Physiological Responses to Salinity Vary with Proximity to the Ocean in a Coastal Amphibian

Gareth R. Hopkins[1,2,3,*]
Edmund D. Brodie Jr.[1,2]
Lorin A. Neuman-Lee[1]
Shabnam Mohammadi[1,2]
George A. Brusch IV[4]
Zoë M. Hopkins[1]
Susannah S. French[1,2]
[1]Department of Biology, Utah State University, 5305 Old Main Hill, Logan, Utah 84322; [2]Ecology Center, Utah State University, 5305 Old Main Hill, Logan, Utah 84322; [3]Behaviour and Evolution Group, School of BioSciences, University of Melbourne, Parkville, Victoria 3010, Australia; [4]School of Life Sciences, Arizona State University, 427 East Tyler Mall, Tempe, Arizona 85281

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ABSTRACT

Freshwater organisms are increasingly exposed to elevated salinity in their habitats, presenting physiological challenges to homeostasis. Amphibians are particularly vulnerable to osmotic stress and yet are often subject to high salinity in a variety of inland and coastal environments around the world. Here, we examine the physiological responses to elevated salinity of rough-skinned newts (*Taricha granulosa*) inhabiting a coastal stream on the Pacific coast of North America and compare the physiological responses to salinity stress of newts living in close proximity to the ocean with those of newts living farther upstream. Although elevated salinity significantly affected the osmotic (body weight, plasma osmolality), stress (corticosterone), and immune (bactericidal ability) responses of newts, animals found closer to the ocean were generally less reactive to salt stress than those found farther upstream. Our results provide possible evidence for some physiological tolerance in this species to elevated salinity in coastal environments. As freshwater environments become increasingly saline and more stressful, understanding the physiological tolerances of vulnerable groups such as amphibians will become increasingly important to our understanding of their abilities to respond, to adapt, and, ultimately, to survive.

*Corresponding author. Present address: Behaviour and Evolution Group, School of BioSciences, University of Melbourne, Parkville, Victoria 3010, Australia; e-mail: gareth.r.hopkins@gmail.com.

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Introduction

Organisms are often exposed to stressors in their environment that cause deviations from homeostasis and may initiate a physiological stress response (French et al. 2006; Wingfield 2013). Increasingly, freshwater organisms are having to cope with stressful elevated salinity in their environments due to road-deicing salt application (e.g., Kaushal et al. 2005), secondary salinization (e.g., Williams 2001), and, in coastal habitats, a combination of stochastic storm events (Gunzburger et al. 2010; Trenberth 2011) and rising sea levels (Nicholls et al. 1999; Lowe and Gregory 2005; Purcell et al. 2008).

Amphibians are particularly sensitive to salt stress due to their highly permeable skin and eggs (Shoemaker and Nagy 1977). As such, the presence of amphibians in brackish and saline environments has traditionally been discounted, despite existing knowledge of select amphibians capable of tolerating saline habitats (e.g., Neill 1958; Gordon et al. 1961; Gordon 1962; Katz 1989; Gomez-Mestre and Tejedo 2003). A recent review (Hopkins and Brodie 2015) reported that amphibians inhabiting saline environments are not as rare as previously thought, although information on how most species achieve physiological tolerance of these environments is lacking. Understanding the physiological responses of amphibians to salinity is becoming increasingly important, as populations face saline stressors both inland (e.g., Collins and Russell 2009; Kearney et al. 2012) and in coastal environments (e.g., Thirion 2002; Gunzburger et al. 2010; Brown and Walls 2013).

Classic work on amphibian osmoregulatory physiology, often examining body-weight changes in response to salinity, has elucidated physiological mechanisms based primarily on cutaneous exchange of water and Na^+ ions and the hypersynthesis and retention of urea for some species living in estuarine environments (e.g., the crab-eating frog [*Fejervarya cancrivora*] in mangrove swamps; e.g., Gordon et al. 1961; reviewed in Shoemaker et al. 1992; Katz 2015). However, the osmoregulatory responses of the majority of amphibian species from a variety of habitats are still unknown (Hopkins and Brodie 2015). In addition, increased salinity may also disrupt other physiological processes that are critical for fitness but largely unexamined in this context, including endocrine and immune responses. In response to a stressor, the vertebrate hypothalamic-pituitary-adrenal (HPA) axis initiates a hormonal cascade, resulting in the production of

adrenal steroid hormones (glucocorticoids; Sapolsky 1992). The production of glucocorticoids (corticosterone or cortisol, depending on the species) is thus considered a general indicator of physiological stress (Sapolsky 1992; Moore and Jessop 2003). In addition to measuring glucocorticoids, there has been a recent push to assay functional physiological responses such as immunity (Hopkins and DuRant 2011; Breuner et al. 2013). Assaying the bactericidal ability of blood plasma immune components (French and Neuman-Lee 2012) can yield important data on the effects of a stressor on components of immune function critical for fitness (Lochmiller and Deerenberg 2000). Finally, while studies involving the effects of stressors on physiology have often focused on either naïve or impacted populations, studies that directly compare a variety of physiological responses of individuals from each type of population are less common but arguably more valuable (Garland and Adolph 1991; Hopkins and DuRant 2011).

Here we test the physiological responses to increased salinity in rough-skinned newts (*Taricha granulosa* Skilton, Caudata: Salamandridae) inhabiting two different areas of a coastal stream on the Pacific coast of North America, including a tidal area where dramatic storm events can wash seawater into newt breeding habitats (fig. 1*c*). We performed multiple laboratory experiments to examine the physiological responses of newts from both tidal and upstream locations to elevated salinity in a common-garden environment. We started with a classic metric for osmoregulatory physiology, change in body weight in response to environmental salinity, and followed this with in-depth analyses of osmotic (Na^+/K^+-ATPase activity of the skin and plasma osmolality), stress (release of corticosterone), and immune (bactericidal ability of blood immune components) physiology, comparing newts from each area of the stream. We hypothesized that newts living in closer proximity to the ocean would be less reactive to salt stress and thus show fewer changes from homeostasis relative to newts found farther upstream.

Methods

Fieldwork

Adult newts were found breeding in Hunter Creek (42° 23′19.60″N, 124°25′21.54″W; fig. 1), a small stream in Curry County, on the southern Oregon coast in May 2012. We collected adult individuals by hand, dip net, and minnow trap from both the tidal mouth area of the stream (hereafter referred to as tidal newts), 100–500 m from the Pacific Ocean, and 3,000 m upstream, where there was no visible tidal influence (hereafter referred to as upstream newts). There were no significant differences in morphometrics (mass, snout-vent length, total length) of newts from tidal versus upstream locations ($P > 0.10$ for all *t*-tests comparing males and females separately from each area). Salinity in Hunter Creek was measured using a handheld YSI EC300 multimeter (YSI, Yellow Springs, OH) and was found to

Figure 1. *a*, The study site, Hunter Creek, Oregon, showing the location of tidal (*a*, *b*) and upstream (*a*) locations where rough-skinned newts (*Taricha granulosa*; *b*) were found. Distances are measured from the Pacific Ocean at high tide in May 2012. *c*, Random storm events, such as was experienced in September 2011, can wash seawater up into the tidal area of the stream where newts are found (white foam from the ocean is visible surrounding the distinctive rock formation [yellow dot]—the closest location where newts were found). *a*, Aerial photograph from Google Earth. *b*, Photograph by G. R. Hopkins. *c*, Photograph by Rose Muenker (used with permission).

be 0.0–0.1 ppt in the upstream habitat and to range between 0.1 and 1.4 ppt in the tidal area, depending on the day, time, tide, and microhabitat measured. In such a potentially volatile environment (fig. 1*c*), salinity measurements at any one point in time should be treated as a snapshot only and not necessarily the potential range that the habitat or its inhabitants may experience. For example, dramatic storm events also wash seawater upstream throughout the tidal area (fig. 1*c*). For the purposes of this study, we chose to examine a wider range of salinity stress that is potentially more indicative of these extreme events faced by newts in the tidal area (see below).

Laboratory Animal Care

Adult newts were taken back to Utah State University (Logan, UT) and used in accordance with Utah State University Institutional Animal Care and Use Committee (IACUC) regulations (protocol 1524). Newts were individually housed in plastic containers (35 cm × 20 cm × 13 cm) with 2.0 L of filtered tap water (0.2 ppt) and a styrofoam perch in a temperature control room set at 5°C with a 12:12 photoperiod, per standard husbandry procedures for long-term storage in our lab. Newts were fed blackworms (*Lumbriculus variegatus*) ad libitum until used for experiments 6–9 months later. All animals were euthanized in MS-222 at the conclusion of experiments, in accordance with the approved IACUC protocol (1524).

Laboratory Salt Challenge 1: Weight Change Experiment

Female and male newts from each stream location were transferred to a chamber set at 14°C (average water temperature in Hunter Creek recorded in the field) in November 2012 and given 10 days to acclimate. Eight days before the beginning of the experiment, each newt was fed ~2 g of blackworms and then fasted until the experiment began. We randomly assigned 4–6 male and 4–6 female newts from each location to a salt solution of either 5 ppt (256.4 mOsm/kg) or 10 ppt (512.8 mOsm/kg) made with lab-grade NaCl (Mallinckrodt Baker, Paris, KY) and filtered tap water or a control (0.2 ppt [10.3 mOsm/kg] filtered tap water). Thus, there were a total of 9–11 newts in each treatment. Salt concentrations were selected based on the general tolerances of freshwater (0–1 ppt) and estuarine (4–25 ppt) organisms (Vernberg and Vernberg 2001) and because 10 ppt has been proposed as a general upper level of salt tolerance for amphibians found in coastal habitats (Gomez-Mestre and Tejedo 2003; Hopkins and Brodie 2015). On the morning of the experiment, newts were removed from their holding containers, patted dry with a paper towel, and weighed on a digital mass balance (±0.1 g). They were then immediately transferred to a container with 1.5 L of randomly assigned test solution where their bodies were completely submersed. After 6 h, each newt was removed from solution, patted dry, and weighed on the same balance. They were then returned to their test solution and weighed again at 24 h. Percent body-weight change from initial mass was calculated.

Laboratory Salt Challenge 2

Salt Stress and Blood and Tissue Collection. Male newts (33 from each stream location) were gradually acclimated from 5°C to 14°C over 13 days at the end of January 2013; they were then held at 14°C for an additional 3 days before the start of experimentation. Newts were randomized to different lab-grade NaCl solutions (5.0 ppt, 10.0 ppt) or a freshwater control (0.2 ppt; 10–11 newts per treatment). Each newt was placed in 1.5 L of solution for 6 h. At 6 h, blood was sampled from the caudal vein (within 3 min) after snipping the end of the tail with a sterile surgical blade (French and Neuman-Lee 2012; Neuman-Lee et al. 2015). Blood was centrifuged at 5°C at 1,106 *g* to separate plasma from cells and stored at −20°C until used in osmolality, corticosterone, and immune assays. All blood sampling was completed between 1500 hours and 1655 hours to ensure that there was no variation in hormone levels due to circadian rhythms. Tail snips were retained for Na^+/K^+-ATPase extraction and assaying and stored at −80°C until assayed.

A total of three newts exposed to control, two newts in 5 ppt, and six newts in 10 ppt in this experiment were previously exposed to salt in laboratory salt challenge 1, held 3 months earlier (only males from the previous experiment were used to eliminate sex-specific hormonal differences). There were no significant differences in responses of newts that had previously been exposed to salt in the first experiment versus those that were only exposed to salt in the second experiment (*t*-tests for all treatments, $P > 0.05$), and all newts were housed in freshwater (0.2 ppt) between experiments.

Plasma Osmolality. Osmolality of plasma samples was determined in triplicate using a vapor pressure osmometer (±6 mOsm/kg; model 5600, Wescor, Logan, UT), as described by Davis and DeNardo (2007). Two individual newts did not have enough plasma sample volume for triplicate readings and were, therefore, not included in the final analysis. When analyzed, it became apparent that one individual upstream animal in 10 ppt exhibited an abnormally low osmolality value (204.25 mOsm/kg; mean ± SEM osmolality for this group = 337.94 ± 7.17 mOsm/kg) and, as a result, was highly skewing normality of the data. Removal of this outlier greatly improved normality of the distribution of data, and we have chosen to present the results of analyses with and without this outlier. Analyses were therefore completed on samples from a total of 11 upstream and 10 tidal newts in control, eight upstream and nine tidal newts in 5 ppt, and seven (six, excluding the outlier) upstream and 11 tidal newts in 10 ppt.

Na^+/K^+-ATPase Extraction and Assay. A 5-mm sterile biopsy punch was used to collect a uniform-sized sample of skin and muscle tissue from newt tail snips for Na^+/K^+-ATPase extraction. Extraction and assaying followed Petschenka et al. (2012). Briefly, samples were homogenized in 500 μL distilled water using a 1-mL all-glass grinder (Wheaton). Homogenates were sonicated in a chilled bath for 15 min to further break apart cell membranes and then centrifuged at 10,000 *g* for 10 min to

precipitate debris. The supernatant was then ultracentrifuged (Beckman Coulter; Optima TLX; rotor: TLA-100.3) at 84,000 *g* for 30 min at 4°C to sediment the membranes containing enzymes. The supernatant was discarded, and the microsomal pellet was washed in distilled water and then resuspended in 300 μL distilled water by vortexing and sonication. After reconstitution, samples were stored at −20°C until assayed. Samples were thawed in a sonicator and thoroughly vortexed before assaying. Assaying was based on methods detailed by Petschenka et al. (2013). In brief, Na+/K+-ATPase activity was determined by the quantification of inorganic phosphate released from ATP after incubation with the samples. Phosphate was stained (Taussky-Shorr) and absorbance measured using a BioRad xMark Microplate Spectrophotometer. Na+/K+-ATPase activity was calculated by regressing the final absorbance (nM) values of the phosphate standards against their corresponding known concentrations and using the resulting linear equation to determine the concentration of phosphate (mM) produced in the assay.

Corticosterone Radioimmunoassay. Circulating corticosterone concentrations were determined following Neuman-Lee et al. (2015) using a radioimmunoassay protocol modified from French et al. (2008). Samples were assayed in duplicate (antibody: MP Biomedicals, Lot 3R3PB-19E) and final concentrations adjusted using individual recoveries. Intra-assay variation was 6.9%. This assay has been used for *Taricha granulosa* previously (Neuman-Lee et al. 2015) but was additionally validated using pooled samples. We tested for parallelism using a serial dilution with the points 1∶1, 1∶2, 1∶3, 1∶5, and 1∶10 for interference by spiking samples with known quantities of corticosterone (40 pg/mL, 250 pg/mL, and 1,000 pg/mL) and for nonspecific binding (including with other steroid species).

Immune Assay. We performed a bactericidal assay to assess immune functioning in newts following salt exposure, using the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1∶5 plasma dilution with CO_2-independent media plus 4 nM L-glutamine and 10^4 colony-producing units *Escheria coli* (EPowerTM Microorganisms 483-237-1, ATCC 8739, MicroBioLogics, St. Cloud, MN) and agar broth on a 96-well microplate. We incubated the plate for 12 h and calculated absorbance using a BioRad xMark microplate reader. Samples were run in duplicate, with means being used for subsequent analyses. Bactericidal ability was calculated by dividing the absorbance for each sample by mean absorbance for the positive controls (containing only media and bacterial solution) and multiplying by 100. This provided the percent bacteria killed relative to the positive controls. Negative controls (containing media only) were also run to ensure that contamination was absent. One upstream individual in 10 ppt displayed much greater than average variation in replicates and was removed from analysis.

Statistical Analyses

For laboratory salt challenge 1, we compared percent body-weight change with a four-way repeated measures ANOVA with salt treatment (fixed-effect factor; three levels: control, 5 ppt, 10 ppt NaCl), stream location (fixed-effect factor; two levels: upstream, tidal), sex (fixed-effect factor; two levels: male, female), time (two time points), and their interactions as independent variables. After determining that sex did not play a significant explanatory role ($F_{1,47} = 0.056$, $P = 0.814$), we analyzed percent change in body weight with a three-way repeated measures ANOVA with salt treatment, stream location, and time (and their interactions) as explanatory variables. Where a significant effect was found, we performed Tukey-adjusted multiple comparisons separately at each time point (6 h and 24 h).

For laboratory salt challenge 2, we analyzed the overall effect of salt treatment (fixed-effect factor; three levels: control, 5 ppt, 10 ppt NaCl), stream location (fixed-effect factor; two levels: upstream, tidal), and their interaction on plasma osmolality, Na^+/K^+-ATPase, and bactericidal ability of newts using two-way ANOVAs. Tukey-adjusted multiple comparisons were conducted to determine differences among groups where overall effects were found to be significant. Bactericidal ability was square-root transformed to meet the assumptions of normality and homogeneity of variance. Corticosterone concentration was analyzed using a generalized linear model with a gamma distribution and log link function, due to an observed pattern of increased variance with the mean. Where a significant factor was found, Holm-simulated multiple comparisons were conducted to determine differences among groups. Finally, to investigate whether there was any correlation between osmoregulation and physiological stress, we conducted nonparametric Spearman correlations of corticosterone concentrations and (i) plasma osmolality and (ii) Na^+/K^+-ATPase for newts from both locations combined and separately for newts from each location. All statistical analyses were completed in SAS, version 9.3 (SAS Institute, Cary, NC), and R, version 3.03 (R Development Core Team 2013), with significance $\alpha = 0.05$.

Results

Laboratory Salt Challenge 1: Weight Change Experiment

Newts from both stream locations maintained their mass over time in control solutions (fig. 2). In contrast, salt treatment caused a significant increase in the mass of newts over time, with the greatest increases occurring in 5 ppt for upstream animals (significantly greater than animals in all other treatments; Tukey-adjusted $P < 0.05$; fig. 2). There was a significant effect of salt treatment ($F_{2,53} = 30.93$, $P < 0.0001$), stream location ($F_{1,53} = 9.13$, $P < 0.01$), and their interaction ($F_{2,53} = 5.18$, $P < 0.01$) on percent body-weight change. There was no significant effect of time ($F_{1,53} = 0.50$, $P = 0.483$), but there were significant interactions between time and stream location ($F_{1,53} = 5.24$, $P < 0.05$) and time and salt treatment ($F_{2,53} = 5.37$, $P < 0.01$). There was not a significant three-way interaction between time, salt treatment, and stream location ($F_{2,53} = 2.59$, $P = 0.084$). By 24 h, there were no significant differences among populations (Tukey-adjusted $P > 0.05$), but animals in salt treatments were all significantly heavier than those in control (Tukey-adjusted $P < 0.05$).

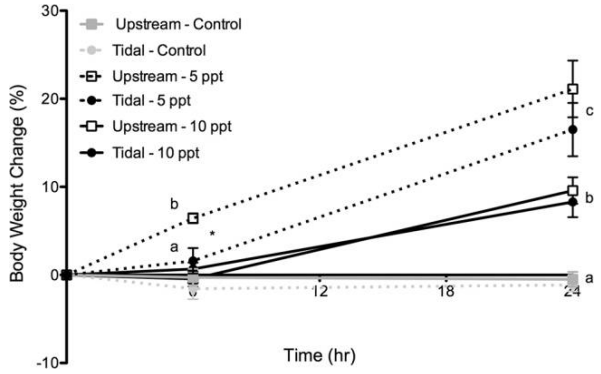

Figure 2. Mean (±SEM) percent body-weight change in rough-skinned newts (*Taricha granulosa*; $n = 9$–11 individuals [50% of each sex] per treatment per stream location) from tidal (filled circles) and upstream (open squares) locations of Hunter Creek after immersion in control (0.2 ppt; gray lines), 5 ppt (dotted black lines), or 10 ppt (solid black lines) NaCl solutions for 6 h and 24 h, respectively, in laboratory salt challenge 1. There was a significant effect of salt treatment ($P < 0.0001$), stream location ($P < 0.01$), and their interaction ($P < 0.01$) on percent body-weight change. There was no significant effect of time ($P = 0.483$), but there were significant interactions between time and stream location ($P < 0.05$) and time and salt treatment ($P < 0.01$). Different letters indicate significant treatment differences at each time point (Tukey-adjusted multiple comparisons for each time point), while asterisks indicate significant stream location-level differences in responses at that time point. Upstream animals gained significantly more weight than all other animals at 6 h (Tukey-adjusted multiple comparisons $P < 0.05$, as indicated by an asterisk), but there was not a significant effect of population at 24 h ($P > 0.05$), with both upstream and tidal animals gaining similar amounts of weight in these salt treatments and all significantly greater than animals in control ($P < 0.05$).

Laboratory Salt Challenge 2

Plasma Osmolality. Plasma osmolality significantly increased with salt concentration, with newts having the highest plasma osmolality in 10 ppt (fig. 3*a*; Tukey-adjusted $P < 0.0001$ for all multiple comparisons among treatments). Including the outlier in the analysis (see "Methods") yielded a significant effect of salt treatment ($F_{2,50} = 66.42$, $P < 0.0001$), though not of stream location ($F_{1,50} = 2.55$, $P = 0.116$) or the interaction term ($F_{2,50} = 0.392$, $P = 0.678$). With the exclusion of the outlier, salt treatment remained significant ($F_{2,49} = 115.67$, $P < 0.0001$), and stream location became a significant factor influencing plasma osmolality ($F_{1,49} = 8.27$, $P < 0.01$). However, the interaction between treatment and location remained insignificant ($F_{2,49} = 1.14$, $P = 0.329$). On average, tidal newts exhibited lower plasma osmolality than upstream newts (fig. 3*a*).

Na^+/K^+-ATPase. There were no significant effects of salt treatment ($F_{2,57} = 1.23$, $P = 0.300$), stream location ($F_{1,57} = 1.93$, $P = 0.170$), nor their interaction ($F_{2,57} = 0.022$, $P = 0.978$) on Na^+/K^+-ATPase activity (fig. 3*b*).

Corticosterone. There was an overall significant effect of salt treatment ($F_{2,56} = 5.01$, $P < 0.01$) and stream location ($F_{1,56} = 7.47$, $P < 0.01$), but not their interaction ($F_{2,56} = 2.24$, $P = 0.116$), on corticosterone levels of newts exposed to salt in the laboratory. Corticosterone levels increased with salinity (Tukey-adjusted $P < 0.05$ for among-treatment comparisons except between 0 and 5 ppt [$P = 0.999$] when populations grouped together). Animals from upstream, however, had significantly higher salt-induced corticosterone levels than tidal animals, especially at 5 ppt (Holm-simulated multiple-comparison between stream locations at 5 ppt, $P < 0.01$, and at 10 ppt, $P = 0.0985$; fig. 4).

There was a significant positive correlation between corticosterone and plasma osmolality (newts from both stream locations combined: $S = 9{,}561.23$, $P = 0.00017$, $\rho = 0.512$; upstream only: $S = 772$, $P = 0.0071$, $\rho = 0.564$; tidal only: $S = 1{,}834.34$, $P = 0.022$, $\rho = 0.440$). There was not, however, between corticosterone and Na^+/K^+-ATPase (newts from both stream locations combined: $S = 18{,}286.2$, $P = 0.647$, $\rho = 0.067$; upstream only: $S = 1{,}356.4$, $P = 0.294$, $\rho = 0.234$; tidal only: $S = 3{,}439.12$, $P = 0.805$, $\rho = -0.049$).

Immune Functioning. Newt immune functioning was significantly affected by salt treatment ($F_{2,59} = 5.20$, $P < 0.01$) but not stream location ($F_{1,59} = 0.899$, $P = 0.347$) or the interaction

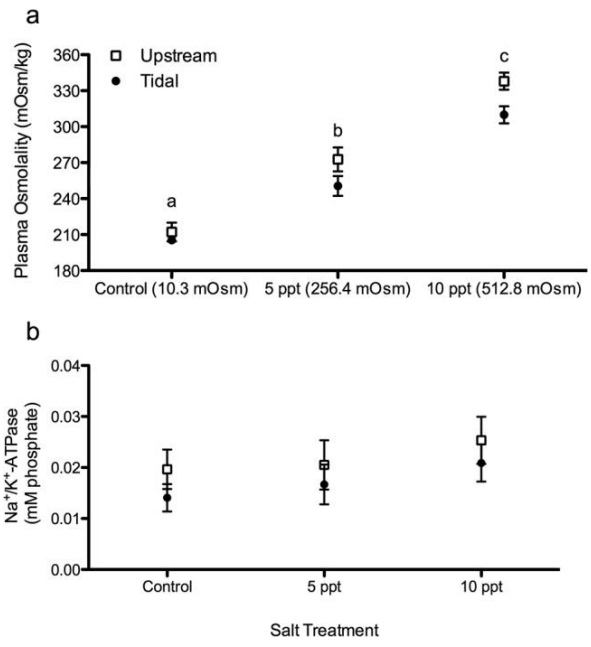

Figure 3. Mean (±SEM) plasma osmolality (mOsm/kg; *a*) and Na^+/K^+-ATPase (mM phosphate produced; *b*) of tidal (filled circles) and upstream (open squares) male newts ($n = 6$–11 animals per treatment per stream location; see text for details) after immersion in control (0.2 ppt), 5 ppt, or 10 ppt NaCl solutions in laboratory salt challenge 2. There was a significant effect of salt treatment ($P < 0.0001$) and stream location ($P < 0.01$), but not their interaction ($P = 0.329$), on plasma osmolality. There was not a significant effect of either factor on Na^+/K^+-ATPase. Different letters indicate significant differences among treatments (Tukey-adjusted multiple comparisons, $P < 0.05$).

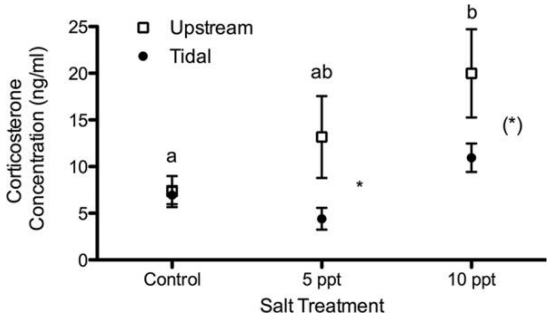

Figure 4. Mean (±SEM) blood plasma corticosterone concentration (ng/mL) of male newts (n = 9–11 animals per treatment per stream location) from tidal (filled circles) and upstream (open squares) locations after immersion in control (0.2 ppt), 5 ppt, or 10 ppt NaCl solutions in laboratory salt challenge 2. There was a significant effect of salt treatment ($P < 0.01$) and stream location ($P < 0.01$), but not their interaction ($P = 0.116$), on corticosterone levels of newts exposed to salt. Different letters indicate Tukey-adjusted significant differences among salt treatments ($P < 0.05$). Asterisks indicate Holm-simulated significant differences among newts from different stream locations at certain salt treatments: a solitary asterisk indicates $P < 0.05$; an asterisk in parentheses indicates $P < 0.10$.

between treatment and location ($F_{2,59} = 0.754$, $P = 0.475$). Exposure to 5 ppt saltwater (Tukey-adjusted $P < 0.01$ comparing control to 5 ppt) appears to have an overall negative effect on newt immune functioning, regardless of where in the stream newts are found (fig. 5).

Discussion

Exposure to saltwater significantly affected the osmotic, stress, and immune physiology of rough-skinned newts (*Taricha granulosa*) from a coastal stream in Oregon. Saltwater caused a disruption of osmotic homeostasis (newts gained weight and increased plasma osmolality), increased plasma corticosterone concentrations, and decreased immune function (at 5 ppt), all indicators of physiological stress. In support of our hypotheses, we found differences in responses to salt from animals living in different areas of the stream; newts found in close proximity to the ocean showed reduced physiological responses to salinity as compared to newts found farther upstream.

Salt exposure disrupted the osmoregulatory homeostasis of upstream newts, as seen in their increase in body mass relative to control. Animals found close to the ocean, in contrast, did not show a large disruption in either solution after 6 h of exposure (only small gains or losses relative to the upstream animals in salt) and only experienced significant disruption after 24 h of exposure (fig. 2). Disruption of osmotic homeostasis in response to salt is well known and expected in amphibians due to their highly permeable skin (Shoemaker and Nagy 1977). Similar patterns of initial increases in mass when placed in salt, possibly due to drinking or the temporary internal accumulation of urea, have been found in a number of other amphibian species, with smaller increases found in saline-adapted species (i.e., *Fejervarya cancrivora* vs. *Fejervarya tigerina* [Gordon et al. 1961]) and populations (i.e., beach vs. inland populations of the salamander *Batrachoseps gavilanensis* [Licht 1975]), similar to our results.

The internal osmolality of newts increased with environmental salinity in experiment 2 (fig. 3*a*). In response to external salinity changes, amphibians will often increase uptake of salts via integumentary sodium channels and pumps, as well as the hypersynthesis and retention of urea, to maintain an internal osmolality iso-osmotic with the external environment (reviewed by Shoemaker et al. 1992; Katz 2015). It appears that while newts from the tidal location were able to achieve this on average at 5 ppt (5 ppt = 256.4 mOsm/kg external osmolality; mean plasma osmolality of tidal newts at 5 ppt = 250.55 mOsm/kg), newts found farther upstream somewhat overshot iso-osmolality, with a mean plasma osmolality of 272.71 mOsm/kg, making their body fluids slightly hyperosmotic to the surrounding environment. If water were to then follow this gradient via osmosis, this might explain why upstream newts experienced a larger increase in body mass after 6 h at 5 ppt than tidal newts (fig. 2). Beach-dwelling salamanders (*B. gavilanensis*) that gained less weight in saltwater than their inland counterparts also maintained lower internal salt concentrations than inland animals when exposed to salt stress (Licht 1975); this was primarily due to lowered plasma Na^+ concentrations (Licht 1975). While we do not know the chemical makeup of the plasma of *T. granulosa* in our study, Brown et al. (1988) found that newts exposed to salt-depleted water decreased their plasma osmolarity primarily due to decreased Na^+ concentrations, which was correlated with a decrease in skin electrical activity (potentially due to decreased Na^+ pump activity). In our study, there was a weak trend of increasing Na^+/K^+-TPase activity at 10 ppt, with tidal newts having an overall lower activity compared to upstream newts regardless of treatment (fig. 3*b*). However, the trends in Na^+/K^+-ATPase activity were not statistically significant. It is likely that this result is due to our choice in assaying skin from the tail. It may

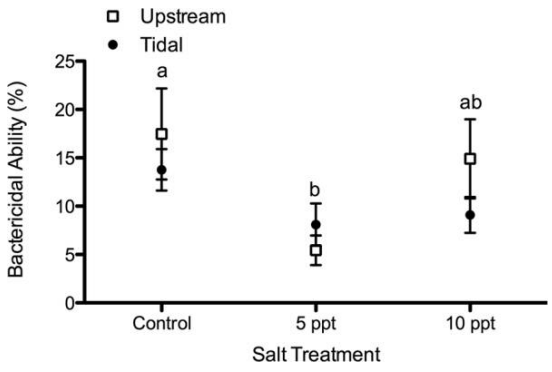

Figure 5. Mean (±SEM) percent of bacteria killed by blood plasma immune components from upstream (open squares) or tidal (filled circles) male newts (n = 9–11 animals per treatment per stream location) immersed in control (0.2 ppt), 5 ppt, or 10 ppt NaCl solutions in laboratory salt challenge 2. Immune functioning was significantly affected by salt treatment ($P < 0.01$) but not stream location ($P = 0.347$) or their interaction ($P = 0.475$). Different letters indicate significant differences among treatments (Tukey-adjusted multiple comparisons).

be that osmotic exchange is more active in other areas of the body, such as the ventral torso (Shoemaker and Nagy 1977; Toledo and Jared 1993). In addition, although many amphibian species tend to primarily use Na^+ ion regulation to osmoregulate (Katz 2015), other species primarily osmoregulate through retaining and oversynthesizing urea (Katz 2015). Although Licht (1975) did not implicate urea as important for the osmoregulation of *B. gavilanensis*, Jones and Hillman (1978) found that it was important in a related *Batrachoseps* species, and it may be that *T. granulosa* also use urea to osmoregulate. Future studies should examine concentrations of specific ions and urea in the plasma to elucidate a more precise osmoregulatory mechanism. In contrast to at 5 ppt, at 10 ppt newts were hypo-osmotic to the external environment (10 ppt = 512.8 mOsm/kg; highest plasma osmolality of any newt at 10 ppt = 360 mOsm/kg). This fact may help explain the otherwise unexpected result of lower body-weight change after 6 h at 10 ppt than 5 ppt, as seen in experiment 1. If the internal osmolality of newts is lower than the external environment, water will not enter the newts' body through osmosis and, therefore, not cause an increase in weight.

The osmoregulatory realities of high salinity exposure for newts from the two areas of this stream may be evident in other physiological metrics, such as the stress response. Indeed, the dose-dependent relationship between salinity and corticosterone (fig. 4) seen in newts from the upstream location is indicative of physiological stress. There was a significant positive correlation between corticosterone and plasma osmolality, suggesting that larger internal changes are indeed more stressful. Increases in corticosterone with salinity have also been seen in larval salamanders (*Ambystoma jeffersonianum*; Chambers 2011), and similar levels of corticosterone increases to those in this study have been seen in newts (Neuman-Lee et al. 2015) and other salamanders (e.g., *Desmognathus ochrophaeus*; Ricciardella et al. 2010) in response to other stressors, such as simulated predation, and capture and handling. The relationship we observed between salinity and corticosterone in upstream newts is not as strong in newts from the tidal location, however, and in fact appears to only manifest at 10 ppt, when osmoregulation becomes more challenging. It may be that tidal newts do not live in an environment that typically reaches this salinity, or it may simply be that this represents a more general limit to amphibian salt tolerance, even for saline-adapted species (Gomez-Mestre and Tejedo 2003; Hopkins and Brodie 2015). Below 10 ppt, concurrent with the apparent trends seen in osmoregulatory physiology, it appears that newts living in close proximity to the ocean do not respond to increased salinity as stressful, and thus their threshold for initiating a physiological stress response may be higher than their conspecifics living farther upstream (Romero et al. 2009).

Our results also indicate that salt-induced physiological stress has the potential to have downstream effects on immune functioning (fig. 5), even with only acute exposure. However, these effects are not as clear-cut as those on osmoregulation and stress physiology, and they vary according to concentration and the area of the stream in which newts are found. Bactericidal ability of plasma from newts found upstream was reduced at 5 ppt but not at 10 ppt. In contrast, tidal newts showed a nonsignificant decrease at 5 ppt and 10 ppt. One possible explanation for our nonlinear immune results is that the effects of corticosterone are context dependent, where they are suppressing the immune system in some instances (e.g., chronic duration) and enhancing it in others (i.e., acute duration; Dhabhar 2009). The increase in corticosterone in newts at 10 ppt (fig. 3) may offset negative immune effects due to salt stress found at 5 ppt. Further research should thus focus on the nuanced effects of different types of stress to partition out the true effects of salt stress itself versus corticosterone on the immune system. Regardless, salt stress does seem to alter immunity, which is critical for individual health and lifetime fitness (Lochmiller and Deerenberg 2000; Ricklefs and Wikelski 2002; Rolff 2002) and is crucial when considering fitness effects on populations.

While there were clearly differences in the salt-induced physiological responses of newts from different areas of the stream, the mechanisms behind such differentiation remain less clear. Differentiation may be the result of local adaptation, genetic drift, phenotypic plasticity, or developmental priming early in life (Garland and Adolph 1991, 1994). We did take steps to minimize phenotypic plasticity by housing newts from the two locations in a common garden environment in the lab for several months before physiological testing. However, as only field-caught adult newts were collected and tested in this study, it is impossible to rule out the effects of early life exposure to potentially increased salinity in the tidal area (such as an event like that seen in fig. 1*c*) affecting the physiological plasticity of tidal individuals later in life (Garland and Adolph 1991; although exposure to transient salinity early in life has recently been shown to have no effect on salinity tolerance later in life in another amphibian [Kearney et al. 2014]). In addition, while there were significant mean differences (or, at least, a trend thereof) in physiological responses of newts from different locations along the stream, it would be prudent to point out that there was also high individual variation in responses from newts within both areas. This may be a product of the small sample sizes used in our laboratory experiments, the relatively brief nature of the experiments or potentially a very real phenomenon characterizing these populations in this environment. A previous study found high intrapopulation variation in salinity tolerance in an inland, salt-naïve newt population (Hopkins et al. 2013), and there also appears to exist high individual variation in salt tolerance in these coastal newts. In addition, while the groups of newts in our study are separated by over 3 km, there is also likely migration and gene flow between the locations, which may act to dampen patterns of differentiation (Garland and Adolph 1994; Lenormand 2002). We currently know very little about the genetic nature of salinity tolerance in amphibians (Hopkins and Brodie 2015), and more work is needed to disentangle the genetic or plastic nature of the patterns of differentiation we see in physiological responses to salt in newts living in different areas of this stream.

Regardless of its ultimate cause, the fact that newts living in close proximity to the ocean do not appear to be as physiologically stressed by salt compared to conspecifics upstream sug-

gests that animals may have some resilience to future increased salinity in this habitat. Gunzburger et al. (2010) also found amphibian communities living in normally freshwater coastal wetlands on the east coast of the United States were remarkably resilient to salinity increases due to hurricane storm surges, and Moreira et al. (2015) found a similar result with numerous species of Brazilian tadpoles subjected to intermittent elevated salinity due to artificial estuary breaching. Amphibians are found in coastal environments around the world, often in habitats affected by storms and sea spray (Hopkins and Brodie 2015). As the rate of storm events, coupled with sea-level rise, increases in these habitats, physiological strategies for coping with increased salinity may be increasingly necessary for many species. More broadly, salinity is impacting freshwater habitats in both coastal and noncoastal areas around the world, including those impacted by road-deicing salts. After road-deicing events, salinity in normally fresh roadside aquatic habitats spikes dramatically for a brief period (Whitfield and Wade 1992, 1996), not unlike a coastal storm event (fig. 1*c*). Thus, amphibians and other osmotically sensitive freshwater organisms living in a variety of habitats increasingly have to cope with salinity in both coastal and inland environments.

As environments become more stressful, understanding the physiological responses of animals and their ability to survive in these habitats will only become more critical to the conservation of biodiversity (Hopkins and DuRant 2011). Our ability to place physiology in the context of these environments, stressors (Wingfield and Kitaysky 2002; Wingfield 2013), and the larger adaptive landscape (Garland and Adolph 1991, 1994; Parsons 2005) will be key to gaining these insights.

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