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Twice as easy to catch? A toxicant and a predator cue cause additive reductions in larval amphibian activity

MARI K. REEVES,[1,2,]† MARGARET PERDUE,[1] GARETH D. BLAKEMORE,[1] DANIEL J. RINELLA,[3] AND MARCEL HOLYOAK[2]

[1]*United States Fish and Wildlife Service, Anchorage Fisheries and Ecological Services Office, 605 West 4th Avenue, Room G-61, Anchorage, Alaska 99501 USA*

[2]*Graduate Group in Ecology and Department of Environmental Science and Policy, University of California, 1 Shields Avenue, Davis, California 95616 USA*

[3]*Environment and Natural Resources Institute and Alaska Natural Heritage Program, University of Alaska, 3211 Providence Drive, Anchorage, Alaska 99508 USA*

Abstract. Toxicants may harm predators or prey differentially, hindering clear determination of multiple stressor effects on predation dynamics in polluted aquatic systems. We built on a prior field study in which we demonstrated that a chemical contaminant, copper (Cu) and odonate predators were correlated with more frequent observations of skeletal abnormalities in Alaskan wood frog (*Rana sylvatica*) tadpoles. Our prior study established a multiple stressor effect linked to an important environmental phenomenon (malformed amphibians) but did not provide clear mechanisms that might guide management. We here investigated behavioral mechanisms because of their potential to produce large changes in predation dynamics, and because in published studies low concentrations of Cu produced behavioral changes in predator-detection in fish. Surprisingly, low but environmentally relevant concentrations of Cu (5 μg/L) combined with chemical cues from a predator (*Aeshna sitchensis*) to produce large changes in the behavior of larval amphibians. Experiments demonstrated that a low concentration of Cu did not inhibit the ability of wood frog tadpoles to detect chemical cues of predators by olfactory means, but produced strong behavioral effects, causing tadpoles to reduce activity and alter microhabitat use. These results occurred with Cu at an exposure level lower than any we could find reported as toxic to amphibians in the literature. When Cu and predator cues were administered together, the activity reduction was additive and stronger at earlier life stages. We suggest that Cu intoxication would be disadvantageous to larval amphibian prey with prolonged exposure to predators during development, and we present field data from 2010 that support this assertion. Our study demonstrates the need to use sensitive behavioral assays and to investigate multiple stressor mechanisms to understand how multiple threats combine to affect organisms in nature.

Key words: Alaska; amphibian; copper; frog; *Lithobates sylvaticus;* multiple stressor; predator; toxicity; trait-mediated interaction; *Rana sylvatica.*

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† **E-mail:** mari_reeves@fws.gov

Introduction

Ecologists and resource managers increasingly recognize that multiple stressors can combine to produce unforeseen consequences for natural communities (Relyea 2010). Environmental contaminants may alter ecological dynamics indirectly by changing individual traits, like an organism's physiology, morphology, or behavior. Such trait changes in individuals may then alter the interactions between that species and other community members, leading to trait mediated indirect effects of contaminants on the whole community (Relyea and Hoverman 2006, Rohr et al. 2006). Multiple stressor effects may be especially important in aquatic ecosystems because they contain proportionally more species of conservation concern than other ecosystems (Wilcove et al. 2000), and because aquatic systems can concentrate pollutants through runoff from terrestrial habitats (Scher and Thiery 2005, Snodgrass et al. 2008). Additionally, predators in aquatic habitats often have large effects on the population dynamics of their prey (e.g., Shurin et al. 2002, Borer et al. 2005). Hence chemicals that modify species traits might alter community dynamics more profoundly in freshwater communities than in other ecosystems.

Behavioral effects of multiple stressors are particularly interesting because of their ability to produce large effects on species interactions (e.g., Preisser et al. 2005). Although studies have independently examined the effects of contaminants on behavior (reviewed in Weis et al. 2001 and Zala and Penn 2004) and predators on behavior (reviewed in Benard 2004 and Relyea 2007), and several theoretical papers or review articles predict how predators and contaminants might interact (Relyea and Hoverman 2006, Rohr et al. 2006), few studies have empirically examined how predators and contaminants may together influence community dynamics through behavioral mechanisms. Non-agricultural, inorganic pollutants have received very little multiple stressor attention. Indeed with one exception, all amphibian studies of contaminants and predator-prey interactions have focused on pesticides (reviewed in Relyea 2010). In these pesticide studies, common behavioral responses of prey to predators and contaminants together included changes in activity level (Bridges 1999*a*, Bridges 1999*b*, Relyea and Edwards 2010), decreased feeding (Broomhall 2004), failure to appropriately use refugia (Bridges 1999*a*), and inability to detect predators by olfactory means (Mandrillon and Saglio 2007). In several studies behavioral changes led to increased vulnerability to lethal predation (Bridges 1999*b*, Broomhall 2002, Broomhall 2004, Relyea and Edwards 2010). In the only study we are aware of to examine how metals affect amphibian predator avoidance behavior, spotted frog (*Rana luteiventris*) tadpoles reared with sediments contaminated with multiple toxic metals showed a lessened fright response to chemical cues from predatory rainbow trout (Lefcort et al. 1998). Because sediments in this study were contaminated with multiple metals, however, it was impossible to isolate which metal or metal combination caused the observed effects.

In this study we concurrently quantified the effects of a contaminant and a predator on behavior, a trait type infrequently addressed in standard toxicology studies. We followed up on an earlier study in which we found strong correlations between chemical contaminants, odonate predators and the occurrence of limb abnormalities in frogs (Reeves et al. 2010). The causes of limb abnormalities in general are a current and controversial topic (Ballengée and Sessions 2009, Johnson and Bowerman 2010, Sessions and Ballengée 2010, Skelly and Benard 2010, Reeves et al. 2010). Possible causes include parasites, predators, UV-B, and contaminants (reviewed in Johnson et al. 2010). The trematode parasite, *Ribeiroia ondatrae* is an established cause of abnormalities where it occurs (Johnson et al. 2010), but there are places where abnormal frogs are frequent but this parasite is not detected (Skelly et al. 2007, Reeves et al. 2008, Bowerman et al. 2010). High frequencies of abnormalities in sites lacking *R. ondatrae* have been correlated with aquatic predators, which may amputate the developing limbs of tadpoles as they prey on them (Ballengée and Sessions 2009, Bowerman et al. 2010), Reeves et al. 2010). Because amputated limbs regenerate only partially before metamorphosis, tadpole limb amputations can cause shrunken or missing limbs in post-metamorphic frogs (Fry 1966, Ballengée and Sessions 2009, Reeves et al. 2010). Nevertheless, the predator hypothesis is controversial because aquatic pred-

Table 1. Water quality and analytical chemistry field data from the Kenai NWR sites in 2010.

	Temperature			Hardness			Copper				
Site	Mean (°C)	N	Range (°C)	Mean (mg/L $CaCO_3$)	N	Range (mg/L $CaCO_3$)	Mean† (μg/L)	N	Range (μg/L)	Mean CCC‡ (μg/L)	% above CCC
8	**13.96**	**12372**	**−0.14–25.57**	**7.23**	**12**	**1.37–11.82**	**0.21**	**12**	**0.15–0.57**	**0.95**	**0**
12	12.14	6186	1.72–24.17	7.14	6	6.18–7.58	3.69	6	2.92–4.25	0.94	100
17	**15.14**	**12372**	**-0.31–22.15**	**1.76**	**12**	**0.81–2.52**	**0.19**	**12**	**0.15–0.48**	**0.28**	**8**
55§	. . .	. . .	. . .	68.42	1	. . .	<5.00	1	. . .	6.75	. . .
56§	. . .	. . .	. . .	71.53	1	. . .	<5.00	1	. . .	7.01	. . .
90	**14.40**	**6186**	**3.59–27.23**	**10.05**	**6**	**2.12–28.63**	**0.73**	**6**	**0.15–1.06**	**1.19**	**50**
97	12.60	12372	0.85–24.02	64.81	12	47.49–81.58	0.15	12	0.15–0.15	6.17	0

Note: Sites in bold are the primary sites used to collect tadpoles for the experimental trials.

† Mean analyte values are calculated using half of the reported detection limit for non-detect data.

‡ Hardness-calculated chronic aquatic life criteria for freshwater using dissolved fraction for current data and total recoverable for historic data (KNA55 and KNA56), (ADEC 2003).

§ Water quality data are historic data from 2004/2005, "<" indicates analyte was not detected at detection limit reported to the right of this symbol.

ators are common in amphibian breeding habitats, yet frogs with missing or shrunken limb abnormalities are not so widely distributed (Johnson and Bowerman 2010, Sessions and Ballengée 2010, Skelly and Benard 2010). Other hypotheses for amphibian limb abnormalities include UV-B radiation, which has largely fallen out of favor (Ankley et al. 2004) and chemical contaminants (reviewed by Johnson et al. 2010).

Contaminants are controversial as proximate or ultimate causes of amphibian limb abnormalities (Sessions and Ballengée 2010). Despite repeated reports of associations between abnormal amphibians and environmental contaminants in the field (Johnson et al. 2010), few studies have convincingly linked pollution to limb abnormalities in nature (but see Bacon et al. 2006 for stronger evidence and Rohr et al. 2008 for one possible mechanism). Importantly, our field observations and experiments spanning 7 years and 5 National Wildlife Refuges in Alaska found that skeletal abnormalities in wood frogs (*Rana sylvatica*) were correlated with multiple stressors in the form of predators, toxic metals, and pesticides (Reeves et al. 2010). When tadpoles were reared in the absence of predators at high malformation sites, the abnormalities did not occur. When frogs were reared in sediment and water containing toxic metals and pesticides from high malformation sites, tadpoles did not develop limb abnormalities, but fewer eggs hatched, tadpoles took longer to metamorphose and were smaller at metamorphosis than controls (Reeves et al. 2010).

Nevertheless, the exact relationships among predators, chemicals, frog size, and frog malformations remain unclear. For example, despite our observation that smaller frogs are more likely to be abnormal (Reeves et al. 2008, Reeves et al. 2010), we do not know whether frogs are small because they are abnormal or whether something about being small makes tadpoles more likely to become abnormal. These size differences are nontrivial: metamorphs in areas with few abnormalities (like remote areas of the Arctic Refuge) are 21% larger on average (mean snout to vent length (μ_{SVL}) = 23 mm, N = 616) than metamorphs in areas where abnormalities are more frequent (Kenai NWR μ_{SVL} = 19 mm, N = 5,716; Reeves et al. 2008). There is a need to understand how chemicals and predators interact to influence frog abnormalities in order to manage ecosystems to limit their occurrence.

We designed the current study to test how Copper (Cu), a primary constituent of road runoff and hard rock mining waste, a known toxicant for aquatic organisms, and one of the four metals correlated with frog abnormalities in our statistical analyses of the Kenai NWR data, interacts with the chemical cues of predators to alter predation risk. Copper is present in Kenai NWR study sites, but at concentrations lower than any previously documented as toxic to amphibians (Table 1). We focus on tadpoles of the wood frog (*R. sylvatica*), which is the only amphibian in most of Alaska and has long been the focus of our abnormality research (Reeves et al. 2008, Reeves et al. 2010). Larval amphibians change behavior in response to predators, competitors, and other environmental cues (Relyea

2001, Van Buskirk 2009), and wood frog tadpoles specifically are known to reduce activity in response to the chemical cues of predators (Relyea 2001, Fraker et al. 2009). The only study we are aware of to test Cu toxicity to wood frogs demonstrated low survival in tadpoles from both acute (7 days) and chronic (28 days) exposure to 15 μg Cu/L (Horne and Dunson 1995). By comparison, in our study sites in 2010, dissolved Cu concentrations ranged from <0.15–4.85 μg/L (Table 1); at these concentrations, Cu effects are largely unknown.

More general evidence suggests that Cu may be an especially important contaminant. Cu is a trace element essential for biochemical activities and enzyme function, but concentrations surpassing biological requirements can be toxic to aquatic life including amphibians (Eisler 1998, Chen et al. 2007). Cu impairs ion exchange at the gill and interferes with osmoregulation (Eisler 1998). It is also a neurotoxin, which inhibits olfaction in fish at low concentrations (Sandahl et al. 2007) and leads to poor homing ability in salmonids. It is plausible but untested that Cu may also inhibit olfaction in larval amphibians. Tadpoles detect predators using olfaction, whereby chemical cues released when a tadpole is injured alert conspecifics to predator presence (Petranka et al. 1987, Fraker et al. 2009, Schoeppner and Relyea 2009).

Our prior studies suggested two hypotheses regarding effects of copper and predators together in nature. First, Cu concentrations at high malformation sites may be toxic enough to inhibit tadpole feeding, which could lead to smaller size over a growing season and thus greater vulnerability to predatory attacks. Second, Cu might interfere with olfaction, thereby altering normal predator detection and avoidance behavior. We made the following predictions about tadpole behavior: (1) Tadpoles exposed to this low concentration of Cu alone might reduce activity (Redick and La Point 2004, Chen et al. 2007) but were not expected to change their microhabitat use; (2) Tadpoles exposed to predator cues alone would reduce activity and seek refuge at the tank bottom to minimize risk of predation (e.g., Peacor 2006, Fraker 2008); and (3) Tadpoles in combined Cu and predator treatments would not detect the predator cue due to olfactory inhibition by Cu; consequently they would behave identically to animals treated with Cu alone (e.g., Mandrillon and Saglio 2007). We elected to focus this study on Cu because of its relevance as a widespread aquatic contaminant, its known toxicological effects in other studies (cited above), and because the role of metals in causing abnormalities or altering aquatic predation dynamics has not been fully considered (Johnson et al. 2010, Relyea 2010).

Materials and Methods

The experiment was conducted between 11 June and 22 July 2010. Tadpoles (n = 227) from 5 sites in the Kenai National Wildlife Refuge (NWR), Alaska, USA, were assayed in 12 trials during this 6-week period (Appendix: Table A1). Because we undertook this study to understand causes of amphibian limb abnormalities, we focused on tadpoles between Gosner stages 26 (beginning of limb development) and 41(beginning of metamorphosis), spanning the entire period of tadpole hind limb development. Each week, tadpoles were caught at field sites in the Kenai Refuge and transported to laboratory facilities in either Kenai or Kodiak, AK. After capture, tadpoles were placed in 2 L of water in individual aquaria, allowed to acclimate for 1–4 days, exposed to a predator cue generated from dragonflies (*Aeshna sitchensis*) consuming conspecific tadpoles, then run through behavioral assays for ~2 hours (Appendix: Table A1).

Experimental aquaria

Experiments were conducted in 2.3 L acrylic aquaria (OXO Bisphenol A-free #7 plastic storage containers, dimensions 16 × 16 × 19 cm; OXO, Chambersburg, PA, USA), each containing 1 tadpole. The outside of each tank was covered with 1-way Mylar film (Energy Saving Platinum window film, Gila Film Products, St. Louis, MO, USA) and aquaria were lit from above to limit tadpole ability to see the observer. The location of aquaria was randomized between trials to account for microenvironmental effects, but aquaria were dedicated to each chemical treatment for the duration of the experiment (e.g., aquaria treated with Cu always contained Cu and aquaria treated with predator cue always contained cue) to limit the possibility of cross-contamination. Aquaria were rinsed between

Table 2. Biological field data from the Kenai NWR sites in 2010.

	Metamorph data				Tadpole data			Odonate data	Periphyton data	
Site	Mean SVL† (mm)	Mean Gosner stage‡	Abnormal§ (%)	N	Development period¶ (days)	Injured# (%)	N	Relative density (N/m^2)	Mean|| (mg/cm^2)	Range
8	**19**	**43**	**25.7**	**66**	**80**	**35.6**	**81**	**16**	**0.15**	**0.14–0.16**
12	14	43	32.0	50	89	...	...	0	0.09	0.07–0.11
17	**20**	**44**	**3.2**	**61**	**88**	**46.0**	**60**	**22**	**0.04**	**0.03–0.05**
55††	16	43	1.9	104	...	68.9	10	2	...	...
56††	20	44	3.0	100	...	62.5	16	4	...	...
90	**18**	**45**	**6.1**	**65**	**71**	**50.6**	**61**	**11**	**0.09**	**0.08–0.10**
97	23	43	1.7 [58]	58	75	...		46	0.18	0.11–0.23

Note: Sites in bold are the primary sites used to collect tadpoles for the experimental trials.
† SVL = snout to vent length.
‡ Gosner 1960.
§ Prevalence of skeletal abnormalities in metamorphs. Percentage is number of abnormal animals divided by total examined in 2010. Abnormality prevalence may be biased high because intensive study effort and instrumentation this season increased the potential for researchers to injure tadpoles, especially at site 12, which is small and shallow.
¶ Time from egg mass observation to metamorphosis.
# Observations of injuries prior to start of the experimental trials.
|| Periphyton data are average of two samples per site.
†† Metamorph size, abnormalities, and odonate abundance data are historic data from 2004/2005.

trials with the same clean water source used in the experiment.

Experimental design

The design was a 2 × 2 × 2 × 5 factorial experiment testing the effects of Cu (0 and 5 μg/L Cu^{2+} added as $CuCl_2$), predator cue (presence or absence), injury status (previously injured or uninjured), and breeding site (5 locations; Tables 1 and 2; Appendix: Tables A1–A5) on tadpole behavior. We also measured two covariates, which might influence tadpole responses to the main effects: tadpole developmental stage, hereafter Gosner stage (Gosner 1960) and time since the predator cue or blank was added in each assay (e.g., Sih et al. 2000, Fraker 2008), hereafter, time since cue addition.

Copper

We set Cu dose based on a review of the toxicology literature, summarized in greater detail in the Appendix. In this literature review, we found an exposure of 5 μg/L to be in the range of a "No Observed Effects Concentration" or well below it for most amphibian species tested. We also considered Cu concentrations in field study sites (Table 1). In Kenai NWR wetlands sampled in 2010, Cu concentrations reached a maximum level of 7.85 μg Cu/L for total recoverable Cu (water hardness in this sample was 8.16 mg/L of $CaCO_3$) and levels in roadside snow were still higher at 11.76 μg Cu/L. In the experiment, half the aquaria in each trial contained water to which 5 μg/L Cu^{2+} was added as $CuCl_2$. The remaining aquaria contained clean water (Water of Life, Kodiak, AK, USA or Alaska's Best Water, Anchorage, AK, USA; [Cu], <0.31 μg/L; specific conductivity, 8–11 μS/cm; salinity, 0 ppt; pH, 6.51–6.89; dissolved oxygen, 92–95%; temperature, 17–22°C). Validation samples of experimental water measured by inductively coupled plasma/mass spectrometry ranged from 4.88–5.12 μg Cu/L (specific conductivity, 8–12 μS/cm; salinity, 0 ppt; pH, 6.57–6.74; dissolved oxygen, 93–99%; temperature, 17–22°C). Cations contributing to water hardness (e.g., calcium (Ca) and magnesium (Mg)) ameliorate Cu toxicity (Eisler 1998). For this reason the chronic aquatic water quality criteria for Cu depends on water hardness (nominal Cu threshold is 9 μg Cu/L at a hardness of 100 mg/L; Buchman 2008, U.S. EPA 2007; e.g., see Table 1 and Appendix: Tables A4 and A5). Experimental water was soft, meant to replicate soft site water in Kenai NWR wetlands (Table 1). Both calcium (Ca) and magnesium (Mg) were below the limit of detection (LOD) in experimental water (Ca limit of detection (LOD) = 16.38 μg/L and Mg, LOD = 41.18 μg/L).

Predator cue

A chemical alarm stimulus was prepared by feeding *R. sylvatica* larvae to odonate predators (*Aeshna sitchensis*) in 2 L of clean water (Fraker et al. 2009). Within 15 minutes of tadpole consump-

tion, 30 mL of predator-cue infused water was injected into each test microcosm with a plastic syringe. Half of the microcosms in each assay were exposed to predator cue and the remaining half received 30 mL of clean water to control for effects of physical but not chemical disturbance. All cues and blanks were administered with syringes dedicated to each treatment to prevent cross-contamination.

Prior injuries

Prior predator conditioning can increase survival probability. In a recent study, tadpoles conditioned to aquatic predators survived subsequent predator encounters better, which increased rates of survival for the predator-conditioned tadpoles over their predator-naïve counterparts (McCoy and Bolker 2008). Poor individual fitness, or other factors we did not measure in this experiment, may also increase vulnerability to injury. For these reasons, we assessed whether individual tadpoles had been injured (cuts, wounds, missing limbs or limb elements, or notched, cut, or torn tails) just prior to placing individuals into treatment aquaria. We observed high injury prevalence in wild-caught tadpoles (Table 2) and believed we should control for this statistically, so we used injury status (injured or uninjured) as a factor in statistical analysis. We hypothesized that animals previously injured would be less active in general and more responsive to the predator cue than uninjured animals, either because of the injury, inherently lower fitness, or because of a behavioral adaptation resulting from prior predator experience (McCoy and Bolker 2008).

Site

We hypothesized that differences in behavior would be measurable across sites from which animals were collected because of environmentally mediated behavioral plasticity in this species (Relyea 2001) and in other larval amphibians (Urban 2007*a*, Van Buskirk 2009). Sites differed in important ways, such as contaminant concentrations, predator abundances, and available food resources (Tables 1 and 2; Appendix: Tables A3–A5). Use of tadpoles from multiple sites also limited the number of tadpoles removed from any one breeding population. We tested a total of 227 animals from 5 ponds in the Kenai NWR (Tables 1 and 2; Appendix: Tables A1–A5). Additional site information including location, water and sediment chemistry and abnormality data can be found in the Appendix and in Reeves et al. (2010).

Developmental stage

Our predictions are complicated by the expectation that tadpole behavior relative to our main effects is expected to change as tadpoles grow older and larger (Fraker 2008, McCoy and Bolker 2008). Developmental stage can also influence how long it takes tadpoles to recover from the predator cue (Fraker 2008). We therefore measured Gosner stage (Gosner 1960) and used this as a quantitative covariate in statistical analyses. Testing began when tadpoles reached early limb development (Gosner stage 26) and terminated when tadpoles began to metamorphose (Gosner stage 41). Tadpole developmental stage is closely related to size in general ($r = 0.89$ in our data). Due to this collinearity, we could have used either size or stage in our models, but not both. We chose stage because prior experience with wood frogs led us to believe that hypothesized behavioral changes in activity or microhabitat use would correlate better with developmental stage than size over the range of variation measured.

Time since cue addition

We measured time since cue addition during the assay as a quantitative covariate for statistical analysis, because tadpoles are known to "recover" from predator cues by gradually increasing activity or returning to the surface of experimental aquaria after exhibiting an initial activity reduction and bottom preference (Sih 1992, Peacor 2006, Fraker 2008). Time since cue addition was measured in minutes, beginning when the predator cue had been added to the last tank. The addition of predator cue and blank took approximately 10 minutes in each trial.

Behavioral assays

Tadpoles were acclimated to the aquaria for 1–4 days, after which the predator cue or blank was added and the trial began. Tadpoles were not fed during this period. To take behavioral data, the observer walked around each tank and recorded tadpole position and activity at a single point in

Table 3. Summary of model statistics and multiple comparisons adjustments.

Model	Parameter	df	χ^2	p	Corrected α	p-value difference†
Activity	Cu	1	38.42	0.0001	0.0036	0.0035
Activity	Gosner	1	23.11	0.0001	0.0038	0.0037
Activity	Time since cue added × Site	4	19.35	0.0007	0.0045	0.0038
Activity	Predator cue	1	11.2	0.0008	0.0050	0.0042
Activity	Time since cue added	1	11.32	0.0008	0.0056	0.0048
Activity	Gosner × Time since cue added	1	9.79	0.0018	0.0063	0.0045
Activity	Injury	1	9.41	0.0022	0.0071	0.0049
Activity	Gosner × Predator cue	1	9.29	0.0023	0.0083	0.0060
Activity	Site	4	14.94	0.0048	0.010	0.0052
Activity	Cu × Predator cue	1	4.46	0.0346	0.017	−0.0179
Vertical position	Cu	1	20.02	0.0001	0.003	0.0030
Vertical position	Gosner	1	16.1	0.0001	0.003	0.0032
Vertical position	Site	4	19.26	0.0007	0.004	0.0035
Vertical position	Time since cue added	1	6.91	0.0086	0.013	0.0039
Vertical position	Injury	1	1.92	0.1653	0.025	−0.1403
Vertical position	Predator cue	1	0.03	0.8644	0.05	−0.8144

† *Note:* Negative values are not significant after sequential Bonferroni correction.

time, then moved to the next tank. Specific behavioral responses included vertical position ("on bottom," i.e., in bottom one-third of the tank, or "above bottom," i.e., in the remainder of tank) and whether the tadpole was moving or still. Ten to twenty replicate observations were made on each tadpole during each trial which lasted 1–2 hours (Appendix: Table A1), for a total of 3,630 observations. Two sets of trials were repeated on the same animals (trials 3 and 5 and 10 and 11) to understand reproducibility of results between trials.

Data analyses

For statistical analyses, we used repeated measures generalized linear models (PROC GENMOD in SAS) that specified that multiple observations were taken on each individual tadpole (using the REPEATED statement). The generalized linear model framework we used is similar to a repeated measures ANOVA, but it allows for both categorical and continuous variables (covariates) and also lets errors follow a binomial distribution (which is appropriate for proportional data). Finally, these models use maximum likelihood-based estimation techniques rather than minimization of sum of squares to determine parameter estimates and p-values. We created a separate model for each response variable (activity and vertical position) and tested the following factors as predictors of tadpole behavior: Cu, predator cue, prior injury, and site. Gosner stage, and time since cue addition were covariates. All factors and covariates were treated as fixed effects. All analyses were conducted with SAS (Version 9.1.3, Cary, NC, USA).

Because of the complexity of our experimental design (with 4 factors and 2 covariates) we only tested interactions that supported a priori hypotheses and made biological sense. In the first modeling step, we tested for significance of all interactions between the main effects (Cu and Predator) and each of the other factors and covariates. We also tested the following interactions we thought might be important based on other studies: Gosner × predator (Fraker 2008, McCoy and Bolker 2008), injury × predator (McCoy and Bolker 2008), Gosner × time since cue addition (Sih et al. 2000, Peacor 2006), and time since cue addition × site (Sih et al. 2000). If interaction terms were not significant in the first model, they were dropped from the next iteration. We then applied a multiple comparisons adjustment to results from the second iteration of each model (Table 3). Only interactions significant in the first model iterations were included in the final models to improve accuracy of parameter estimates and limit the number of parameters used for multiple comparisons adjustment. We adjusted for multiple comparisons with a sequential Bonferroni procedure (Holm 1979) that included all parameters tested in both models (Table 3).

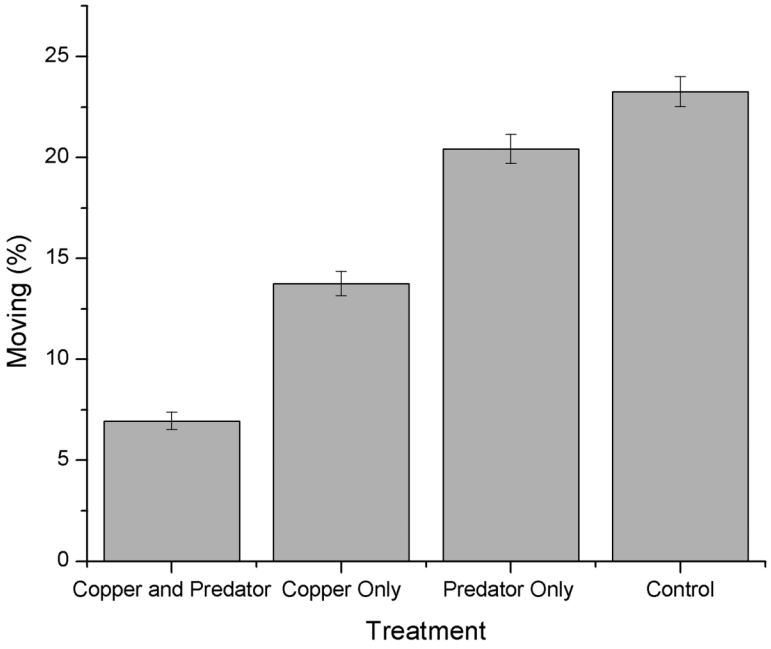

Fig. 1. Proportion of observations in which a tadpole was moving summarized by treatment. Copper and Predator = Tadpoles exposed to 5 μg/L Cu^{2+} as $CuCl_2$ and 30 mL predator cue-infused water; Copper Only = Tadpoles exposed to 5 μg/L Cu^{2+} as $CuCl_2$; Predator Only = Tadpoles exposed to 30 mL predator cue-infused water prior to assay; Control = Tadpoles exposed to clean water and predator blank. Error bars were calculated based on the underlying binomial distribution.

Results

Activity

Copper significantly reduced tadpole activity. Tadpoles exposed to copper were half as likely to be moving as those in copper-free aquaria (Fig. 1; Odds Ratio (OR) = 0.50; 95% confidence interval (CI) = 0.33–0.75; $p < 0.0001$). Predator cues also reduced tadpole activity (Fig. 1; OR = 0.011; CI = 0.001–0.151; $p = 0.0008$). There was a marginally significant interaction between Cu and the predator cue before Bonferroni correction, where tadpoles reduced activity more when treated with both stressors together (OR = 0.52; CI = 0.29–0.94; $p = 0.0298$), but sequential Bonferroni correction gave $\alpha = 0.016$, and hence the effect was not significant at $p < \alpha$ after correction for the number of comparisons was made (Table 3).

Later stage tadpoles were more active than earlier stage tadpoles (Fig. 2A; OR = 1.15; CI = 1.06–1.25; $p < 0.0001$) and were less responsive to the odonate predator cue than earlier stage tadpoles. Older tadpoles challenged with predator cues were more likely to remain active, indicated by a positive predator × Gosner interaction (Fig. 2A; OR = 1.13; CI = 1.05–1.23; $p = 0.0023$). These later stage tadpoles also recovered more quickly from the physical disturbance of adding predator cues and blanks (Fig. 3A; indicated by a significant Gosner × time since cue addition interaction; OR = 0.998; CI = 0.997–0.999). The earliest stage tadpoles (26–30; mean snout to vent length (SVL) ± standard deviation = 11 ± 2 mm) responded strongly to the initial disturbance of cue/blank addition and remained inactive for the duration of the trials, the medium stage class (31–35; mean SVL = 17 ± 2 mm) responded initially but recovered from this disturbance during the trials, and the latest stage tadpoles (36–41; mean SVL = 20 ± 3 mm) showed little response to the predator cue or blank at all (Fig. 3A). Injured tadpoles were 64% less likely to be active than their uninjured counterparts (Fig. 4; OR = 0.64; CI = 0.48–0.84; $p = 0.002$), and activity also differed significantly by breeding site (Fig. 4; $p = 0.004$).

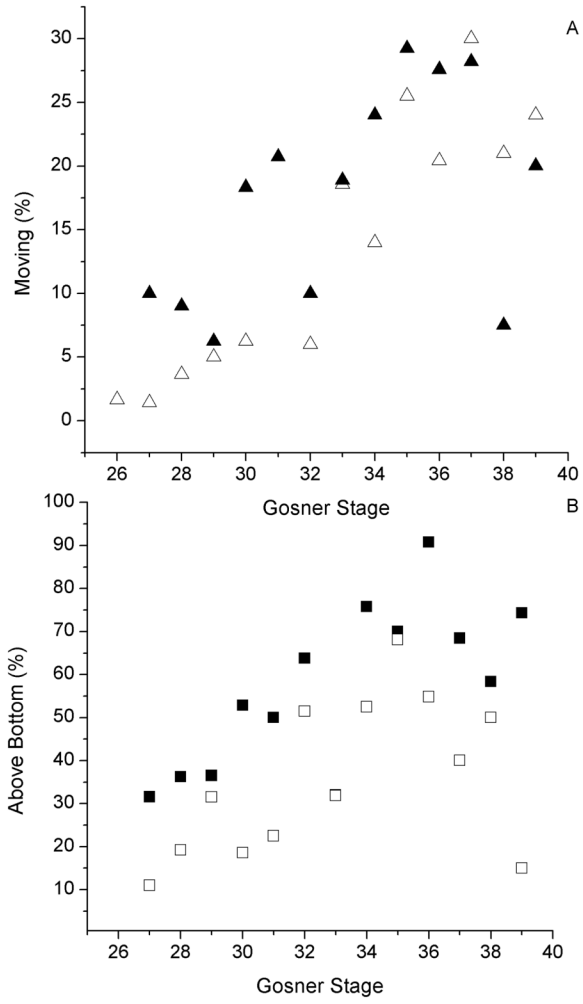

Fig. 2. Interactions with Gosner stage. Horizontal axes are developmental stage (Gosner 1960). (A) Proportion of observations in which tadpoles were active. White triangles are animals treated with predator cue and black triangles are animals treated with clean water blanks at the time of cue addition. (B) Proportion of observations in which tadpoles were above the bottom of the mesocosm. Black squares are animals treated with Cu and white squares are animals not treated with copper. Data are summarized across trials.

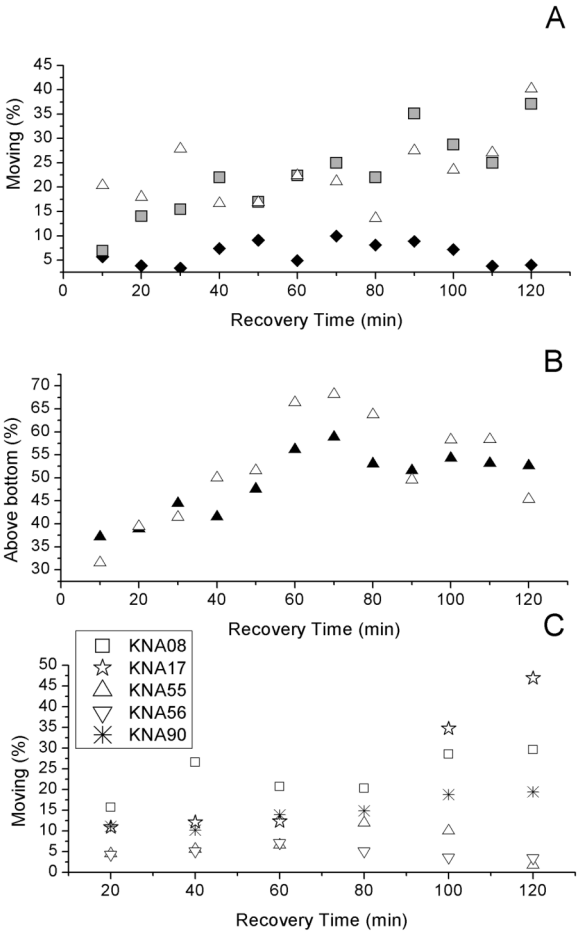

Fig. 3. Interactions with time since cue addition. Horizontal axes are time since predator cue or blank was added. A. Proportion of observations in which tadpoles were moving. Diamonds are earliest stage tadpoles (Gosner 26–30; mean snout vent length (SVL) ± sd = 11 ± 2 mm). Squares are intermediate stage tadpoles. (Gosner 31–35; SVL = 17 ± 2 mm). Triangles are latest stage tadpoles (Gosner 36–41; SVL = 20 ± 3 mm). B. Proportion of observations in which tadpoles were above the bottom of the tank. Black triangles are animals treated with a predator cue and white triangles are animals treated with clean water blank. C. Proportion of observations in which tadpoles were moving. Symbols are different sites, summarized in legend. Data are summarized across trials and across the predator and Cu treatments.

Vertical position

Contrary to our hypotheses, copper but not predator cue significantly affected the vertical position of tadpoles. Copper caused tadpoles to spend less time at the bottom of the aquaria (Fig. 2B; OR = 0.39; CI = 0.26–0.57; $p < 0.0001$). Later stage tadpoles also spent less time at the bottom of the aquaria (Fig. 2B; OR = 0.86; CI = 0.79–0.93; $p < 0.0001$). The predator cue had no measurable

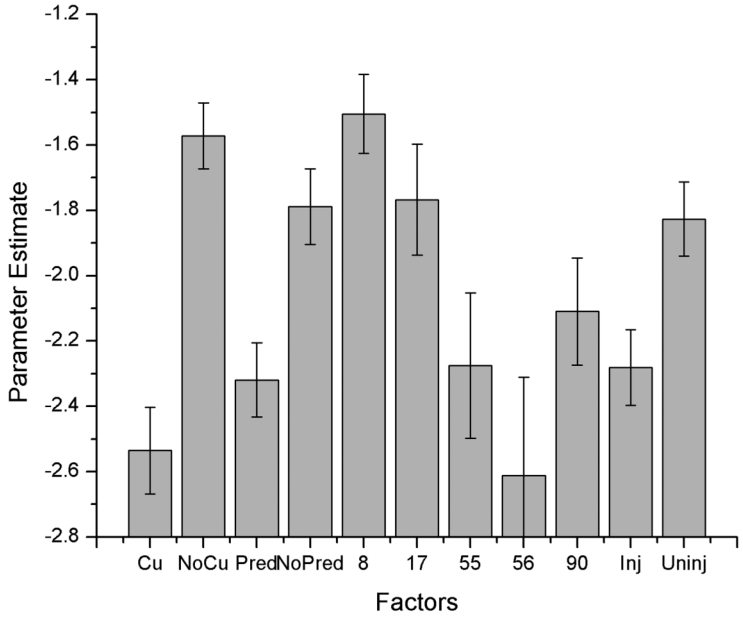

Fig. 4. Model generated parameter estimates for factors in the Activity model. Values are parameter means and standard errors calculated with the LSMEANS procedure in SAS v. 9.1.3. Cu = Copper; NoCu = No Copper; Pred = Predator Cue; NoPred = Blank; Numbers are sites; Inj = Injured; Uninj = Uninjured.

effect on vertical position (Fig. 3B; $p = 0.86$), however the physical disturbance of adding the cue or blank caused a slight but significant tendency for tadpoles to spend more time at the bottom of the aquaria after the cue was added (Fig. 3B; OR = 0.995; CI = 0.991–0.999; $p = 0.009$). For every minute after cue addition, tadpoles were 0.5% more likely to be above the bottom of the tank (Fig. 3B). Prior injuries did not influence vertical position ($p = 0.16$) and there were no significant interactions in this model (Table 3). There were, however, significant differences in vertical position preference among animals from different sites ($p = 0.0007$).

Discussion

Our prior studies (Reeves et al. 2008, Reeves et al. 2010) suggested two different hypotheses regarding how copper and predators may interact to produce abnormal frogs in nature, and we found evidence to support only one of these in this experiment. Even the relatively low Cu concentrations in some high malformation sites may be toxic enough to reduce tadpole activity enough to inhibit feeding, which could lead to smaller size over a growing season or a longer duration of exposure to gape-limited predators, like dragonfly larvae. Our field results from 2010 corroborate this interpretation (Tables 1 and 2), with smaller frogs found in sites with the highest Cu concentrations, especially when site water was soft. These size differences would be difficult to explain based solely on measured periphyton food resources (Table 2), but inference is limited because we sampled a limited number of field sites in 2010. Our second hypothesis, that Cu might interfere with olfaction and inhibit normal predator detection and avoidance behavior, was not supported by the results of this experiment.

In this experiment, Cu affected tadpole behavior at concentrations lower than any previously documented as toxic to larval amphibians and at approximately half of the nominal chronic water quality criterion of 9 μg/L (Buchman 2008). We found the effects of Cu and predators to combine additively (Fig. 1), but did not detect a significant synergistic interaction when we controlled for the number of comparisons made (Table 3). These findings fit broad expectations about the effects of Cu and predators. Tadpoles challenged

with predator cues commonly reduce activity to avoid predation (Relyea 2007) and less active tadpoles may suffer fewer predator attacks in the short term (Skelly 1994). Nevertheless, long-term reductions in activity as a result of either Cu (Redick and La Point 2004, Chen et al. 2007) or predators (Benard 2004, Relyea 2007) can result in a reduction in time spent foraging, resulting in smaller size or a longer time to metamorphosis over the course of a season (reviewed in Relyea 2007). These growth reductions or developmental delays may be due to either reduced foraging or higher metabolic costs of responding to toxicant or predator stress (Chen et al. 2007, Relyea 2007). Copper in particular has been shown to cause lethargy, loss of equilibrium, apparent loss of appetite, reductions in growth, deformities, delayed metamorphosis, and mortality, but at concentrations at least 3–20 times higher than we tested here (Horne and Dunson 1995, Redick and La Point 2004, Chen et al. 2007).

Because activity reduction is a common behavioral response to predators (Relyea 2007), one might interpret our results to mean that Cu exposure may benefit tadpoles by reducing their predation risk, and others have proposed this (Redick and La Point 2004), but an alternative explanation is also possible. Whereas short term activity reduction in response to predators has a clear risk-reduction benefit (Skelly 1994), short term activity reduction due to Cu has no fitness benefit, rather it is likely to just limit foraging and growth (Chen et al. 2007). Long term activity reduction from Cu exposure could increase the time it takes for tadpoles to grow beyond the gape-limitation of the odonate predators in these systems, lengthening the time during which tadpoles risk predator attack (Urban 2007*b*). Indeed, in our field investigations, three of five wetlands exceeded the hardness-based chronic Cu toxicity threshold for aquatic life; these sites also had smaller frogs at metamorphosis and high abnormality prevalence (Tables 1 and 2). At Site 12, 100% of the water samples were over the chronic toxicity threshold, and this site exhibited the smallest tadpoles at metamorphosis, the longest time to reach metamorphosis, and the highest percentage of skeletal abnormalities. Neither temperature nor available periphyton food resources adequately explained these patterns in size or development rate in the field (Tables 1 and 2). Moreover, in earlier controlled experiments during which we exposed tadpoles to sediment and water from six sites from eggs to metamorphosis, Sites 12 and 90 had the lowest hatching success (less than 10% for both sites compared to 78% for controls) and the lowest survival to metamorphosis (25% and 50% of tadpoles, respectively, compared to 71% for controls) in the experiment (Reeves et al. 2010). Both our field results (Tables 1 and 2) and the earlier experimental work corroborate the findings of this experiment and support the hypothesis that high Cu concentrations may lead to reduced activity and smaller size, and possibly also an increased prevalence of predation injuries and malformations, if predators are also present. An additional environmental contingency in the case of some metals may also be soft water, which allows lower metal concentrations to exert a greater toxic effect.

Simple experiments are of course limited in how directly they can be extrapolated to complex interactions in nature, especially in discussing vulnerability to predators. Both Cu and predators reduced tadpole activity, but Cu also led tadpoles to spend more time at the water surface, a behavioral response to low oxygen documented in other studies (Moore and Townsend 1998, McIntyre and McCollum 2000). Even this low concentration of Cu may have inhibited gill function (Eisler 1998) so tadpoles were unable to obtain adequate oxygen, despite measured dissolved oxygen concentrations being near saturation in both treatments (range of 92–95% dissolved oxygen saturation for control water and 93–99% for Cu-treated water). Yet, a preference for the water bottom has been shown to be an antipredator response in previous studies (Peacor 2006). The results of our experiment suggest that wood frog tadpoles sought refuge beneath the water surface when younger (Fig. 2B) and when threatened (Fig. 3B). A lack of bottom preference may make Cu-intoxicated tadpoles more vulnerable to attack from odonate predators like the climbing libellulid dragonflies, which hunt visually, clinging to vegetation below the water surface (Pritchard 1965). This type of odonate specifically has been shown to cause amphibian limb malformations by preferentially chewing the hind limbs off of tadpoles during non-lethal attacks (Ballengée and Sessions 2009).

Although we found libellulid dragonfly larvae in only four of 21 study sites (19%) in earlier work (Reeves et al. 2010), we found the larger aeshnid dragonfly larvae used in this experiment in all but three study sites (86%). When generating the predator cue for this experiment, we observed these aeshnid larvae chewing the legs off of later stage *R. sylvatica* tadpoles—those approaching the limit of their gape-limitation—yet leaving the remainder of the tadpole alive in the tank. We propose that if Cu at this concentration does lead tadpoles to spend more time at the water surface, rather than camouflaged against pond bottom sediments, then they may be spending more time in a more vulnerable position with respect to dragonfly attack. In other studies, increased time at the surface has been shown to increase (Moore and Townsend 1998) or decrease (McIntyre and McCollum 2000) tadpole predation risk, depending on the predator. Therefore, more research is clearly needed on this topic. An obvious next step is to challenge Cu-intoxicated tadpoles with the predators themselves, rather than just their chemical cues.

Our results showed an activity reduction, but not a bottom preference in response to chemical cues from aeshnid dragonfly larvae (Fig. 1 and Fig. 3B). Our failure to detect an effect of the predator cue on vertical position may mean that wood frog tadpoles do not respond to threats from aeshnid dragonflies specifically by seeking bottom refuge, or that our aquaria did not provide a realistic substrate to suggest safety in the face of this predator. This lack of significant result may also be due to the fairly striking changes in behavior with increasing size and developmental stage (Fig. 2). Consistent with theory on the relationship between prey growth and degree of threat from the gape-limited predators that dominate our study system (Urban 2007*b*), later stage tadpoles responded less strongly to the odonate predator cue (Fig. 2) and recovered more quickly from it (Fig. 3A), suggesting they may have had some method to assess the changing risk as they outgrew the gape-limitation of the odonate predator used to generate the chemical alarm (Fig. 2). This result is consistent with other studies that have explicitly examined how response to predator cue changes with size (Fraker 2008, McCoy and Bolker 2008) and with research showing that larger tadpoles can more easily escape odonate predators (Richards and Bull 1990). Indeed, in this experiment as tadpoles got larger, it was increasingly difficult for the large aeshnids to kill or injure tadpoles used to generate the alarm cues.

Finally, tadpole behavior in the experiment varied with breeding site (Fig. 3C and Fig. 4). Larval amphibians are known to be physically and behaviorally plastic in response to environmental conditions (Urban 2007*a*, Van Buskirk 2009), and the reasons for site variation we documented in this study need more investigation. It is interesting that activity levels were highest and recovery times were shortest in tadpoles from sites with the highest odonate densities (Fig. 3C, Fig. 4, Table 2). This observation provides empirical support for the idea that prey from riskier sites (those with higher densities of gape-limited predators like odonate larvae) may engage in riskier foraging behaviors that allow them to attain a size threshold beyond the predator's gape faster (Urban 2007*a*). Enough other parameters varied across the different sites (Tables 1 and 2; Appendix: Tables A2–A5), that we refrain from further interpreting site patterns in the experimental data here. Similar field research incorporating 30 additional sites in the Kenai NWR will begin in 2011.

Our experiment, like the majority of toxicity studies, examined the effects of a single toxicant, although we investigate the interaction between a toxicant and predation. In nature, the co-occurrence of different contaminants in a single area creates a specific form of multiple stressor scenario. As shown in Appendix: Tables A3–A5, at these high-malformation wetlands from which experimental animals were collected, multiple metals including aluminum, barium, copper, iron, manganese, and zinc exceeded aquatic toxicity criteria in multiple samples. Moreover, several of these sites also received measurable inputs of road salt, known to be toxic to aquatic organisms (Corsi et al. 2010). These sites are located on a National Wildlife Refuge in Alaska and some metals exceeded both acute and chronic hardness-based water quality criteria (U.S. EPA 2007) in part because these waters are so soft.

Water hardness exerts an important control on metals toxicity—soft water causes metals to be more toxic at lower concentrations (Meyer 1999).

The main cations that control water hardness are Ca and Mg. It is possible that application of $MgCl_2$ as a road surface de-icer, instead of the less expensive and more commonly used NaCl, could increase the hardness of natural waters that receive road runoff, thereby ameliorating the toxicity of the other heavy metal ions road runoff contains (e.g., Boxall and Maltby 1997). Other water characteristics including pH, dissolved organic carbon, and other ions in solution can also ameliorate Cu toxicity by other mechanisms (e.g., the Biotic Ligand Model reviewed in Niyogi and Wood 2004). Whether the numerous chemicals in road runoff interact additively, synergistically, or antagonistically (Moriarty 1999), the multiple stressor nature of contaminants in anthropogenic mixtures cannot be stressed enough for its relevance to environmental management.

Johnson and Bowerman (2010) suggest multiple stressors, environmental cofactors, and ecological contingencies are critical to fully understanding the abnormal frog problem, or more broadly, predation dynamics in aquatic systems. Other environmental co-factors these authors suggest are invasive species, availability of alternate prey sources, habitat refugia, and the timing of predator development relative to prey, which in invertebrates and ectothermic vertebrates is coupled with temperature and climate. We reiterate these contingencies here. We further argue that the type of reductionist strategies exemplified by traditional dose-response toxicology, with estimation of the concentration of a single pollutant that kills 50% of the population (LC_{50}) as an endpoint are of limited utility to managers responsible for allowing permissible amounts of chemical pollution into natural systems. Such reductionism may even be counterproductive if a primary management goal is conservation of animals and their habitats.

Finally, our results demonstrate that 5 μg Cu/L did not interfere with olfaction in wood frog tadpoles sufficiently to inhibit their ability to detect chemical predator cues, but rather the two stressors together had additive effects on behavior. A growing body of research with agricultural chemicals and larval amphibians has shown similar results: that natural stressors like predators and parasites can combine with anthropogenic stressors like biocides to make these chemicals more lethal to amphibians (Relyea 2004, Relyea 2005, Relyea and Diecks 2008, Rohr et al. 2008). More work remains before we can really resolve the question of whether toxicants in general interfere with olfaction in anurans as they do in salmonids (Sandahl et al. 2007). This remains a viable hypothesis that should be tested for other species of amphibians, other metals and biocides, and for the suite of chemicals that co-occur in road or agricultural runoff (Scher and Thiery 2005, Corsi et al. 2010).

We conclude that a single chemical contaminant (Cu) and the chemical cue of a single predator acted additively to alter behavior in larval amphibians. We argue that exposure to Cu, even at low concentrations, could be disadvantageous to larval amphibian prey confronted by odonate predators. This hypothesis should be tested with future work. Our research presents several methodological considerations for future predation studies, including the need to explicitly test animals at different sizes and life stages and account for time since cue addition during assays to get a whole understanding of predator effects, which may change with the life history of the organism. Finally our field and experimental research combines to support two hypotheses for Cu toxicity in nature. First, Cu intoxication may reduce foraging and lead to smaller size and developmental delays, which in turn increase the time during which tadpoles are vulnerable to gape-limited predators. Second, Cu intoxication may reduce tadpole ability to obtain sufficient oxygen by impairing the gills, thereby driving tadpoles to the surface of the water. These hypotheses warrant further testing for the abnormal frog problem specifically, and as drivers of predation dynamics in aquatic systems.

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Appendix

Additional Methodological Information

Literature review

A query of the EPA ECOTOX database with keywords "Copper" and "Amphibian" resulted in 239 results from 50 publications, with a huge variability in the results for what concentration of copper is toxic. These studies contained a range of effects from accumulation in *Bufo arenarum* tadpoles at 0.4 μg/L (Herkovitz and Pérez-Coll 2007) to lipid peroxidation in adult *Rana ribunda* at 100,000 μg/L (Papadimitriou and Loumbourdis 2005) and included all life stages of anuran amphibians. Of 78 results in this query reporting a concentration that killed 50% of test organisms (LC_{50}), the values for this endpoint ranged from 39 μg/L to 35,990 μg/L, with a mean of 1,868 μg/L. Concentrations reported as either No Observed Effects Concentrations (NOEC) or No Observed Effects Levels (NOEL) ranged from 5 to 100,000 μg/L with means±standard deviations as follows: NOEC = 16,574 ± 32,837 μg/L and NOEL = 68,765 ± 45,780 μg/L.

Experiment methods

Site. We collected tadpoles from two high elevation sites (KNA55 and KNA56) and three low elevation sites (KNA08, KNA17, and KNA90) to separate the effects of developmental stage (real effect) versus date of trial (experimental artifact). Tadpoles from high elevation sites were developmentally about a month behind low elevation sites. On the same dates, tadpoles from high elevation sites were Gosner 27–28, whereas their lower elevation counterparts were 35–38. Fewer tadpoles were tested from the two high elevation sites, because tadpoles from high elevation sites required us to hike a 10-km round-trip and no other sampling was planned at these sites in 2010. Additional experimental data are presented in Table A1.

Table A1. Additional experimental information.

Trial	Date	Sites	No. tadpoles	Obs. per tadpole (N)	Mean SVL† (mm)	Mean Gosner stage‡	Length of trail (min.)	Moving (% obs.)	Above bottom (% obs.)
1	11/06/2010	8 and 90	29	10	11	28	112	9	28
2	17/06/2010	8 and 90	27	10	14	31	104	14	36
3	01/07/2010	8 and 90	32	10	18	35	120	33	81
4	01/07/2010	8	8	10	17	33	32	14	46
5	04/07/2010	8 and 90	32	10	18	35	112	13	85
6	05/07/2010	17	14	20	16	32	52	9	35
7	08/07/2010	17	16	10	18	33	28	13	24
8	14/07/2010	8 and 90	31	10	17	37	106	16	78
9	16/07/2010	17	15	10	21	35	27	15	58
10	17/07/2010	55 and 56	26	20	10	28	120	4	20
11	20/07/2010	55 and 56	25	10	10	28	85	9	44
12	22/07/2010	8 and 17	31	20	21	37	123	31	47
Means					16	33	85	16	48

Note: Trials 3 and 5 and 10 and 11 repeated observations on the same animals.
† SVL = snout to vent length.
‡ Gosner 1960.

Table A2. Additional site information.

Site ID	Latitude	Longitude	Road type	Deep or shallow
KNA08	60.62650 N	−150.80745 W	Intermediate use gravel	Deep
KNA12	60.71423 N	−150.81541 W	Intermediate use gravel	Shallow
KNA17	60.732830 N	−150.619140 W	Intermediate use gravel	Deep
KNA55	60.526960 N	−150.069530 W	Remote wilderness	Shallow
KNA56	60.523690 N	−150.064090 W	Remote wilderness	Shallow
KNA90	60.46187 N	−151.09351 W	High use paved	Shallow
KNA97	60.53160 N	−150.42769 W	High use paved	Deep

Field methods. The following sections detail methods used in 2010 to collect environmental data at the five study sites for which data are presented in Tables A2–A5: KNA08, KNA12, KNA17, KNA90, and KNA97. Site locations, hydroperiods, and adjacent road types are presented in Table A2. No field data were collected for sites KNA55 and KNA56 in 2010, but we present historic data from these remote sites from 2004–2006. Additional historic information about all sites may be found in Reeves et al. 2010.

1. Wetland habitat and hydroperiod. We assigned each site to either a "Deep" or "Shallow" category, to describe gross differences in wetland habitat and hydroperiod. "Deep" wetlands are permanent wetlands. They are characterized by sphagnum vegetation at the edge and a high organic matter peat bottom, which will generally not support the weight of a standing person. "Shallow" wetlands are ephemeral and may dry over the course of the summer. Although some shallow wetlands retain water through the summer some years, they dry completely in others. Shallow wetlands tend to have bottoms that will support a person's weight, with fine sediments higher in inorganic content than deep wetlands. The primary vegetation types in the shallow wetlands are aquatic grasses, rushes, and sedges. Wetland type for each site is listed in Table A2.

2. Roads. We chose sites based on the type of road the wetland borders: "High Use Paved" and "Intermediate Use Gravel" (Table A2). The high use paved road was the Sterling Highway in both cases, but the two sites were separated by approximately 40 km. The intermediate use gravel roads were Swanson River Road (KNA08, KNA12), which serves recreational and industrial users (there is an oil field at the end of this road that has operated since the 1950s) and Swan Lake Road (KNA17), which serves only recreational users.

3. Temperature. We measured temperature continuously between early May (when data loggers were deployed) and mid-September (when loggers were collected). Temperature was measured at the near (close to road) and far (from

Table A3. Additional water quality field data from the Kenai NWR sites in 2010.

	Specific Conductivity			pH			Chloride†		
Site	Mean (μS/cm)	N	Range (μS/cm)	Mean	N	Range	Mean (mg/L)	N	Range (mg/L)
8	**19.9**	**2129**	**17.7–23.7**	**5.77**	**208**	**5.26–6.86**	**1.73**	**208**	**0.29–4.61**
12	26.9	**2129**	14.1–42.1	6.54	36	6.18–6.93	1.91	36	0.46–11.96
17	**10.6**	**2129**	**9.6–12.8**	**6.59**	**177**	**5.65–7.20**	**1.43**	**177**	**0.30–2.76**
90	**165.9**	**2129**	**139.0–222.2**	**6.09**	**36**	**5.45–7.18**	**32.16**	**36**	**7.20–116.70**
97	152.3	2009	129.2–172.7	6.88	72	6.42–7.39	1.48	72	0.19–3.90

Note: Sites in bold are the primary sites used to collect tadpoles for the experimental trials.
† Chronic aquatic life criteria for Cl = 230 mg/L, four-day average for freshwater (ADEC 2003).

road) edges of the wetland, both near the surface and at depth (surface only at the shallow sites) with Hobo tidbit loggers (Onset Data Corporation, Pocasset, MA, USA) set to log every hour. Shallow loggers were floated 10 cm below the water surface, hanging from a piece of foam that was tied to an anchor with plastic-covered wire cable, and secured to the foam by plastic zip-ties. Deep loggers were floated 10 cm off the pond bottom. Data presented in Table 1 are mean and range of all recorded measurements at a site between 1 May 2010 and 7 Sept 2010.

4. Specific conductivity. We measured specific conductivity continuously between mid-June (when data loggers were deployed) and mid-September (when loggers were collected). Specific Conductivity was measured at the near (close to the road) edge of the wetland only at a depth of 10 cm below the water surface with HOBO U24 conductivity loggers (Onset Data Corporation, Pocasset, MA, USA) set to log every hour. The unit was suspended inside a perforated PVC pipe as recommended in the deployment guidelines. Data presented in Table A3 for specific conductivity are mean and range of all recorded measurements at a site between 10 June and 7 Sept 2010.

5. pH and chloride. We measured pH and chloride ion concentration (Cl^-) once every 7–10 days at each study site between April 26 and September 7, 2010 with a YSI water quality meter. A standard confidence solution was used to test readings weekly and the meter was calibrated at least biweekly for all parameters. Measurements were taken at the points nearest to and farthest from the road in the wetland, within 1 m of the temperature loggers. We took surface measurements and depth profiles each week. The first measurement was taken at 10 cm depth, after which recordings were in 30 cm increments until the YSI reached the bottom of the wetland. Values in Table A3 are mean and range for all measurements at all depths within a site taken over the summer.

6. Water chemistry. We collected water samples for metals analysis from the following locations in each study site in late spring and mid-summer. Samples were collected in certified chemically clean HDPE plastic sampling bottles.

6. A. Near and far edge. The locations of these samples were determined by wetland shape. Samples were taken from the points nearest to and farthest from the road edge in both sampling events. In the mid-summer sampling event, we also collected a water sample that was a composite of 3 sampling locations to compare to the point measures. This sample was scaled to the size of the wetland with one sample roughly in the "middle" and the other two samples at the "edges" of the near and far area. The sample was taken by a person standing at the wetland edge, in a location where they did not disturb the bottom sediments. The exact sampling locations necessarily changed in some wetlands that dried over the summer.

6. B. Shallow and deep. In shallow wetlands, the samples collected from 10 cm below the surface at the near and far locations were the only water samples. In deep wetlands, however, samples were also taken at depth at the near and far wetland edges. The deep samples were taken from 10 cm above the bottom of the wetland using a sub-surface grab sampler device (Wheaton Science Products, Millville, NJ, USA). Locations of deep samples were at least 1 m below the water surface, to determine how oxidation/reduction potential (which can vary with depth) affected metals solubility in these wetlands. All metal concentrations were measured at the Applied Science Engineering and Technology

Table A4. Additional analytical chemistry data from the Kenai NWR sites in 2010: Al, Ba, Fe.

		Aluminum			Barium			Iron		
Site	N	Mean† (μg/L)	Range (μg/L)	% above CCC	Mean† (μg/L)	Range (μg/L)	% above CCC	Mean† (μg/L)	Range (μg/L)	% above CCC
8	**12**	**208**	**36–357**	**83**	**5.8**	**2.5–9.5**	**83**	**622**	**161–1585**	**16**
12	6	135	114–155	100	29.4	18.0–35.6	100	286	111–420	0
17	**12**	**6**	**4–9**	**0**	**1.4**	**1.0–2.3**	**0**	**15**	**6–45**	**0**
55‡	1	<50	...	...	5.0	...	...	60	...	...
56‡	1	<50	...	...	13.0	...	...	80	...	...
90	**6**	**51**	**38–74**	**0**	**7.3**	**3.0–15.3**	**66**	**108**	**42–285**	**0**
97	12	17	5–35	0	16.5	10.7–23.0	100	1325	195–3789	50

Note: Sites in bold are the primary sites used to collect tadpoles for the experimental trials. Criterion Continuous Concentration is the chronic limit for priority pollutants in freshwater surface waters using the dissolved fraction (ADEC 2003). Al CCC = 87μg/L; Ba CCC = 3.9 μg/L; Fe CCC = 1000 μg/L.

† Mean analyte values are calculated using half of the reported detection limit for non-detect data.

‡ Analytical chemistry data are historic data from 2004/2005, "<" indicates analyte was not detected at detection limit reported to the right of this symbol.

Table A5. Additional analytical chemistry data from the Kenai NWR sites in 2010: Mn, Zn.

		Manganese			Zinc			
Site	N	Mean† (μg/L)	Range (μg/L)	% above CCC	Mean† (μg/L)	Range (μg/L)	Mean Zn chronic criteria‡ (μg/L)	% above criteria
8	**12**	**64**	**16–142**	**41**	**18**	**9–41**	**13**	**75**
12	12	37	22–54	0	18	8–28	13	83
17	**12**	**9**	**2–30**	**0**	**14**	**4–49**	**4**	**100**
55§	1	20	...	...	<5	...	88	...
56§	1	<5	...	...	<5	...	90	...
90	**6**	**39**	**5–77**	**0**	**20**	**15–27**	**16**	**66**
97	12	82	7–203	41	13	4–25	82	0

Note: Sites in bold are the primary sites used to collect tadpoles for the experimental trials. Criterion Continuous Concentration - chronic limit for priority pollutants in freshwater surface waters using dissolved fraction (ADEC 2003). Mn CCC = 80 μg/L.

† Mean analyte values are calculated using half of the reported detection limit for non-detect data.

‡ Hardness-calculated chronic aquatic life criteria for freshwater using dissolved fraction for current data and total recoverable for historic data from sites 55 and 56 (Buchman 2008).

§ Analytical chemistry data are historic data from 2004/2005, "<" indicates analyte was not detected at detection limit reported to the right of this symbol.

Laboratory, University of Alaska Anchorage by inductively coupled plasma-mass spectroscopy (ICP-MS) by EPA method 200.8.

6. C. Dissolved metals. We sampled water for the dissolved metal fraction by filtering the sample through a 0.45 micron syringe filter prior to collection and preserving the sample with 1% concentrated nitric acid (HNO_3). The filtration step eliminates small sediment particles in the water, to which metals can sorb, and therefore eliminates particle-bound metals from quantitative ICP-MS analysis, thus sampling only metals that are dissolved. Although we also collected samples of the "total recoverable" fraction, only dissolved samples were included in Tables 1, A4 and A5.

7. Metamorphs for abnormality assessment. Methods for metamorph abnormality assessment were fully described in Reeves et al. 2010. Briefly, 50–100 metamorphic frogs, stage 42–46 (Gosner 1960), were assessed for abnormalities at each site. This method controls tadpole developmental stage by limiting sampled animals to recent metamorphs between forelimb emergence and complete tail resorption. Snout-to-vent length (SVL) and tail length were measured, and developmental stage recorded. All abnormalities were classified by a single researcher. All frogs were released at the capture site after abnormalities were documented with digital photographs.

8. Macroinvertebrate sampling. Macroinvertebrates were collected from 10 stations within each wetland with a D-frame dipnet (500 μm mesh). One linear meter was sampled in each

station. The sampler disturbed the vegetation or substrate with the net, dislodging the organisms present while sweeping the net through the water. All samples were labeled inside and out with site ID and date sampled, and preserved in 95% ethanol until identified in the laboratory.

9. Periphyton sampling. Periphyton was collected from suitable substrate at each station. Large stable substrate, (e.g., cobble) was sampled while holding it over a funnel inserted into a clean graduated 1000 mL bottle. A sampling delimiter was applied to the substrate to standardize the area sampled; periphyton was sampled only from inside the rubber washer of the periphyton delimiter. If the periphyton layer was thick, it was scraped with a spoon or laboratory spatula and rinsed into the funnel using a wash bottle filled with water from the wetland. The delimited substrate was then scrubbed with a clean toothbrush and all loosened periphyton was again rinsed into the funnel. We alternated scrubbing using the periphyton scrubber and rinsing using the wash bottle until all periphyton within the delimited area was removed and rinsed into the funnel. If no large substrate was present in the littoral plot, we sampled periphyton from the sediment by pushing a small inverted (open side down) petri dish into the sediment, and sliding a spatula underneath. The captured sediment and associated periphyton were then washed into the funnel. Two samples were collected at each station and samples were composited into "near road" and "away from road" samples. Sample collection methods were recorded so that total sample area could be calculated. The volume of the composite sample was recorded before an aliquot was removed for analysis. A well-mixed 25-ml aliquot was filtered through a glass filter (Whatman GF/F or equivalent). The filter was then folded, placed into a centrifuge tube, and stored on ice. This sample was analyzed for ash free dry mass, a measure of periphyton standing stock.