concentrations. Using "Wes", I will quantify the abundance of proteins in essential osmoregulatory tissues in adults. I focus on NKA and NKCC abundance in the kidney, bladder, and pelvic skin as these proteins and tissues are implicated as the primary targets involved in maintaining osmotic balance in adult amphibians[24]. I will analyze protein abundance data using regression-based approaches, such that inferences will be based on the differences in slopes and variances around the relationships that emerge between protein quantity, species and location, and salinity (e.g. Figs. 2 and 3).

Figure 3. Individuals from coastal populations of *Hyla cinerea* (right panel) maintain lower blood plasma osmolality than inland *Hyla cinerea* (middle panel). Both populations of inland and coastal *Hyla cinerea* maintain lower blood plasma osmolality compared to a closely related sister taxa, *Hyla chrysoscelis* (left panel).

Products

The results produced from the germinal projects strongly suggest that North Carolina's coastal frogs are becoming locally adapted to tolerate significantly higher salinities than ever recorded for treefrogs. This discovery and my position on North Carolina's increasingly saltwater-intruded coast has presented me with the unique opportunity to investigate organismal adaptation at the land-sea interface. I expect that the data collected from "Wes" (this study), along with complementary immune-histological work being completed by an undergraduate researcher (Sarah Gill), will produce a major publication, especially considering that this will be among the first studies to examine mechanisms of adaptation to environmental change. Further, I anticipate this study will fuel multiple presentations by both Sarah and I (ECUs Research and Creative Activities Week, Ecological Society of America, Southeastern Ecology and Evolution Conference, Society for Integrative and Comparative Biology). The findings of this study are important for the completion of my dissertation, and when combined with my other findings, will represent one of the first and most complete studies of environmental adaptation in macrofauna that has been conducted to date.

Proposed Budget

Item	Price per Unit	Number needed	Total Cost
Simple Western Kit	$1,250.00	3 kits	$ 3,750.00
NKA Antibodies	$40.00 per mL	5 mL	$ 200.00
NKCC Antibodies	$40.00 per mL	5 mL	$ 200.00
Laboratory consumables	$500.00		$ 500.00
Total Request:			**$ 4,650.00**

Budget Justification

Although the tissues have already been collected and preserved, I require antibodies, laboratory supplies, and "Wes kits" in order to complete this investigation. A single kit for the Simple Western contains eight plates, which can run up to 26 samples (total of 208 samples per kit). I plan to quantify the amount of two different proteins (NKA, NKCC) for each of the three

populations/species (*Hyla cinerea* coastal, *Hyla cinerea* inland, *Hyla chrysoscelis*) in three osmoregulatory tissues (kidney, bladder, pelvic skin) across five salinity doses (0ppt, 4ppt, 8ppt, 12ppt, 16ppt) for a total of 450 samples. Thus, I will require three kits that each cost approximately $1,250.00 (total = $3,750.00). In addition, primary antibodies for NKA and NKCC cost $40.00 per milliliter from Hybridoma Bank. I anticipate needing approximately 5 mL for each antibody for a total of $400.00. I also request $500.00 to purchase necessary laboratory consumables such as pipette tips, vials, gloves, buffers, etc. In total, I am requesting $4,650.00 to complete this project.

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