

Salted roads lead to edema and reduced locomotor function in wood frogs

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1 **Abstract**

2 Human activities have caused massive losses of natural populations across the globe. Like many groups,
3 amphibians have experienced substantial declines worldwide, driven by environmental changes such as
4 habitat conversion, pollution, and disease emergence. Each of these drivers is often found in close
5 association with the presence of roads. Here we report a novel consequence of roads affecting an
6 amphibian native to much of North America, the wood frog (*Rana sylvatica*). Across 38 populations
7 distributed from southern to central New England, we found that adult wood frogs living adjacent to roads
8 had higher incidence and severity of edema (bloating caused by fluid accumulation) during the breeding
9 season than frogs living away from the influence of roads. This effect was best explained by increased
10 conductivity of breeding ponds, caused by runoff pollution from road salt used for de-icing. Edema
11 severity was negatively correlated with locomotor performance in more northerly populations.
12 Interestingly, northern populations experience more intense winters, which tends to result in more de-
13 icing salt runoff and increased energetic demands associated with overwintering cryoprotection needs.
14 Thus, this emerging consequence of roads appears to impose potential fitness costs associated with
15 locomotion, and these effects might be most impactful on populations living in regions where de-icing is
16 most intense.

17

18 **Introduction**

19 Human activities are a leading driver of wild population declines, extirpations, and extinctions, culminating
20 in the loss of global biodiversity (Ceballos et al. 2015, Leigh et al. 2019, Rosenberg et al. 2019). These
21 losses shorten the branches of the tree of life and leave lasting if not permanent alterations to long-
22 evolved interactions among species (Emer et al. 2019), modifying ecosystem function and the services
23 they provide (Symstad et al. 1998, Cardinale et al. 2012).

24 Among the many groups of organisms declining across the globe, amphibians have captured a
25 special place in the attention of researchers, fueling a surge in scientific inquiry over the past three
26 decades (Green et al. 2020). Causes of declines in amphibian populations are complex and dependent
27 on both regional context and species-specific adaptations, but unsurprisingly share a common theme of
28 human activity (Grant et al. 2020). Drivers include habitat loss and alteration, pollution, climate change,

29 and disease. These and many other human-mediated drivers can act synergistically, confronting
30 populations with conditions far exceeding the capacity of existing trait variation or the potential for
31 adaptive evolutionary or plastic responses (reviewed in Green et al. 2020).

32 Lurking among the numerous drivers of amphibian declines lies a common but often overlooked
33 thread: roads. The planet is swathed in some 64 million km of roads (Central Intelligence Agency 2013),
34 with 25 million km more expected by 2050 (Dulac 2013). This vast network contributes an extensive and
35 generally negative set of impacts on natural populations (Trombulak and Frissell 2000, Fahrig and
36 Rytwinski 2009), even as some may be adapting (Brady and Richardson 2017). Perhaps most direct and
37 apparent, roads result in huge numbers of road kill – dispatching up to 1 million vertebrates per day in the
38 U.S. alone (Lalo 1987) – a phenomenon that disproportionately affects amphibians (Glista et al. 2008).
39 Roads can facilitate the spread of both disease (Urban 2006) and invasive species (Mortensen et al.
40 2009); they fragment habitats (Reed et al. 1996), reduce connectivity (Shepard et al. 2008), and affect
41 dispersal and gene flow (Riley et al. 2006). Roads are also substantial sources of pollution. Leaching and
42 stormwater runoff have deposited countless contaminants into the environment for decades (Huber et al.
43 2016). Many contaminants can reach toxic levels in soil, surface waters, and groundwater (Marsalek et al.
44 1999, Tang et al. 2013, McIntyre et al. 2018), and these effects can extend to habitats located well
45 beyond the road (Forman and Deblinger 2000). Some contaminants are fleeting, broken down by
46 chemical or biological processes, but others are long-lived or inert and tend to increase over time
47 (Kaushal et al. 2005). In short, this singular and physically narrow change to the landscape caused by
48 roads spurs numerous and wide-reaching drivers of population decline. Identifying and understanding
49 these drivers and their effects is essential for conservation efforts.

50 In areas with cold winters, a chief pollutant threatening amphibian populations is road salt. Due to
51 de-icing practices and the pervasiveness of roads, salt pollution of surface and groundwaters has become
52 common and widespread (Mullaney et al. 2009, Corsi et al. 2010). Even in large waterbodies (e.g.,
53 permanent ponds, lakes), runoff pollution can result in substantial salt concentrations exceeding national
54 water quality criteria for aquatic life (Dugan et al. 2017, Kaushal et al. 2018). In smaller waterbodies
55 however (e.g., vernal pools, wetlands), levels can be many times higher, approaching brackish conditions
56 typical of coastal waters, a stark contrast to the generally salt-free conditions of small, inland surface

57 waters. For many pool-breeding amphibians, these smaller waters are critical sites for reproduction,
58 where embryos and larvae develop before metamorphosing and dispersing into terrestrial habitats.
59 Across the complex life-history cycle common to many amphibians, sensitivity to salt tends to be highest
60 for embryos and larvae (Gordon and Tucker 1965, Uchiyama et al. 1990, Karraker et al. 2008, Albecker
61 and McCoy 2017). Thus, pool-breeding amphibians tend to face salt pollution at peak sensitivity, and this
62 timing often coincides with annual peaks in salt concentration that occur after winter. At high enough
63 concentrations, salt causes outright mortality in amphibians (Sanzo and Hecnar 2006, Brady et al. 2017).
64 At lower concentrations, sublethal effects are numerous. Aquatic-stage exposure to salt affects behavior
65 (Denoël et al. 2010) and modifies growth and developmental rates (Dananay et al. 2015, Brady 2017), it
66 increases malformations and susceptibility to disease (Karraker and Ruthig 2009, Brady 2013, Hall et al.
67 2020), and it causes ‘carry-over’ effects, negatively impacting post-metamorphic survival in the terrestrial
68 environment (Dananay et al. 2015); all of which can contribute to observed population declines (e.g.,
69 Karraker et al. 2008).

70 In the present study, we investigated an additional and novel physiological consequence of roads:
71 severe gross edema found in adult frogs breeding in polluted, roadside ponds. Although edema is
72 recognized as a disorder in veterinary and disease literature (e.g., Hadfield and Whitaker 2005, Miller et
73 al. 2011), its occurrence and causes in natural amphibian populations have been overlooked (but see Hall
74 et al. 2017). For the wood frog (*Rana sylvatica*) – the focus of our study – temporary edema can be found
75 among breeding adults in early spring, apparently resulting naturally from overwintering physiology.
76 During spring thaw, the reversal of the process whereby water is sequestered out of cells to prevent intra-
77 cellular ice formation, is thought to cause temporary edema (Kling et al. 1994, Irwin et al. 1999). However,
78 our preliminary observations suggest that spring edema is more severe in populations from polluted,
79 roadside ponds compared to those from unpolluted, woodland ponds.

80 Here, we quantified the severity of edema in wood frogs from 38 ponds in New England, where
81 road salt pollution is common. We posited that road salt might be causing severe edema, and therefore
82 asked whether variation in edema prevalence and severity correlates with road adjacency and water
83 conductivity, and if edema prevalence and severity increases with age, a proxy for lifetime exposure. We
84 also investigated directly the effect of roadside pond water on edema by estimating body mass gain for

85 animals exposed to water from roadside versus woodland ponds and compared to spring water. Next, we
86 used dissections and histological preparations to characterize the relation between edematous outward
87 appearance and potential underlying signs and causes. Since edema could be associated with winter
88 physiology and spring thaw, we also asked if blood glucose levels correlated with edema severity. Finally,
89 we investigated a potential fitness cost of edema. Specifically, we asked how jumping performance, a
90 commonly used fitness proxy for frogs, is affected by edema severity.

91

92 **Methods**

93 *Natural history*

94 Wood frogs are widespread in North America, ranging in distribution from the eastern southern
95 Appalachian Mountains and extending north- and westward to the Alaska-Yukon region within the Arctic
96 circle (Martof and Humphries 1959, Green et al. 2014). To survive winter conditions, particularly in higher
97 latitudes, wood frogs rely on physiological adaptations to freezing (Storey and Storey 1988, Costanzo et
98 al. 2015). Wood frogs typically use ephemeral or other small, fishless ponds for breeding in early spring.
99 Across our study area, breeding occurs typically between early March and mid-April, lasting about 1-2
100 weeks in any given pond. Immediately prior to breeding, adults migrate from upland terrestrial habitat to
101 mate in ephemeral ponds. Males amplex females and fertilize eggs externally during oviposition. Each
102 female lays a single clutch as an egg mass containing approximately 800–1100 eggs. Embryos develop
103 over 2–3 weeks before hatching and continue to develop as aquatic larvae throughout spring and early
104 summer until they metamorphose into terrestrial juveniles, which disperse into upland habitat. Adults can
105 live for 5-6 years (Berven 2009, Brady et al. 2019), and apart from annual breeding, tend to spend most
106 of their lives in terrestrial habitat. Juvenile wood frogs show strong natal site fidelity and, as adults, strong
107 breeding site fidelity (Berven and Grudzien 1990).

108

109 *Edema severity/prevalence and environmental variation*

110 We quantified edema by compiling data for 935 individual frogs from 38 breeding ponds (putative
111 populations, referred interchangeably as “populations” hereafter) in New England (N=20 roadside, 18
112 woodland; Fig. 1). Following Brady (2013), ponds were categorized as either ‘roadside’ (< 10-15 m from a

113 paved road) or 'woodland' (> 150 m from any road). Frogs were captured during four breeding seasons in
114 three different study areas (spanning from 41.2-43.8 degrees North), each containing a mix of roadside
115 and woodland populations: 1) Southern Connecticut (N=9 roadside, 9 woodland ponds in 2018), 2)
116 Northeastern Connecticut (N=6 roadside, 5 woodland ponds in 2010), and 3) the Upper Valley of Vermont
117 and New Hampshire (N=5 roadside, 4 woodland ponds in 2016 and 2017). In Northeastern Connecticut,
118 frogs were captured on their inbound breeding migration using drift fences with pitfall traps placed
119 adjacent to breeding ponds. Frogs from these populations were therefore not exposed to pond water
120 immediately prior to capture. In the other two study areas, drift fences with pitfalls were supplemented
121 with minnow traps. Thus, frogs from these populations had mixed histories of exposure to pond water
122 immediately before capture. Both types of traps were checked daily for new captures. Captured frogs
123 were returned to the lab and dorsal photographs were taken within two days. Edema severity was scored
124 from these photographs on a 1-3 integer scale, corresponding to 1) non-edematous, 2) moderate edema,
125 and 3) severe edema. For the analysis of prevalence, an individual was considered edematous if it had
126 an edema score of either 2 or 3. The following environmental variables were measured during the spring
127 breeding / rearing seasons in a subset of ponds: specific conductivity (N=31 ponds), dissolved oxygen
128 (N=29 ponds), and pH (N=18 ponds).

129 We used separate mixed models to evaluate the effect of population type (roadside vs. woodland)
130 on 1) edema severity score and 2) edema prevalence. Edema severity was fit using a linear model with a
131 Gaussian distribution while edema prevalence was fit using a binomial model with a logit link. In each
132 case, we first used AIC model selection to compare three candidate models fit with maximum likelihood
133 and the following random effect structures: population, population and region (i.e. Southern Connecticut,
134 Northeastern Connecticut, or Upper Valley of Vermont and New Hampshire), and population nested
135 within region. Using a threshold for delta AIC value of 2, the model with the single random effect of
136 population was preferred for both edema severity and prevalence. Fixed effect inference of severity was
137 made after refitting the selected model with restricted maximum likelihood estimation. Here and
138 elsewhere when linear mixed models were used, p-values were based on degrees of freedom calculated
139 with Satterthwaite's approach implemented in the R package 'lmerTest' (Kuznetsova et al. 2017).
140 Because our edema severity data could potentially be considered ordinal, we also composed a mixed

141 model using the R package 'ordinal' (Christensen 2019). Inference was similar between these two
142 modeling approaches ($P < 0.001$ in each case) and we therefore interpreted results from the linear model.
143 For the binomial responses of prevalence, inference was made using a likelihood ratio test between the
144 selected model and a reduced model in which the response mean was used as the sole fixed effect in
145 place of population type (i.e. intercept-only model). In a separate suite of standard linear models, we
146 analyzed edema in response to conductivity, dissolved oxygen, and pH. Pond-level averages of edema
147 score were used as the response variable in each model. Conductivity was log-transformed because of
148 its wide range (26-984 $\mu\text{S}/\text{cm}$) and bimodal distribution between woodland and roadside ponds.

149 We analyzed the relation between adult age and both edema severity and prevalence using data
150 from 74 individuals collected in 2014 from 9 populations (N=4 roadside, 5 woodland) in the Upper Valley
151 of Vermont and New Hampshire. Individuals used in this analysis were collected during the inbound
152 breeding migration (prior to arriving at ponds) using drift fences with pitfall traps. Edema was estimated
153 from photos as described above and age was estimated using skeletochronology of toe clips (methods
154 described in Brady et al. 2019). We used a linear model with either prevalence or severity as the
155 response variable, and the interaction between age and population type for fixed effects.

156

157 *Exposure-induced change in mass*

158 We examined the effects of pond water exposure on adult wood frog water uptake, a potential cause of
159 edema. We analyzed data from a prior study conducted in the same Northeastern CT populations,
160 designed originally to test the effects of early-embryo exposure to polluted pond water on larval survival
161 (see Brady 2017). Briefly, adult frogs were captured during the breeding season using drift fences with
162 pitfall traps adjacent to breeding ponds. All frogs were captured on their inbound migration and thus were
163 not yet exposed to breeding pond water at the time of capture. At each breeding pond, captured frogs
164 were measured for snout-vent length (SVL) and weighed prior to being paired haphazardly to breed in 5.1
165 L plastic aquaria filled with 1 L of either breeding-pond water or spring water. For roadside populations,
166 this design resulted in approximately half the treatments being roadside water and the other half being
167 spring water. For woodland populations, about half the treatments were woodland pond water and the
168 other half were spring water. Across 11 ponds (N=6 roadside, 5 woodland), an average of 8.5 (range: 4-

169 14) breeding pairs were stocked into each treatment (i.e. breeding-pond water or spring water). Exposure
170 period was determined by the time required to breed. Following breeding, adults were re-weighed.

171 We analyzed change in body mass relative to SVL for males prior to and after breeding. Females
172 were excluded from this analysis because change in mass caused by water is confounded with change in
173 mass due to oviposition. Specifically, we calculated a body condition index (BCI) as mass per unit SVL
174 (i.e. mass/SVL), both before and after exposure to pond or spring water that occurred while breeding. We
175 then calculated delta BCI as the ratio of post-breeding BCI to pre-breeding BCI. Thus, delta BCI values >
176 1 indicate body mass gain whereas those < 1 indicate body mass loss. We used separate linear mixed
177 models with population as the random effect to analyze both delta BCI and exposure duration across the
178 interaction of population type and exposure water type. We used the R package 'emmeans' (Lenth et al.
179 2018) to estimate marginal means for each of these models and to compute 95% confidence intervals
180 with respect to a null value of 1 (i.e. no change in body mass). We applied Tukey contrasts to make
181 pairwise comparisons of both delta BCI and exposure duration across this interaction.

182

183 *Morphology, histology and blood glucose*

184 In spring 2020, we necropsied six frogs from Southern Connecticut populations (N=1 with severe edema,
185 N=5 non-edematous, all male) to identify the underlying source of edema and to evaluate potential
186 changes in gross morphology associated with edema. Following prosection and gross dissection, soft
187 tissues (including gastrointestinal tract, liver, spleen, kidneys, lung, heart, reproductive tissues and
188 remainder of body) were fixed in 10% neutral buffered formalin, while long bones, associated muscle,
189 nerve and skin, as well as brain and eyes (in skull), were fixed in Bouin's solution for one week. Tissues
190 were processed using standard paraffin processing sectioned at 5 μ m, and stained with hematoxylin and
191 eosin for light microscopic evaluation. Images were taken with a Zeiss Axioskop and Zeiss AxioCam MRC
192 camera. We also measured blood glucose in subset of 54 male frogs from 6 roadside ponds (N = 35
193 frogs) and 4 woodland ponds (N = 19 frogs) in Southern Connecticut. We obtained a blood sample using
194 a 30.5 gauge needle to pierce the facial vein (Forzán et al. 2012). A drop of blood was then placed on a
195 testing strip and assayed using a Bayer Contour® Next blood glucose meter. We used a mixed model
196 with population as a random effect to analyze blood sugar in response to edema and population type. We

197 included body mass as a covariate in this analysis because of its potential effect on blood glucose
198 irrespective of edema related to pollution. The two highest blood glucose observations were removed
199 from analysis because they were 12 and 6.8 times the median absolute deviation.

200

201 *Jumping performance*

202 To evaluate whether jumping performance might be compromised by edema, we conducted jump trials on
203 a subset of frogs scored for edema from Southern Connecticut (N = 270 in 2019) and the Upper Valley of
204 Vermont and New Hampshire (N = 258 in 2016 and 2017). Frogs were placed on one end of a 0.91 X 5 m
205 piece of brown paper and at least 5 consecutive jumps were recorded, or frogs were placed between two
206 plastic walls (height: 34 cm; length: 3.4 m) separated by approximately 30 cm, such that frogs could only
207 jump forward. Frogs were motivated to jump as needed by tapping their urostyle with a finger (Brady et al.
208 2019). Marks were placed where frogs landed and the maximum distance between marks was recorded.
209 We used a mixed model with population as the random effect to analyze the relation between jumping
210 performance and edema. For each frog, we used maximum jump distance from among all jumps in a
211 given trial as the response variable. We analyzed jump performance collectively and separately for the
212 two regions. We also used a mixed model with population as the random effect to analyze whether jump
213 performance varied between the two regions.

214

215 **Results**

216 *Edema severity/prevalence and environmental variation*

217 Across 38 ponds, edema was more severe and more prevalent in roadside than woodland ponds (Fig. 1).
218 Specifically, model estimates of edema score were 1.84 in roadside ponds compared to 1.46 in woodland
219 ponds ($F_{1,30.0} = 14.77$, $P < 0.001$). Variance between ponds (regardless of type) estimated from the
220 random effect accounted for 12.3% of total edema severity variance. The probability of finding edema in
221 roadside ponds was estimated at 0.605 compared to 0.367 in woodland ponds. Edema was therefore
222 65% more prevalent in roadside versus woodland ponds. Among environmental variables, edema
223 severity was correlated with log-transformed conductivity ($F_{1,29} = 17.72$, $P < 0.001$; Fig 1) but not pH ($F_{1,16}$
224 $= 0.603$, $P = 0.45$) or dissolved oxygen ($F_{1,27} = 0.00$, $P = 0.996$). Conductivity averaged 545 $\mu\text{S}/\text{cm}$ in

225 roadside ponds (range: 170-984 $\mu\text{S}/\text{cm}$), representing a 14-fold increase over the average 38 $\mu\text{S}/\text{cm}$
226 (range: 26-53) found in woodland ponds ($F_{1,29} = 38.57$, $P < 0.001$).

227 Neither edema severity ($X^2 = 0.146$, $\text{df} = 1$, $P = 0.702$) nor prevalence ($X^2 = 0.01$, $\text{df} = 1$, $P =$
228 0.921) varied across the interaction of age and population type. Similarly, when main effects were
229 considered, we found no effect of age or population type on edema severity (age: $F_{1,71} = 1.85$, $P = 0.178$;
230 population type: $F_{1,71} = 0.207$, $P = 0.651$) or prevalence (age: $X^2 = 0.957$, $\text{df} = 1$, $P = 0.328$; population
231 type: $X^2 = 0.003$, $\text{df} = 1$, $P = 0.958$).

232

233 *Exposure-induced change in mass*

234 Delta BCI, or the change in body mass relative to SVL, varied across the interaction of water type X
235 population type (Fig. 2; $F_{1,339.3} = 18.02$, $P < 0.001$). Specifically, wood frogs exposed to roadside water
236 had the highest relative change in mass compared to those in all other treatments. Model estimates
237 indicated that roadside wood frogs exposed to roadside water maintained their mass, whereas wood
238 frogs in all other combinations of water and population type lost mass (Supplemental Table 1). The mean
239 duration of exposure was 5.94 days (range of 1-13 days) and differed across the interaction of water type
240 X population type ($F_{1,290.23} = 3.97$, $P = 0.047$). Tukey post-hoc pairwise comparisons revealed that the
241 only difference in exposure time was between woodland populations in woodland water versus spring
242 water ($P = 0.026$; woodland populations exposure duration: 5.91 days for woodland pond water compared
243 to 4.96 days for spring water; all other pairwise comparisons: $P > 0.505$), indicating differences in
244 exposure time did not drive the effect found in delta BCI for roadside populations.

245

246 *Morphology, histology and blood glucose*

247 On gross examination, marked subcutaneous edema was evident in the frog with severe edema (Fig. 2).
248 Upon dissection, edema fluid rapidly dissipated from the subcutis, and was evident to a much lesser
249 degree in the coelomic cavity. In all frogs, the gastrointestinal tract was devoid of ingesta or digesta, no
250 fat stores were present, and testes were well developed. On histology, the grossly edematous animal
251 exhibited subcutaneous edema. Adipose tissue was absent, and bone marrow was characterized by
252 marked serous fat atrophy (or 'gelatinous bone marrow transformation') a condition associated with

253 starvation and characterized by loss of both fat and red-blood stem ('hematopoietic') cells, and the
254 deposition of extracellular mucous (Fig. 2). In contrast, grossly non-edematous animals had absent or
255 mild subcutaneous edema and greater retention of intact adipocytes within bone marrow. Remaining
256 tissues were unremarkable, with the exception of a trematode segment characterized by an acoelomic
257 body, tegument and intestinal tract within the liver on a non-edematous animal. This was considered an
258 incidental finding. In all animals, spermatogenesis was vigorous.

259 Blood glucose varied with respect to edema severity ($F_{1,43.8} = 7.00$, $P = 0.011$) and body mass
260 ($F_{1,27.4} = 5.06$, $P = 0.033$) but not population type ($F_{1,10.0} = 0.05$, $P = 0.831$). When accounting for
261 population type and mass, along with random effects of population, blood glucose increased 13.16 mg/dL
262 with each one-unit increase in edema score and decreased 4.2 mg/dL for each gram of body mass.

263

264 *Jumping performance*

265 When all populations from the two regions with jumping data were analyzed together, edema was not
266 correlated with maximum jumping distance in (male) frogs ($F_{1,183.2} = 0.278$, $P = 0.097$). However, separate
267 analyses for each region revealed that edema negatively affected maximum jump distance for frogs in the
268 Upper Valley ($F_{1,131} = 4.92$, $P = 0.016$) but not for frogs in Southern Connecticut ($F_{1,199.4} = 0.50$, $P =$
269 0.479). Specifically, maximum jump distance for Upper Valley frogs declined by 4.3 cm for each unit
270 increase in edema. Thus, on their best jumps, the frogs with severe edema jumped approximately 9 cm
271 less than non-edematous frogs. Jumping performance also varied between regions independent of
272 edema ($F_{1,11.7} = 71.05$, $P < 0.001$). Southern Connecticut frogs jumped an estimated 71 cm compared to
273 49 cm for Upper Valley frogs. Thus, maximum jump for Upper Valley frogs was about 68% that of
274 Southern Connecticut frogs.

275

276 **Discussion**

277 Edema was both more prevalent and more severe in roadside populations subject to salt runoff compared
278 to unsalted woodland populations across three different regions in the northeastern USA. In support of
279 salt runoff being a causative agent of edema, we found that edema severity was correlated with
280 conductivity in breeding ponds, and that frogs experimentally exposed to roadside pond water during

281 breeding increased fluid retention. Edema also correlated with increased blood glucose, as well as with
282 decreased jumping performance in northerly (but not southerly) populations. Together, these results
283 reveal that pollution from roads appears to be increasing the severity of edema in amphibians, and that
284 this relatively unexplored consequence of roads imposes a potential fitness cost through its effect on
285 locomotion.

286 Our gross morphological and histological findings indicate that fluid accumulation in the subcutis
287 was indeed responsible for the edematous appearance of frogs. Several factors identified from the
288 veterinary literature are known to cause subcutaneous edema, including fungal, bacterial, or viral
289 infection, exposure to low solute water, and kidney diseases (reviewed by Pessier 2009). Interestingly,
290 high salinity water might in some instances help prevent some diseases in amphibians (e.g., Stockwell et
291 al. 2015). However, the consequences of salt runoff for disease susceptibility in amphibians are complex
292 and not fully understood, with some studies suggesting that osmotic stress caused by salt might be
293 detrimental to the survival of individuals in populations with ranavirus infections (Hall et al. 2020).
294 Although we cannot completely rule out ranavirus as a factor, infected individuals typically present
295 ulcerative and hemorrhagic syndromes in multiple tissues (Cunningham et al. 1996, Docherty et al. 2003),
296 which we did not observe in the edematous animal dissected or among the animals scored for edema.

297 In our study, exposure to low solute water can definitively be ruled out as a possible factor, since
298 animals presenting edema were more frequently those exposed to high solute water. Frogs have highly
299 permeable skin and must osmoregulate in aquatic environments. For frogs in freshwater, the tendency is
300 for water to move from the environment into the frog. Curiously, from an osmotic perspective, adding salt
301 to freshwater should decrease the osmotic potential of the external environment relative to that of the
302 frog. That is, by adding ions to the pond, water pressure exerted on frog skin decreases. In contrast, in
303 unpolluted woodland ponds, external water pressure is higher than that found in polluted roadside ponds.
304 Thus, based purely on osmotic pressure, there should be a greater tendency for woodland, not roadside,
305 water to move into frogs. And yet we found edema was more severe in salty, roadside environments,
306 pointing to dysfunction in osmoregulation. Therefore, it is likely that the culprit of edema is kidney
307 disorder, potentially incurred from osmoregulatory stress from prior exposure to high salinity.

308 Increased edema severity in roadside ponds might also be linked to overwintering physiology.
309 Beginning in fall as temperatures decline, wood frogs accumulate urea (Costanzo and Lee 2005) and
310 continue to increase liver glycogen stores. During freezing, glycogen is metabolized into glucose
311 (Costanzo and Lee 2013, Costanzo et al. 2015) and distributed to cells and organs. Water is moved to
312 the coelomic cavity and lymph system (Lee et al. 1992), and cytosol moves osmotically to extracellular
313 spaces. These physiological changes help prevent cells from freezing while permitting coelomic and
314 subdural ice formation to occur with little damage to organs and tissue. Estimates indicate that up to
315 about 2/3 of body water can become frozen in wood frogs during the winter (Layne Jr and Lee Jr 1987,
316 Storey and Storey 1988, Costanzo and Lee 2013). Thawing in the spring can be rapid and appears to be
317 a source of fleeting edema (Kling et al. 1994, Irwin et al. 1999) during which time there might be a lag
318 period for water resorption from coelomic and subdural spaces, consistent with our histological and gross
319 dissection findings. Although it is unclear why road proximity might increase severity of thaw-induced
320 edema, conceivably, pollution might trigger a hyper-reactive freeze tolerance response and/or a reduced
321 capacity for resorption. In which case, it is not surprising that we found elevated levels of glucose in the
322 spring, suggesting either that edematous frogs have an amplified cryoprotective response (concentrating
323 more glucose in their cells) and/or they metabolize glucose more slowly after thawing.

324 Regardless of whether edema is linked to winter physiology or a direct consequence of kidney
325 disease, the severity we detected in roadside populations prompts consideration of what conditions in
326 roadside environments increase edema severity. For instance, is edema exacerbated by the aquatic
327 environment, terrestrial environment, or both? For adults in our study, one such exposure could have
328 occurred during embryonic and larval stages (as wood frogs show strong natal site fidelity; Berven and
329 Grudzien 1990) while another exposure could have occurred in adult stages but in prior breeding years
330 (as wood frogs also show strong breeding site fidelity; Berven and Grudzien 1990). In either case, some
331 degree of carry-over effect is implied. Exposure could also occur in terrestrial roadside habitats, for
332 instance through consumption of saltier food items or exposure to saltier soils.

333 The edema we describe occurred in breeding frogs captured either on route to or soon after
334 arriving in their breeding ponds. This means that for at least a subset of frogs, the onset of edema
335 occurred before they were exposed to pond water. This finding indicates that edema sets in either during

336 the short migration from upland hibernacula to breeding ponds, or that frogs are edematous prior to
337 migration. In late fall, frogs tend to choose overwintering sites close to their breeding ponds (O'Connor
338 and Rittenhouse 2016), such that salt runoff exposure in the terrestrial environment might be
339 exacerbating edema that forms naturally following the reversal of winter cryoprotection. Interestingly, we
340 found no relation between age and edema severity or prevalence, suggesting that a single exposure to
341 the roadside environment might be just as likely to generate edema as a history of adult breeding in
342 roadside ponds.

343 Our study additionally suggests a significant impact of salt runoff exposure in the aquatic
344 environment. We found higher edema severity with increased conductivity of the aquatic environment, a
345 variable widely used to measure road salt pollution. In New England, like many temperate regions with
346 cold winters, roadside ponds have become salinized due to runoff from road salt (Kaushal et al. 2005,
347 Karraker et al. 2008, Brady 2012, Dugan et al. 2017). Although a variety of salts are used in de-icing
348 practices, NaCl is the agent applied most abundantly, both in our study region and throughout the U.S.
349 (Mullaney et al. 2009). Conductivity values in roadside ponds averaged 545 $\mu\text{S}/\text{cm}$, and individual values
350 ranged up to 2050 $\mu\text{S}/\text{cm}$. Based on prior calibrations (G. Benoit unpublished data), we estimate these
351 values correspond to 300 and 1128 mg/L of total NaCl, or 182 mg/L and 684 mg/L of Cl^- , respectively.
352 Moreover, we found that frogs experimentally exposed to roadside water during breeding maintained
353 mass on average while those exposed to woodland or spring water lost mass. We interpret this change in
354 mass as being driven primarily by water gain or loss, thus representing a relative increase in water
355 retention or uptake in roadside water, because frogs did not have access to food during this period.
356 Saltier aquatic environments should result in frogs taking up more ions for osmoregulation (Greenwald
357 1972), which could lead to higher water retention (Park and Do 2020) consistent with our findings (Fig. 2).
358 For example, Park and Do (2020) found that exposure of black-spotted frogs (*Pelophylax nigromaculatus*)
359 to about 9,400 $\mu\text{S}/\text{cm}$ resulted in increased blood electrolytes and that frogs became edematous. The
360 authors attribute this effect to renal dysfunction resulting from the increase in electrolytes. Future studies
361 would be required to disentangle the relative importance of pre- and post-metamorphosis exposure, as
362 well as exposure through aquatic versus terrestrial environments.

363 Finally, we found that jumping performance was compromised by edema in the more northerly
364 populations of Vermont and New Hampshire, but not those of Southern Connecticut. Here again,
365 overwintering conditions might mediate this effect. For instance, in northern populations, more severe and
366 longer winters should produce more intense cryoprotective physiological changes (Costanzo et al. 2015).
367 This investment in cryoprotection might bear a cost to muscle performance needed for locomotion,
368 consistent with our findings that Upper Valley frogs jumped just 68% the distance of Southern
369 Connecticut frogs. Costanzo et al. (2015) report that muscle catabolism is an important component of
370 freeze tolerance observed in northern (Alaska) wood frog populations but absent in Ohio populations, and
371 that it results in skeletal muscle atrophy. The authors suggest that this mechanism is essential to increase
372 nitrogen availability for urea production given the more extreme winters in Alaska. Conceivably, harsher
373 winters in Vermont and New Hampshire induce this same mechanism and cause muscle atrophy that
374 diminishes jumping performance. Also, the serous fat atrophy we found with severe edema suggests that
375 edematous frogs consume more energy for cryoprotection. Given the importance of energy and
376 locomotion during spring breeding, particularly in the context of road crossings and swimming
377 performance during mating, these consequences could bear impacts on fitness components including
378 survival and reproductive success and, therefore, on the viability of higher latitude populations.

379 In this study, we investigated possible causes and fitness consequences of increased edema
380 severity, representing an amphibian population threat from roads and runoff pollution that has remained
381 largely unexplored. Increased edema severity in wood frogs appears to be a wide-ranging phenomenon
382 in road-adjacent habitats, driven by road salt pollution. Given the regional distribution of edema reported
383 here, we expect that this phenomenon likely affects wood frogs across much of their range, although the
384 consequences of edema might be most severe for populations experiencing more intense winters.
385 Interestingly, northern areas where winters are more intense tend to use more road salt. Thus, the
386 presence of these multiple, correlated stressors suggests that wood frogs in more northerly populations
387 might be both most likely to experience higher edema severity near roads and locomotor consequences
388 as a result. Many important questions remain about the mechanisms and consequences of severe edema
389 in roadside wood frogs. Indeed, while road salt is a prevalent contaminant and almost certainly the most
390 common pollutant by mass in these ponds, other contaminants could be contributing to edema, such as

391 heavy metals and aromatic hydrocarbons, and such effects might be interactive. Identifying the causes of
392 severe edema and its impacts on populations should guide future investigations.

393

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401

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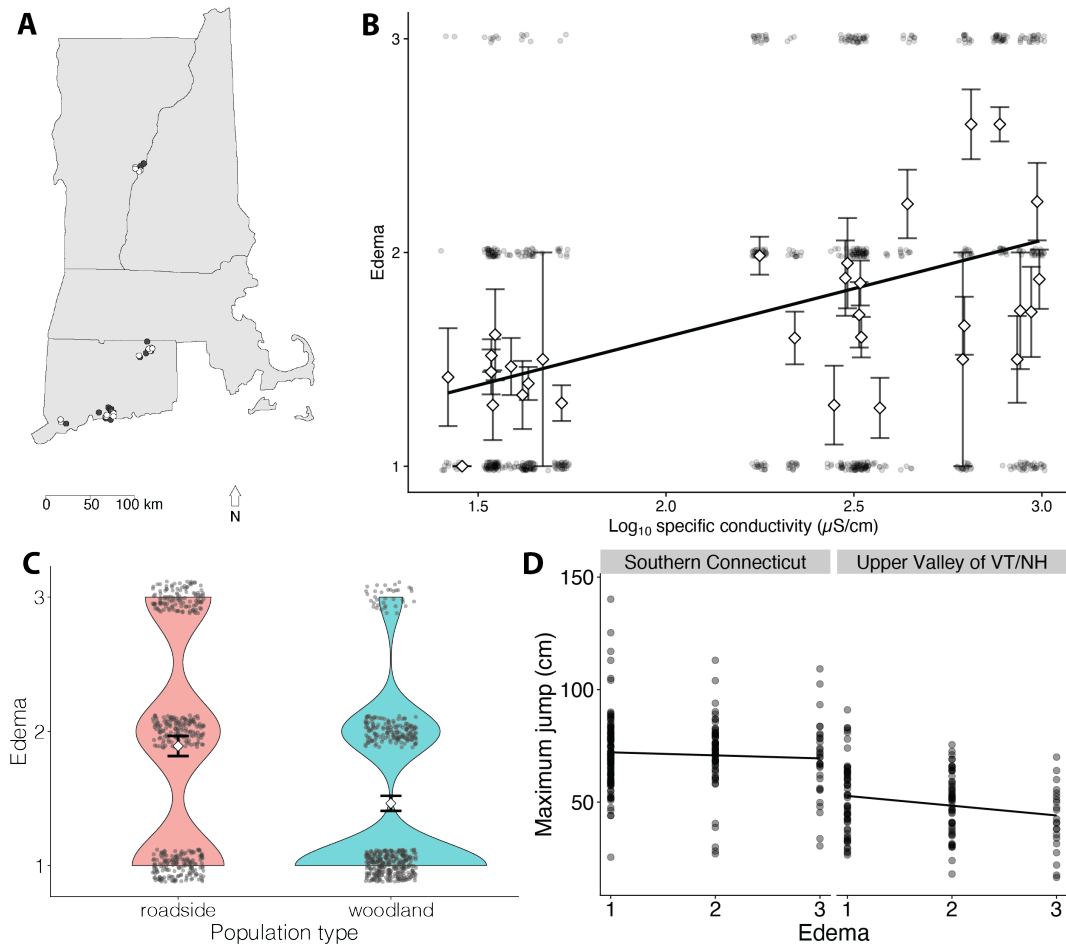
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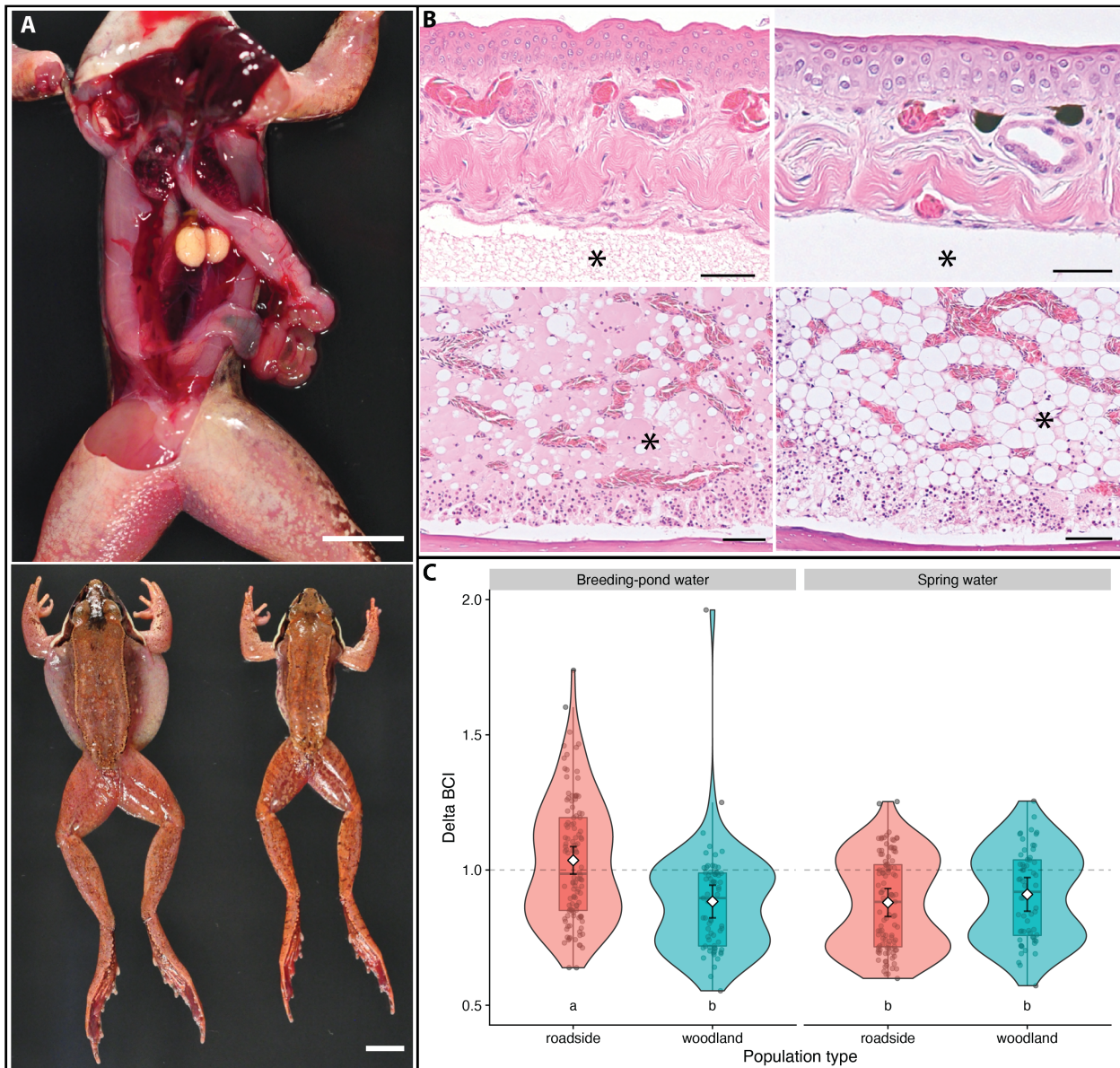
562 **Figures**

563 **Figure 1. Study sites, edema prevalence, and jump performance.** Panel A: Edema was scored in
564 frogs from 38 ponds located within three regions in New England, from south to north: Southern
565 Connecticut, Northeastern Connecticut, and the Upper Valley in Vermont/New Hampshire. Locations of
566 roadside ponds (filled circles) and woodland ponds (open circles) are shown; points were jittered to
567 reduce overlap. Panel B: Edema increased with increasing conductivity (a proxy for road salt). Diamonds
568 (± 1 SE) represent pond-level average edema. Circles show individual data points, jittered to reduce
569 overlap. Regression line was estimated with a linear model of pond-level averages for both edema and
570 conductivity. Panel C: Edema was more severe in roadside ponds. Each point represents edema for a
571 unique frog. Points are jittered vertically and horizontally to reduce overlap. Violin plots show density
572 distribution. Means ($\pm 95\%$ CI) for each population type are indicated by open diamonds. Panel D: Edema
573 compromised jumping performance for (right) more northerly ($P = 0.016$) but not (left) more southerly
574 populations ($P = 0.479$). Regression lines represent fixed effect of population type from mixed model.
575



576

577 **Figure 2. Edema and water balance.** (A) On gross dissection of the edematous frog (top), edema fluid
578 rapidly dissipated from the subcutis (not visible), and was evident to a much lesser degree in the coelomic
579 cavity. Testes were well developed. Marked subcutaneous edema is evident in an edematous frog
580 (bottom left) compared to a non-edematous frog (bottom right). Bars = 1cm. (B) On histology, the grossly
581 edematous animal exhibited subcutaneous edema (asterisk, top left) not evident in the non-edematous
582 animal (asterisk, top right). Marked serous atrophy of fat was evident in bone marrow of the edematous
583 animal (asterisk, bottom left) in comparison to non-edematous animals with greater retention of intact
584 adipocytes within bone marrow (asterisk, bottom right). Bars = 50 μ m, hematoxylin and eosin. (C) Change
585 in adult male mass relative to body size (i.e. body condition index, or 'BCI') during a controlled breeding
586 experiment is shown for different exposure waters. Mass was measured before and after breeding. Delta
587 BCI of 1 (dashed line) indicates no change. Experimental exposure to (far left) roadside pond water
588 caused frogs from roadside ponds gain or maintain water on average during the breeding period. In
589 contrast, on average, frogs from woodland ponds exposed to (left center) woodland water lost mass.
590 Similarly, individuals exposed to spring water – whether from (right center) roadside ponds or (far right)
591 woodland ponds – lost mass on average. Raw data are shown as gray circles. Violin plots and boxplots
592 are overlain. White diamonds and error bars represent estimated marginal means and corresponding
593 95% confidence intervals.



594