

Continental-extent patterns in amphibian malformations linked to parasites, chemical contaminants, and their interactions

Sarah E. Haas¹  | Mari K. Reeves² | Alfred E. Pinkney³ | Pieter T. J. Johnson⁴

¹Texas Parks and Wildlife Department, Austin, TX, USA

²US Fish and Wildlife Service, Honolulu, HI, USA

³US Fish and Wildlife Service, Annapolis, MD, USA

⁴Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA

Correspondence

Sarah E. Haas, Texas Parks and Wildlife Department, Austin, TX, USA.
Email: Sarah.Haas@tpwd.texas.gov

Funding information

USFWS; National Science Foundation, Grant/Award Number: DEB-0841758, DEB-1149308; National Institutes of Health, Grant/Award Number: R01GM109499; David and Lucile Packard Foundation

Abstract

Widespread observations of malformed amphibians across North America have generated both concern and controversy. Debates over the causes of such malformations—which can affect >50% of animals in a population—have continued, likely due to involvement of multiple causal factors. Here, we used a 13-year dataset encompassing 53,880 frogs and toads from 422 wetlands and 42 states in the conterminous USA to test hypotheses relating abnormalities and four categories of potential drivers: (i) chemical contaminants, (ii) land use practices, (iii) parasite infection, and (iv) targeted interactions between parasites and pesticides. Using a hierarchically nested, competing-model approach, we further examined how these associations varied spatially among geographic regions. Although malformations were rare overall (average = 1.6%), we identified 96 hotspot sites with 5%–25% abnormal individuals. Using the full dataset of 934 collections (without data on parasite infection), malformation frequency was best predicted by the presence of oil and gas wells within the watershed. Among collections also examined for parasite infection ($n = 154$), average parasite load and its interaction with pesticide application positively predicted malformations: wetlands with a greater abundance of the trematode *Ribeiroia ondatrae* were more likely to have malformed amphibians, but these effects were strongest when pesticide application was also high, consistent with prior experimental research. Importantly, however, the influence of these factors also varied regionally, helping explain divergent results from previous studies at local scales; parasite infection was more influential in the West and Northeast, whereas pesticide application and oil/gas wells correlated with abnormalities in the Northeast, Southeast, and western regions of the USA. These results, based on the largest systematic sampling of amphibian malformations, suggest that increased observations of abnormal amphibians are associated with both parasite infection and chemical contaminants, but that their relative importance and interaction strength varied with the spatial extent of the analysis.

KEYWORDS

amphibian malformations, chemical contaminants, land use, *Ribeiroia ondatrae*, spatial nonstationarity

1 | INTRODUCTION

Widespread observations of malformed amphibians in North America have prompted concern as well as controversy among scientists and

the general public (Johnson, Reeves, Krest, & Pinkney, 2010; Reeves et al., 2013; Rohr, Schotthoefer, et al., 2008; Skelly & Benard, 2010; Souder, 2000). To date, at least 70 species of amphibians have been reported with abnormalities, for which the majority of observations

involve recently metamorphosed frogs and toads (anurans) from wetland habitats (Johnson, Reeves, et al., 2010). Of particular concern are recent observations that document substantially higher levels of severe malformations (defined as permanent structural defects resulting from abnormal development, whereas abnormalities refer to any gross deviation from the normal range in morphology), ranging from 5% to 100% of sampled individuals (Johnson & Bowerman, 2010; Johnson, Reeves, et al., 2010; Lannoo, 2008). While some small fraction of abnormalities may occur in any amphibian population, available data indicate this baseline frequency is <5%, with populations exhibiting significantly higher frequencies classified as "hotspots" (Johnson et al., 2002; Johnson, Reeves, et al., 2010; Ouellet, 2000). The most frequently reported abnormalities involve the hind limbs, including partially missing limbs, completely missing limbs, extra limbs, and misshapen limbs (Johnson, Reeves, et al., 2010; Reeves et al., 2013). Most recent reports involve North American amphibians, although mass malformations have also been observed in other countries (Bacon, Linzey, Rogers, & Fort, 2006; Flyaks & Borkin, 2004; Gurushankara, Krishnamurthy, & Vasudev, 2007; Laurentino, Pais, & Rosa, 2016; Ouellet, 2000). Several retrospective studies based on museum samples and historical resurveys further indicate that limb malformations have increased over time (Hoppe, 2005; Johnson & Lunde, 2005; McCallum & Trauth, 2003), highlighting the importance of investigating environmental changes associated with morphological abnormalities through space and time.

Debate over the causes of abnormalities and their relative importance has been extensive (Ballengée & Sessions, 2009; Johnson & Bowerman, 2010; Skelly & Benard, 2010). Attention has focused on factors with the potential to disrupt development of amphibians during their aquatic phase (i.e., tadpoles), for which the most commonly advanced hypotheses include parasite infection, chemical contaminants, changes in land use practices (e.g., roads, agriculture, or urbanization), and injuries from introduced predators (Ankley, Degitz, Diamond, & Tietge, 2004; Johnson, Reeves, et al., 2010; Lunde & Johnson, 2012; Reeves, Jensen, Dolph, Holyoak, & Trust, 2010; Taylor et al., 2005). For instance, both experimental studies and field-based correlations have linked infection by the digenetic trematode *Ribeiroia ondatrae* to amphibian limb abnormalities in multiple regions of North America (Johnson & Hartson, 2009; Johnson et al., 2002; Kiesecker, 2002; Lunde, Resh, & Johnson, 2012; Roberts & Dickinson, 2012). These parasites specifically encyst around the developing limb buds of tadpoles, often disrupting growth leading to extra limbs, missing limbs, skin webbings, and other malformations (Johnson, Preston, Hoverman, & Richgels, 2013; Johnson, Sutherland, Kinsella, & Lunde, 2004; Johnson et al., 2012; Sessions, Franssen, & Horner, 1999). However, other wetlands with a high frequency of abnormal frogs do not support *R. ondatrae* infection (Bowerman, Johnson, & Bowerman, 2010; Reeves, Doplh, Zimmer, Tjeerdema, & Trust, 2008; Skelly, Bolden, Freidenburg, Freidenfelds, & Levey, 2007), reinforcing the need to investigate additional factors. Extensive research has analyzed the role of chemical stressors in disrupting amphibian development (summarized in Johnson, Reeves, et al., 2010), including evidence that abnormalities are associated with a wetland's

proximity to agricultural (e.g., pesticides and fertilizer; Taylor et al., 2005; Gurushankara et al., 2007; Rohr, Schotthoefer, et al., 2008) or industrial activities (e.g., coal combustion waste, petroleum hydrocarbons; Hopkins, Congdon, & Ray, 2000; Flyaks & Borkin, 2004). While laboratory studies suggest that water and sediment extracts from contaminated sites can induce amphibian abnormalities (Bridges, Little, Gardiner, Petty, & Huckins, 2004), linking field-observed patterns to specific compounds is complicated by the diversity of chemicals released into the environment and their potential to interact with one another or additional factors (Johnson, Reeves, et al., 2010). Finally, observational field surveys and experimental manipulations indicate that sublethal predation by aquatic predators, such as larval dragonflies, introduced fishes, and leeches, can also contribute to abnormal limb growth (Ballengée & Sessions, 2009; Bowerman et al., 2010; Johnson & Bowerman, 2010). When such attacks involve tadpoles still undergoing limb development, the resulting abnormalities may not appear to be the obvious outcome of trauma (Bowerman et al., 2010), challenging efforts to determine the causative agent by abnormality type alone.

Despite progress in identifying the causes of abnormalities in some localities, opportunities to broadly evaluate the importance of multiple causal factors and how they vary spatially has been hindered by the narrow spatial extent of previous surveys, an overall tendency to focus on single-factor explanations, and inconsistent methodologies among studies (Johnson, Reeves, et al., 2010; Lunde & Johnson, 2012). Given the large geographic extent over which abnormalities have been reported, it is likely that multiple causative agents are involved, the importance of which will vary spatially in form and magnitude. Studies that explicitly consider spatial nonstationarity (i.e., parameter values that vary in space) are therefore increasingly essential for understanding and forecasting complex ecological phenomena (Bini et al., 2009; Cohen et al., 2016). For instance, the factors underlying the risk of human-caused wildfires in the Mediterranean Basin are highly region-specific: while the amount of forested area is a broad predictor of risk, fires in agrarian areas are associated with land abandonment and increased fuel build-ups, whereas those near more urbanized regions are often linked to land encroachment (Koutsias, Martínez-Fernández, & Allgöwer, 2010). Moreover, the factors underlying amphibian malformations have inherent potential to interact; exposure to pesticides, for instance, can suppress amphibian immunity and increase infection by parasites (Hayes, Falso, Gallipeau, & Stice, 2010; Jayawardena, Rohr, Navaratne, Amerasinghe, & Rajakaruna, 2016; Pochini & Hoverman, 2017; Rohr, Schotthoefer, et al., 2008), whereas toxic metals have been hypothesized to interfere with larval amphibians' ability to evade aquatic predators such as larval dragonflies (Hayden et al., 2015; Reeves, Perdue, Blakemore, Rinella, & Holyoak, 2011; Reeves et al., 2010). These observations underscore the potential for regional variation in causes and emphasize the need for large-scale, systematic surveys of abnormalities combined with multifactorial measurements of possible causative agents.

Here we use data collected across 42 states in the conterminous USA to evaluate drivers of amphibian malformations both at national

and regional spatial extents. Between 1999 and 2012, we assessed abnormalities in 53,880 amphibians representing 41 species across 422 wetlands and 127 U.S. Fish and Wildlife Service (USFWS) National Wildlife Refuges (see Reeves et al., 2013). Using a hierarchically nested, competing-model approach, we evaluated the explanatory power of targeted causative agents that have received prior research support: parasitic trematodes, chemical contaminants, and anthropogenic forms of land use (Johnson et al., 2002; Johnson, Reeves, et al., 2010; Rohr, Schotthoefer, et al., 2008; Taylor et al., 2005). Specifically, we compiled and combined spatially explicit information on land cover, the distribution of oil and gas wells, application of pesticides and fertilizers, and abundance of the trematode *R. ondatrae* (collected at a subset of wetlands as part of this project) to test the capacity of these variables to predict patterns of skeletal malformations in amphibian populations across North America. On the basis of previous research, we expected a higher prevalence of morphological abnormalities in wetlands adjacent to agriculture or industrial activities, or those with greater *R. ondatrae* infection (Johnson et al., 2002, 2013; Lunde et al., 2012). We also tested the influence of targeted interactions between parasite infection and pesticide application on the basis of prior experimental studies and region-specific field research (Rohr, Raffel, Sessions, & Hudson, 2008; Rohr, Schotthoefer, et al., 2008). Importantly, we examined how these response-environment associations varied spatially by comparing the identity and explanatory power of hypothesized factors at national vs. regional spatial extents, which has important implications for how potential threats are studied and managed.

2 | MATERIALS AND METHODS

2.1 | Site and species selection

We selected wetlands distributed across National Wildlife Refuges based on the known or suspected presence of amphibians and the availability of accessible habitat. In total, our sampling included 422 wetlands on 127 Refuges in 42 states across the conterminous USA, encompassing a broad range of ecoregions and land use histories (Figure 1). We focused on frogs and toads with a particular emphasis on lentic-breeding species within the genera *Rana* and *Lithobates* (see Appendix S1 for a list of the 41 species), owing to their widespread distribution, prior history of abnormalities, and the value of focal taxa for broad-scale comparisons (Lunde & Johnson, 2012; Ouellet, 2000). Potential amphibian breeding areas were identified based on preliminary site visits and discussions with Refuge personnel. Sites were usually small wetlands or other water bodies, such as agricultural ponds, marshes, or roadside ditches, located within Refuges, Wetland Management Districts (WMD), or Waterfowl Production Areas (WPA). However, while Refuges are managed “to conserve, protect, and enhance fish, wildlife, plants, and their habitats for the continuing benefit of the American people” (USFWS Mission Statement), they vary considerably in habitat, water quality, biological diversity, and land use history. Some of the sampled Refuges represent nearly undisturbed natural habitats, whereas others have been affected by prior land use practices associated with significant habitat degradation or contamination, thereby creating broad variation well-suited for our survey of amphibian abnormalities.

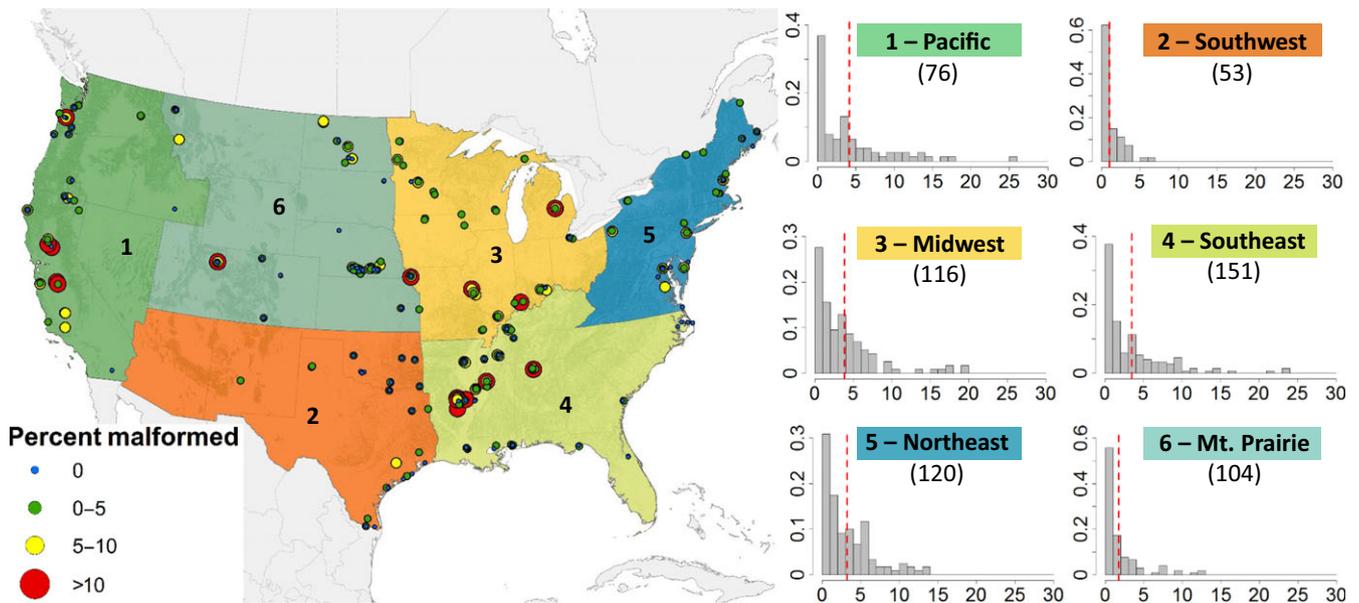


FIGURE 1 Geographical distribution of the percentage of amphibians exhibiting skeletal malformations from field collections across the conterminous USA. Here we only show data from the 620 collections with at least 30 individuals surveyed. Averages are shown for wetlands with multiple collection events. Histograms display relative frequencies of the percentage of malformed individuals across all collections within USFWS administrative regions (we adopted the former USFWS classification scheme that combined Region 1 and 8 to achieve greater balance in sampling coverage across the USA). Dashed lines show the average percentage of malformed individuals across all collections within each region (the number of collections is shown in parentheses)

2.2 | Field sampling

Sampling of wetlands was conducted between 1999 and 2012; individual wetlands were typically visited once or twice per season, with a late-spring or early summer visit to detect amphibian activity and guide the timing of a second visit to catch animals near metamorphosis. While most if not all USFWS regions were sampled each year, specific wetlands were generally visited over the course of one to two (often consecutive) years, thereby maximizing the total sampling coverage within and among regions. During each collection event, we aimed to capture 50 individual amphibians of a given species and examine them for external abnormalities following established protocols (USFWS, 1999). We focused on developmental stages from forelimb emergence through full tail resorption (i.e., metamorphosis), given that abnormalities are more easily observed at these stages (rather than during early larval development) and that abnormal animals often die before reaching sexual maturity (Lunde & Johnson, 2012). Although numerous types of amphibian abnormalities were recorded across sites (Reeves et al., 2013; USFWS, 1999), we focus here on skeletal malformations involving the limbs, including: extra limbs (polymelia) and/or digits (polydactyly); missing limbs (amelia), limb segments (ectromelia), or digits (ectrodactyly); and other miscellaneous types of skeletal malformations (e.g., abnormal skin webbing). Abnormalities that did not affect the skeleton, such as open wounds, scarring, or eye abnormalities, were excluded. Abnormality data used in this paper have been posted to Data Dryad (<https://doi.org/10.5061/dryad.dc25r>).

To evaluate patterns of parasite infection, we selected a subset of normal and malformed (when present) individuals from collections in each region. Collections were chosen for parasite analysis based on (i) the presence of malformed frogs in prior collections and (ii) attempts by regional biologists to obtain broad spatial coverage within USFWS regions. Both normal and abnormal animals were necropsied to determine the abundance of *R. ondatrae* cysts or metacercariae, which are typically found within or just beneath the skin around the developing hindlimbs or, less often, around the mandible (Johnson et al., 2004). *Ribeiroia ondatrae* has a complex life cycle involving sequential transmission among ram's horn snails, larval amphibians, and birds or mammals (Johnson, Lunde, Ritchie, & Lauer, 1999; Johnson et al., 2002; Lunde & Johnson, 2012; Roberts & Dickinson, 2012). Parasite encystment within the developing tadpoles can disrupt normal limb growth, which is ultimately hypothesized to increase parasite transmission by amplifying predation by avian hosts (Johnson et al., 2004). Whenever possible, we examined between 10 and 20 freshly caught animals to facilitate accurate identification of living parasites and minimize errors in parasite detection and abundance (Lunde & Johnson, 2012). Investigators used a stereo-dissecting microscope to examine the major organ systems, digestive tract, and skin (external and internal) of each frog to quantify all visible parasites. Once excysted, metacercariae of *R. ondatrae* were identified based on the presence of esophageal diverticula and other diagnostic morphological features (Lunde & Johnson, 2012). Because infection can vary strongly among years and species,

we calculated *R. ondatrae* abundance—or the average number of metacercariae per frog in a collection (Bush, Lafferty, Lotz, & Shostak, 1997)—only within the same year and species of the corresponding collection event. We used abundance rather than presence/absence because previous studies have shown that *R. ondatrae* infection load correlates positively with malformation frequency (Johnson & Hartson, 2009; Johnson et al., 2002, 2004). Only sites with at least five dissected individuals of the same host species that could also be linked to full malformation assessments from the same year were included. One hundred and fifty-four of the total 934 collections (16%) fulfilled these criteria (corresponding to 102 wetlands on 63 Refuges in 31 states; see Appendix S5 for a map displaying the geographic location of these sites).

2.3 | Variable construction

An important analytical challenge in conducting analyses at a continental extent is obtaining standardized environmental variables measured at a scale compatible with that of the response yet still spanning the relevant geographic extent (Levin, 1992). To quantify the influence of anthropogenic forms of land use adjacent to wetlands where amphibians were surveyed, we estimated the proportion of land cover types within HUC-12 watersheds (USGS, 2017) by averaging values from the 2001 and 2006 National Land Cover Data products (at a 30 × 30 m resolution; Homer, Huang, Yang, Wylie, & Coan, 2004), which broadly captured our sampling period. We consolidated the low intensity, medium intensity, and high intensity developed land cover categories into one class of developed land. We grouped woody wetlands, emergent herbaceous wetlands, and open water into a single category to encompass the amount of nearby wetland area, which was included because of its expected influence on amphibian occupancy and abundance (Gould et al., 2012). Cultivated crops and pasture/hay were combined to represent agricultural activity as a broad category frequently invoked as a potential driver of amphibian malformations (Taylor et al., 2005). Consolidation of land cover types was performed to improve classification accuracy and simplify the environment for our evaluation. We selected the HUC-12 watershed as preferable to arbitrary distance classes that may have omitted information about the directional flow of water.

To obtain information on chemical contaminants, we extracted publicly available county-level data on pesticide and fertilizer use for all counties in the conterminous USA (field-based water samples were not collected as part of this study). Estimates of annual, county-level pesticide use—for selected herbicides, insecticides, and fungicides applied to agricultural crops grown in the USA from 1992 to 2009—were obtained from the USGS National Water-Quality Assessment Program (Thelin & Stone, 2013). We narrowed the list of chemicals for inclusion in our pesticide exposure variable to nine compounds that have been shown to pose risks to amphibians: atrazine, carbaryl, chlorpyrifos, chlorothalonil, copper, diazinon, endosulfan, glyphosate, and malathion (see Appendix S2 for details on the pesticide selection process). We excluded data prior to 1998 to match the temporal extent of amphibian surveys (1999–2012),

retaining the previous year to capture possible lag effects of pesticides remaining in the environment. For each unique compound-year combination (which is how the raw data were obtained), we weighted pesticide application (kg) by the geographic area (km²) of each county. We then applied a natural log ($x + 1$) transformation to help normalize the distribution prior to scaling. Because pesticides vary in their toxicity per unit volume, we scaled pesticide application by dividing these values by the standard deviation of each respective compound-year combination from all counties, and then summed these scaled values among all years and compounds for each county. This effectively identified counties with relatively high (or low) application values for the selected pesticides, concurrently accounting for variation in toxicity per unit volume. To ensure these results were insensitive to the pesticides and scaling approach, we verified that similar effects were generated with the unscaled values (i.e., non-transformed but weighted by county area) of all 433 insecticides, herbicides, and fungicides (applied during 1998–2012), and that effects persisted even with removal of the well-studied herbicide atrazine from the nine focal compounds (see Appendix S2). We also obtained county-level estimates of nitrogen and phosphorus fertilizer applied commercially (farm and nonfarm uses) in the conterminous USA from 1987 to 2006 (Gronberg & Spahr, 2012). We calculated fertilizer exposure for each county by summing the cumulative amount of fertilizer applied (kg) from 1998 to 2006 and weighted by county area. Previous research has highlighted the potential for nutrient inputs to have both direct effects on amphibians (Marco & Blaustein, 1999) as well as indirect effects mediated through trematode infection (Johnson et al., 2007; Rohr, Schotthoefer, et al., 2008).

Finally, we assessed oil and gas development in the vicinity of wetlands as a possible source of contaminants. Ramirez and Mosley (2015) documented chronic pollution at oil and gas production sites on National Wildlife Refuges across the USA, ranging from localized oil or brine spills to flowline leaks releasing large volumes of pollutants. Moreover, region-specific field surveys have suggested links between abnormal vertebrate development and the presence of petroleum and heavy metal-based contamination, which is also supported by toxicological research (Bacon, Fort, Todhunter, Mathis, & Fort, 2013; Hayden et al., 2015; Mahaney, 1994; Reeves et al., 2010, 2011). To generate our oil/gas well variable, we collected publicly available data on the geographic location, type, and status of oil and gas wells for all states that had petroleum production and included our amphibian survey sites ($n = 21$ states). These data were amassed on a state-by-state basis and then trimmed by removing wells that were initially drilled after 2009 and those classified as “dry” wells (i.e., wells that never produced oil or gas; see Appendix S3 for additional data cleaning steps of this variable). We used the trimmed data to construct estimates of: (i) well presence/absence within each HUC-12 watershed, and (ii) well density (number of wells divided by the watershed area). We used well presence rather than density in our statistical models because the distribution of the latter variable was highly bimodal: at the national extent, about half of the watersheds with study sites had no wells.

2.4 | Analytical procedures

To evaluate potential drivers of skeletal malformation prevalence, we fit generalized linear mixed models (GLMMs) using the “lme4” library, v 1.1-7 (Bates, Maechler, Bolker, & Walker, 2015) in R v. 3.2.0 (R Development Core Team, 2010). GLMMs explicitly allow for non-normal error distributions (such as binomial for malformations in this case) and inclusion of “grouping variables” as random terms, alongside advantages in accommodating unbalanced designs, correcting for sources of autocorrelation (including spatial autocorrelation), and using “shrinkage” to improve parameter estimation, which is especially important for subgroups with limited sampling intensity (Gelman & Hill, 2007; Zuur, Ieno, Walker, Saveliev, & Smith, 2009). The sampling unit in all analyses was the individual collection event (collection of the same species at the same site on the same date) and the response variable was the number of malformed amphibians relative to the number of normal frogs, which was modeled as a binomial error distribution with a log-link function. Using the “cbind” function in R to combine the number of abnormal and normal frogs in the response metric, our models weighted collection events by the total sample size, helping to avoid the use of arbitrary sample size thresholds and the discounting of potentially meaningful data. We standardized numerical covariates by subtracting their mean and dividing by their standard deviation, thereby centering each at zero and placing values on a standard deviation scale to facilitate comparisons of regression coefficients. We also applied a $\log_{10}(x + 1)$ transformation to parasite abundance and pesticide exposure.

Prior to model construction, we used pairwise correlation coefficients to identify collinearity among candidate predictor variables and examined the variance inflation factors of multivariate models to further ensure model validity. We omitted fertilizer exposure because it was highly correlated with pesticide exposure (Pearson $r > .74$) and led to high variance inflation when both terms were included together (VIF = 3.8). For some region-specific models, we also had to exclude agricultural land use or developed area owing to high correlations with pesticide use (Table 1; see Appendix S4 for more detail). To accommodate nonindependence among collections in space and time, we included random intercept terms for wetland identity, sampling year, HUC-12 watershed, and the USFWS administrative region (see Appendix S4 for further justification). We did not include a random effect for amphibian species identity both because of the large geographic extent of the study (such that most species did not extend beyond specific regions) and because exploratory analyses suggested that neither a species- nor family-level random effect accounted for significant variation in the response, consistent with the findings of Reeves et al. (2013). We tested for overdispersion in all models (using the “overdisp_fun” function in R) by calculating the sum of squared Pearson residuals and comparing it to the residual degrees of freedom (Venables & Ripley, 2002). To address overdispersion detected in some models (see Appendix S4), we included an observation-level (collection-level) random effect (OLRE). This method involves giving each observation a unique level of an added random intercept term, thereby helping absorb the

TABLE 1 Model-averaged regression coefficients for analysis of amphibian skeletal malformation prevalence at national and regional spatial extents (only coefficients with $p \leq .10$ are shown; otherwise “—”). Coefficients with 95% confidence intervals (shown in parentheses) that do not include zero are denoted by bold format. The number of collection events included in each model is shown in parentheses. For continuous predictors, variables were scaled such that coefficients range between 0 and 1; for the factor predictor (oil/gas well presence), the variable was not scaled (such that coefficients cannot be directly compared between continuous and categorical variables). Significance codes are as follows: “***” $\leq .001$; “**” $\leq .01$; “*” $\leq .05$; “.” $\leq .10$. The models using all data ($n = 934$ collections) do not include the parasite abundance covariate nor the parasite-by-pesticide interaction term

	Parasite abundance	Parasites × pesticides	O/G well presence	Pesticide exposure	Agriculture	Developed	Wetland
National							
Parasite data ($n = 154$)	0.27** (0.08–0.46)	0.20* (0.03–0.37)	—	0.22 ^c (–0.02 to 0.47)	—	—	—
All data ($n = 934$)	NA	NA	0.37* (0.05–0.68)	—	—	—	—
Regional (parasite data)							
Pacific ($n = 26$)	0.46*** (0.27–0.64)	—	1.22* (0.04–2.41)	—	—	—	—
Southwest ($n = 2$) ^a	—	—	—	—	—	—	—
Midwest ($n = 30$)	—	—	—	—	—	—	—
Southeast ($n = 13$) ^b	NA ^c	NA ^c	1.78*** (0.82–2.74)	0.49* (0.04–0.94)	NA ^d	—	—
Northeast ($n = 61$)	0.47*** (0.22–0.71)	—	–1.48*** (–2.36 to –0.60)	0.51* (0.09–0.92)	NA ^d	NA ^d	—
Mountain Prairie ($n = 22$)	—	—	—	—	NA ^d	NA ^d	—
Regional (all data)							
Pacific ($n = 99$)	NA	NA	0.83* (0.12–1.54)	—	—	—	—
Southwest ($n = 117$)	NA	NA	—	—	—	—	—
Midwest ($n = 176$)	NA	NA	—	—	—	—	NA ^d
Southwest ($n = 233$)	NA	NA	—	—	NA ^d	—	–0.49*** (–0.78 to –0.23)
Northeast ($n = 189$)	NA	NA	—	0.30^c (–0.02 to 0.61)	NA ^d	—	—
Mountain Prairie ($n = 120$)	NA	NA	—	—	NA ^d	—	—

^aThe Southwest regional model was omitted because too few sites included data on parasite infection in this region.

^bWe used a generalized linear model rather than a generalized linear mixed model because the random intercept terms had variance values approaching zero, coupled with the small sample size in this region.

^cParasite infection was not detected in the Southeast region.

^dVariables excluded due to multicollinearity (see Appendix S4).

“extra” binomial variation, providing more robust estimates of parameters, and more accurately partitioning explained and unexplained variation in the data (Elston, Moss, Boulinier, Arrowsmith, & Lambin, 2001; Harrison, 2014, 2015); in other models, overdispersion was negligible and no OLRE was included.

Building upon our hypotheses, we constructed the following set of candidate models: (i) a null model containing the intercept and random effects only; (ii) six univariate models for each fixed effect (pesticide exposure, oil/gas well presence, parasite abundance, and proportion of agricultural, developed, and wetland land use within each watershed); (3) three multivariate models corresponding to either “chemical contaminants” (pesticide exposure + well presence), “land use” (agriculture + developed + wetland), or “parasite-by-pesticide interactions” (parasite abundance-by-pesticide exposure); and (4) a global model containing all six fixed effects and a parasite-by-pesticide interaction term. Because parasite infection was only assessed in a subset of 154 collections from 102 wetlands, which were chosen in part based on detection of malformations (protocols available: <https://doi.org/datadryad.org/resource/10.5061/dryad.dc25r>), we performed this analysis both on two datasets: one with the parasite covariate ($n = 154$ collections from 102 sites) and one without (934 collections from 422 sites). Thus, the dataset with parasite infection was a nonrandom subset of all collections that allowed us to examine the individual and combined influence of *R. ondatrae*, chemical contaminants, and land use variables. To examine spatial nonstationarity in environment-response associations, we also examined the influence of these variables at a regional spatial extent using the USFWS administrative regions (Figure 1); once again, these models were run both with and without the parasite infection covariate to allow maximal use of the data. We omitted the parasite-by-pesticide interaction term from the regional models owing to the reduction in sample size and a desire to avoid over-fitting. Because some of the predictors were collinear within regions, the specific covariates included varied slightly among regions (Table 1; Appendix S4). Residuals from the best-supported models for each analysis (national and region-specific extents, both with and without parasite infection) were examined for evidence of overdispersion, spatial autocorrelation, and other diagnostics to help assess model validity (see Appendix S4).

We used an information-theoretic approach coupled with model averaging to evaluate the importance of land use, chemical contaminants, parasites, and targeted interactions in explaining the distribution of amphibian skeletal malformations. Candidate models were ranked by Akaike's Information Criterion corrected for small sample size (AIC_C), and model-averaged parameter estimates, standard errors, and 95% confidence intervals were obtained based on weighted support from candidate models (Burnham & Anderson, 2002). This approach has the advantage of providing a formal “relative strength of evidence” for each of the alternative hypotheses (Burnham, Anderson, & Huyvaert, 2011). Model-averaging was performed by calculating a weighted-average of parameter estimates across all models in the full set of candidate models considered. Thus, parameter estimates of more likely models count more toward parameter estimates of the averaged model. We used the “natural-average method” for parameter estimation (Burnham & Anderson,

2002), whereby models that did not include a given parameter were excluded from the averaging of that particular estimate. Analyses were performed using the MuMIn (Multi-Model Inference) R-package (Barton, 2015).

3 | RESULTS

3.1 | Sampling overview

Over 13 years we obtained 934 collections from 422 wetlands and 127 National Wildlife Refuges to examine skeletal malformations. In total, 53,880 amphibians representing 41 species were surveyed. When examining average malformation prevalence across space, we first examined collections with at least 30 individuals ($n = 620$ collections) to increase confidence in our estimates (Figure 1). Whereas abnormalities were rare in most of these collections, the percentage of malformed amphibians ranged from 0% to >25%, with approximately 47% ($n = 292$) of those collections exhibiting $\geq 2\%$ malformed amphibians and 22% ($n = 138$) at or exceeding the oft-cited baseline frequency of 5% (Lunde & Johnson, 2012). When using the binomial mixed-effects model to account for variation in sample size, study location, and sampling year (allowing us to use all 934 collections), we estimated a model-adjusted mean malformation frequency of 1.6% [95% CI: 1.0%–2.3%]. Among regions, the Midwest (model-adjusted mean = 2.5% [1.8%–3.4%], $n = 176$ collections), Northeast (model-adjusted mean = 2.1% [1.3%–2.9%], $n = 189$ collections), and Pacific (model-adjusted mean = 2.0% [1.1%–3.6%], $n = 99$ collections) regions exhibited the highest frequencies of skeletal malformations, followed by the Southeast (model-adjusted mean = 1.8% [1.2%–2.5%], $n = 233$ collections), Mountain Prairie (model-adjusted mean = 0.9% [0.5%–1.4%], $n = 120$ collections), and Southwest regions (model-adjusted mean = 0.6% [0.3%–1.0%], $n = 117$ collections). *Ribeiroia ondatrae* infection was detected within 39 of 102 sampled wetlands (38%) with corresponding malformation surveys; average infection load per collection ranged from 0.1 to 55.8 metacercariae per frog (10.3 ± 1.7 SEM). Infection was highest in the Northeast (average load among collections with at least one infected frog = 13.1 metacercariae; $n = 37$ collections), followed by the Pacific (7.1 metacercariae; $n = 9$ collections), Midwest (6.9 metacercariae; $n = 15$), and Mountain Prairie regions (2.1 metacercariae; $n = 3$); there were no parasite detections in the Southwest and Southeast regions. Across all regions, the highest parasite loads were observed at Great Swamp NWR (55.8 metacercariae per frog, 11.9% abnormal of 59 sampled) and Erie NWR (47.8 metacercariae per frog, 5.5%, $n = 55$) in the Northeast and Ellicott Slough NWR in the Pacific (47.3 metacercariae per frog, 25.2% abnormal, $n = 107$). Maps illustrating the spatial variability in each predictor variable across the USA can be found in Appendix S5.

3.2 | National scale analysis

At the national extent using the subset of collections in which parasites were examined, the best-performing model included a main

effect of *R. ondatrae* abundance as well as a parasite-by-pesticide interaction, such that malformations increased more steeply at sites with high levels of both parasites and pesticides (Tables 1, 2, Figure 2). The model-averaged, standardized coefficient estimates for parasite abundance and the parasite-by-pesticide interaction were $\hat{\beta} = 0.27$ (95% CI: 0.08–0.46) and $\hat{\beta} = 0.20$ (95% CI: 0.03–0.37). No other covariates had 95% confidence intervals that did not

include zero. Inclusion of fertilizer application—rather than pesticides—was also associated with higher malformations ($\hat{\beta} = 0.23$; $p = .04$), but no interaction with parasite infection was found ($\hat{\beta} = 0.04$; $p = .63$; see Appendix S4 for more detail). Repeating these analyses on the full dataset representing 934 collection events, the presence of oil/gas wells within the watershed was associated with higher malformations in the best-supported model (Table 2). The model-

Spatial extent	Model	k	AIC _C	ΔAIC _C	w _i
National					
Parasite data (n = 154)	Parasite-by-pesticide	3	697.28	—	0.71
All data (n = 934)	Oil/gas well presence	1	2866.7	—	0.31
	Chemical contaminants	2	2868.3	1.63	0.14
	Global	5	2868.5	1.78	0.13
	Wetland	1	2868.7	1.94	0.12
Regional—parasite data					
Pacific (n = 26)	Parasite abundance	1	137.51	—	0.98
Mountain Prairie (n = 22)	Null (intercept only)	—	94.39	—	0.56
Southwest (n = 2) ^b	—	—	—	—	—
Midwest (n = 30)	Null (intercept only)	—	142.57	—	0.30
	Oil/gas well presence	1	143.91	1.34	0.15
	Development	1	144.21	1.64	0.13
Southeast (n = 13) ^c	Chemical contaminants	2	63.21	—	0.84
Northeast (n = 61)	Global	4	268.69	—	0.74
Regional—all data					
Pacific (n = 99)	Oil/gas well presence	1	397.76	—	0.42
	Chemical contaminants	2	399.24	1.47	0.20
Mountain Prairie (n = 120)	Development	1	358.38	—	0.33
	Null (intercept only)	—	358.95	0.57	0.25
Southwest (n = 117)	Null (intercept only)	—	168.32	—	0.30
	Agriculture	1	169.76	1.43	0.15
	Development	1	169.79	1.46	0.14
	Wetland	1	170.02	1.70	0.13
	Pesticide exposure	1	170.23	1.91	0.11
Midwest (n = 176)	Null (intercept only)	—	616.97	—	0.32
	Development	1	618.21	1.24	0.17
	Pesticide exposure	1	618.72	1.74	0.13
	Agriculture	1	618.78	1.80	0.13
Southeast (n = 233)	Wetland	1	686.79	—	0.49
	Global	4	687.65	0.86	1.32
Northeast (n = 189)	Pesticide exposure	1	640.30	—	0.40
	Null (intercept only)	—	641.79	1.48	0.19
	Chemical contaminants	2	642.21	1.90	0.15

TABLE 2 Best-supported information-theoretic models (within 2 ΔAIC_C) for amphibian malformation prevalence. For each model, “k” is the number of parameters^a, AIC_C is Akaike’s information criterion corrected for small sample size, ΔAIC_C is the difference in AIC_C between the lowest ranked model and the model under examination and w_i is the Akaike weight. The models using all 934 collections do not include the parasite abundance covariate nor the parasite-by-pesticide interaction term. See Appendix S6 for all models within 4 ΔAIC_C

^aThe number of parameters within a candidate model category (e.g., global) can vary due to some variables being omitted following multicollinearity checks.

^bThe Southwest regional model was omitted because too few sites included data on parasite infection in this region.

^cWe used a generalized linear model rather than a generalized linear mixed model because the random intercept terms had variance values approaching zero, coupled with the small sample size in this region.

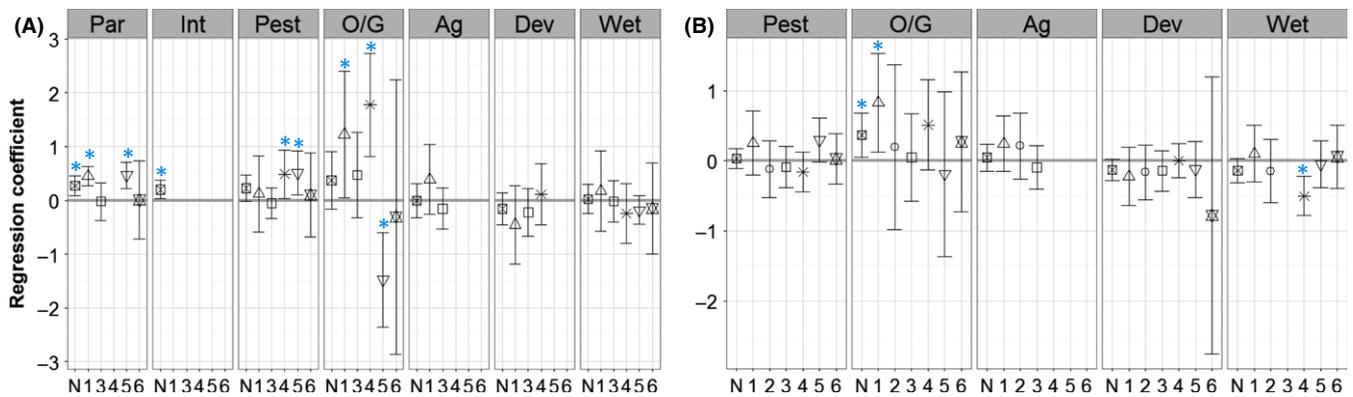


FIGURE 2 Model-averaged regression coefficients for analysis of amphibian skeletal malformation prevalence at national and regional extents, using (a) the 154 collections containing parasite infection data, and (b) all 934 collections (excluding the parasite abundance and parasite-by-pesticide interaction covariates). Point estimates for each spatial extent are shown with 95% confidence intervals; asterisks denote 95% CIs that exclude zero. Covariates include parasite abundance (“Par”), a parasite-by-pesticide interaction term (“Int”), pesticide exposure (“Pest”), oil/gas well presence (O/G), and proportion of agricultural (“Ag”), developed (“Dev”) or wetland land use/cover (“Wet”). Spatial extents include national (“N”), Pacific (“1”), Southwest (“2”), Midwest (“3”), Southeast (“4”), Northeast (“5”), and the Mountain Prairie (“6”) regions. For continuous predictors, variables were scaled such that coefficients range between 0 and 1; for the factor predictor (oil/gas well presence), the variable was not scaled (such that coefficients cannot be directly compared between continuous and categorical variables). The Southwest regional model was omitted when using the parasite dataset due to small sample size. For some regions, certain covariates were excluded owing to high correlations with other variables (see Table 1, Appendix S4)

averaged coefficient for oil/gas wells was also the only coefficient in the national dataset for which the 95% confidence interval excluded zero ($\hat{\beta} = 0.37$; 95% CI: 0.05–0.68; Tables 1, 2, Figure 2). Residuals from the best-supported models both with and without parasite infection showed no evidence of overdispersion or spatial autocorrelation (see Appendix S4).

3.3 | Regional scale analysis

By geographically decomposing the national models, we evaluated the region-specific influence of candidate variables on malformation patterns in the datasets both with and without parasite examination (Tables 1, 2, Figure 2). These analyses identified model similarities and differences as a function of scale (national vs. regional) and the specific dataset (full vs. subset with parasite examination). For the dataset with parasites examined ($n = 154$ collections), the best-supported model for the Pacific region included parasite abundance (model-averaged regression coefficient: $\hat{\beta} = 0.46$; 95% CI: 0.27–0.64), whereas the second best-performing model included oil/gas well presence ($\Delta\text{AIC}_c = 9.74$; model-averaged coefficient: $\hat{\beta} = 1.22$; 95% CI: 0.04–2.41). Similarly, in the Northeast, malformation frequency increased with parasite abundance ($\hat{\beta} = 0.47$; 95% CI: 0.22–0.71) and pesticide exposure ($\hat{\beta} = 0.51$; 95% CI: 0.09–0.92), but decreased with well presence ($\hat{\beta} = -1.48$; 95% CI: -2.36 to -0.60; although well presence and pesticide application were marginally collinear [$r = .6$], see Appendix S4). In the best-supported models for the Southeast region, where *R. ondatrae* infection was not detected among any of the samples processed for parasites ($n = 13$), malformations associated positively with oil/gas wells ($\hat{\beta} = 1.78$; 95% CI: 0.82–2.74) and pesticide exposure ($\hat{\beta} = 0.49$; 95% CI: 0.04 – 0.94). Models for the Mountain Prairie and Midwest regions failed to

identify influential covariates, such that all of the 95% confidence intervals around the model-averaged coefficients included zero, and too few sites ($n = 2$) in the Southwest included data on parasite examination (see below for the analysis without parasite infection).

When analyzing region-specific models without the parasite covariate (the larger dataset with 934 collections), we found that malformation frequency was higher within watersheds that contained one or more oil/gas wells in the Pacific region ($\hat{\beta} = 0.83$; 95% CI: 0.12–1.54), in broad parallel to findings using the parasite data subset for this region as well as the national-scale model with all collections. In the Northeast, malformations were marginally higher in counties with more pesticide application ($\hat{\beta} = 0.30$; 95% CI: -0.02 to 0.61), consistent with the analysis using the parasite dataset. In the Southeast region, only the amount of wetland area was associated with malformation frequency ($\hat{\beta} = -0.49$; 95% CI: -0.78 to -0.23), without the observed links to pesticides and oil/gas wells detected among the subset of sites examined for parasites in this region. No other regional models conducted with this dataset—including those for the Midwest, Mountain Prairie, and Southwest—contained model-averaged regression coefficients excluding zero from the 95% confidence intervals.

4 | DISCUSSION

The widespread nature and suspected increase in amphibian malformations have prompted interest from scientists and alarm from the general public (Burkhart et al., 2000; Kaiser, 1997; Rohr, Schotthoefer, et al., 2008; Souder, 2000). Despite numerous studies examining the potential causes of malformed amphibians (see reviews by Ouellet, 2000; Ankley et al., 2004; Johnson, Reeves, et al., 2010; Lunde

& Johnson, 2012), the factors responsible for recent observations of abnormalities in North American amphibians remain controversial (Johnson & Bowerman, 2010; Sessions & Ballengée, 2010; Skelly & Benard, 2010). Given the large spatial extent over which abnormalities have been reported, it is highly likely that multiple factors are involved—either individually or through interactions—and that their effects vary with geographic location. As such, one of the greatest challenges to this investigation is the difficulty of disentangling the relative contributions of multiple factors, particularly those that co-occur on the landscape or vary spatially and temporally in their influence on amphibian development (Johnson, Reeves, et al., 2010; Reeves et al., 2013).

Here, we coupled results of a continental-extent, systematic survey of skeletal malformations in 41 species of amphibians across the USA with a hierarchically-nested assessment of hypothesized drivers, including parasite infection, pesticide use, oil and gas contaminants, land use, and targeted interactions between parasites and pesticides. This entailed a uniquely extensive compilation of geospatial data on watershed-level exposure to chemical threats as well as empirical collection of covariates such as *R. ondatrae* infection abundance. While malformations were rare in most amphibian populations (1.6% model-adjusted average prevalence), we identified 96 “hotspot” locations with skeletal malformations affecting 5%–25% of examined individuals (when ≥ 30 individuals examined; see also Reeves et al., 2013). Consistent with our hypotheses and prior surveys conducted at local extents, results of the national-extent analyses identified roles of both parasite infection and chemical contaminants in predicting geographic variation in malformation risk.

While contaminants have frequently been hypothesized to contribute directly to amphibian limb abnormalities, consistent with an extensive history of ecotoxicological research in the laboratory, few studies have thus far identified a link between measured or applied contaminants in the field and observed patterns of abnormalities in natural systems, particularly at large spatial extents (Bacon et al., 2013; Ouellet, Bonin, Rodrigue, DesGranges, & Lair, 1997; Reeves et al., 2010; Rohr, Schotthoefer, et al., 2008; Taylor et al., 2005). In examining nine pesticides known to be harmful to amphibians across the conterminous USA, we found evidence for a main effect of pesticide application in predicting patterns of amphibian abnormalities in a wetland, which was significant in the Northeast and Southeast regions and marginally significant at the continental scale. Moreover, by carefully assembling a national-extent database on the distribution of oil and gas wells within the USA, we also detected a positive association between oil/gas activity and the prevalence of malformations at both the national extent and in the Pacific and Southeast regions specifically (although this association was reversed in the Northeast). Whether this pattern is causal or reflects links with unmeasured factors is unclear, but the connection between petroleum byproducts and amphibian development warrants further investigation. In a survey of National Wildlife Refuges, Ramirez and Mosley (2015) reported chronic oilfield and brine leaks, which may result in complex mixtures of petroleum hydrocarbons leaching into the environment (see also Vidic, Brantley, Vandenbossche,

Yoxtheimer, & Abad, 2013). Ongoing research at a series of wetlands in Bermuda further suggested a link between petrochemical and metal contamination in pond sediment and elevated frequencies of morphological abnormalities in toad and killifish populations (Bacon et al., 2013).

Among the subset of collections in which we also examined amphibians for trematode infection, average infection load by *R. ondatrae* predicted the frequency of malformations at the national extent and within the Pacific and Northeast regions specifically. These effects were intensity-dependent, such that higher levels of average infection were associated with a greater risk of malformation, which has also been reported experimentally and in studies from the western USA (Johnson et al., 2012, 2013). *Ribeiroia ondatrae* infection has been linked to severe limb malformations in amphibians in previous experimental research and regional field surveys (Johnson et al., 1999, 2002, 2013; Lunde et al., 2012; Roberts & Dickinson, 2012; Stopper, Hecker, Franssen, & Sessions, 2002). Parasite-induced limb malformations are hypothesized to adaptively enhance transmission of *R. ondatrae* between its intermediate (amphibian) and definitive (bird) hosts, which depends on predation (Johnson et al., 2002, 2004), although considerable debate has focused on the potential for factors such as nutrient runoff or pesticides to amplify infection or its effects on frogs (Johnson & Chase, 2004; Rohr, Schotthoefer, et al., 2008).

Importantly, parasite load also interacted synergistically with the national-extent patterns of pesticide application to predict skeletal malformations in amphibians. While many studies have focused on specific factors, emerging evidence has highlighted the potential for additive or synergistic interactions among putative causes of abnormalities (Hayden et al., 2015; Johnson et al., 2013; Reeves et al., 2010, 2011; Rohr, Schotthoefer, et al., 2008). Several studies have identified such effects between parasites and pollutants, whereby increased runoff of biocides or nutrients amplifies parasite infection through complex ecological mechanisms (Jayawardena et al., 2016; Johnson et al., 2007). Rohr, Raffel et al. (2008) and Rohr, Schotthoefer et al. (2008) found that the commonly applied herbicide atrazine and the fertilizer phosphate jointly predicted trematode abundance in amphibians from 18 agricultural wetlands in Minnesota. These synergistic effects were hypothesized to stem from pesticide-mediated decreases in host immunocompetency and nutrient-mediated amplification of trematode-infected snails, each of which were supported by corresponding experimental studies (Johnson et al., 2007; Rohr, Schotthoefer, et al., 2008). Similarly, Reeves et al. (2010) reported an association between toxicants and abnormal amphibians in Alaska, from which they suggested that contaminants (metals and pesticides) may inhibit larval amphibians' capacity to avoid attacks by dragonfly larvae that cause limb abnormalities (see also Ballengée & Sessions, 2009; Bowerman et al., 2010). Additional studies have shown how the threat of predation (visual and chemical cues) can indirectly increase trematode infections in amphibians through reductions in escape behaviors or stress-induced immune suppression (Marino & Werner, 2013; Thiemann & Wassersug, 2000). However, macroinvertebrate predators may also decrease

trematode infections by consuming infected snails or the free-swimming parasite stages (cercariae) infectious to amphibians (Johnson, Dobson, et al., 2010; Rohr et al., 2015), highlighting the potential complexity of ecological interactions in driving abnormal development in amphibians.

The large spatial extent of the current survey, which included 422 wetlands from 42 states, emphasized regional variation in the identity and relative influence of putative drivers of amphibian malformations. For instance, whereas infection by *R. ondatrae* has been linked to malformation hotspots in Canada, the western, midwestern, and northeastern United States (Johnson & Hartson, 2009; Johnson et al., 2002, 2013; Lannoo et al., 2003; Lunde et al., 2012; Roberts & Dickinson, 2012), it is absent from other sites and regions with a history of abnormalities, including Alaska, Vermont, and Bermuda (Bacon et al., 2013; Reeves et al., 2013; Skelly et al., 2007). For example, Reeves et al. (2008) examined 576 abnormal wood frogs in Alaska for *R. ondatrae* and found that none were infected. Such spatial nonstationarity is consistent with the findings of our region-specific analyses: while *R. ondatrae* infection was among the most significant correlates of malformations in the western and northeastern US, it was relatively unimportant in models for the Mountain Prairie region and Midwest, despite being widespread in the Midwest. *Ribeiroia ondatrae* was also not detected in any sites in the southwestern or southeastern USA, although the lower sample sizes in these regions leave open the question of whether it is absent or under-sampled (see also Johnson & McKenzie, 2009). Similar, region-specific effects associated with both pesticide application (in the Southeast and Northeast) and oil/gas well activity (Pacific, Southeast and Northeast regions) further help to characterize the malformation issue as a series of discrete phenomena rather than a single problem with a single solution. Whether the negative effect of oil/gas wells on malformations in the Northeast represents another example of spatial nonstationarity or instead stemmed from only two of the 10 Refuges having wells within their watershed will require further investigation.

Alongside the effects of spatial extent (national vs. regional), our results also identified variation in supported covariates as a function of the dataset used: the full dataset involving all 934 collections or the subset of 154 that included examination for parasite infection. Specifically, the “reduced dataset” in which we tested for parasite infection was also associated with stronger covariate effects from pesticide application and oil/gas well presence. These differences likely stem from at least two factors. First, by accounting for the influence of *R. ondatrae* infection on observed abnormalities, models involving the reduced dataset were able to partition out the added links with contaminants. Second, and perhaps most importantly, the subset of collections with parasite examination were not randomly selected; often they were from wetlands or populations at which abnormalities were detected, such that the mean malformation frequency was ~1.5 times greater (4.8% abnormal among parasite-examined collections vs. 3.1% in the full dataset, when restricting to collections with ≥ 30 frogs). Thus, while the full dataset provided broader but coarser information into factors associated with

abnormal amphibians, the parasite subset allowed us to specifically examine the influence of additional, site-specific hypotheses (e.g., *R. ondatrae* infection and its interaction with pesticides) among wetlands with nonzero malformation prevalence. An inherent challenge to this approach is that many of the potential causes of abnormal development in amphibians are themselves correlated in space and time. While we used multiple approaches to address collinearity, this nonetheless required that some covariates be removed from specific analyses (e.g., fertilizers or agricultural land use). The overall tendency in some regions for malformations to occur predominantly at wetlands with concurrent chemical and biological threats limited the capacity of models to rigorously discriminate among hypotheses, and especially their potential interactions (only a subset of which were tested here). For instance, some sites with high abnormality frequencies in the Southeast and Pacific regions had relatively high levels of agrochemicals (e.g., pesticide and fertilizer use) alongside oil/gas development in the watershed.

In light of the necessarily correlational relationships presented for analyses at such large geographic extents, we caution against strong causal inference and emphasize the potential for alternative explanations. Given the number of sampled wetlands it was not possible to quantify specific contaminants in the water, sediment, or frogs, such that our predictor variables represent proxies for contaminant exposure. Many studies have reported substantial variation in pesticide concentrations among aquatic sites, even at small scales, which is highly dependent upon watershed connectivity, drainage from agricultural lands, the types of crops cultivated, rainfall events, and the timing of amphibian activity (Smalling et al., 2015). Moreover, particular types of landscapes (e.g., agricultural areas) or county-level application of pesticides could correlate with other, unmeasured factors that influence observed abnormalities. For instance, we excluded fertilizer application from the analysis owing to its high collinearity with pesticide exposure and agricultural land use, leaving open the possibility that inferred linkages between abnormal frogs and pesticides were mediated through nutrient-rather than pesticide-based effects on parasites or tadpoles (Marco & Blaustein, 1999). And while we specifically aimed to incorporate variables previously shown or suspected to be linked to abnormalities in wildlife, some candidate factors do not have readily available data. For instance, detailed geographic information on the distribution and abundance of aquatic predators with the potential to cause amphibian limb abnormalities, including larval dragonflies, small fishes, and leeches (Ballengée & Sessions, 2009; Bowerman et al., 2010), are lacking for the continental USA generally and these wetlands specifically. Future efforts should also aim to make a more detailed examination as to how malformation types and frequencies vary with amphibian species or phylogenetic relationships, which are likely to influence both exposure and susceptibility to potential teratogens. Finally, despite the large scope of the geographic sampling program, all sites were from National Wildlife Refuges, Wetland Management Districts (WMD) or Waterfowl Production Areas (WPA), which may not be representative of other types of lands or properties.

Severe skeletal malformations, as well as their underlying drivers, have the potential to contribute to population declines of amphibians (Adams et al., 2013; Goodman & Johnson, 2011; Johnson et al., 2013), which have become the most imperiled class of vertebrates on earth (Hoffmann et al., 2010; Stuart et al., 2004; Wake & Vredenburg, 2008). Given that wildlife habitats are often simultaneously subjected to multiple threats, understanding the relative effects and interactions of co-occurring factors is essential for developing conservation and management plans to mitigate such declines and abnormalities. Amphibian limb development is an extremely plastic process subject to disruption by a wide variety of natural and anthropogenic agents (Ouellet, 2000). To our knowledge, this represents the first systematic, continental-extent analysis to evaluate the relative influence of multiple factors in driving amphibian abnormality patterns in nature. Our findings, while correlative, implicated additive and synergistic effects of trematode parasite infection and chemical contaminants, including both application of agrochemicals and presence of oil/gas wells in the watershed. We hope these findings catalyze additional investigations into the regional factors associated with above-baseline frequencies of malformations that ultimately identify how and why causative factors vary through space and time.

ACKNOWLEDGEMENTS

We thank the over 100 USFWS biologists, refuge managers, and volunteers who conducted field work, logistics, and quality control, especially J. Bettaso, B. Cain, K. Dickerson, L. Eaton-Poole, R. Hinzman, L. Irwin, D. Kodama, C. Kravitz, S. Krest, L. Lyon, R. McWilliams Munson, and K. Trust. Database creation and management was conducted by K. Nguyen. Additional diagnostic analyses were performed by D. Green, the late D. Sutherland, M. Lannoo, K. Medley, and L. Guderyahn. We appreciate the contribution of K. Smalling in the analysis of the pesticide database. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service. This work was supported, in part, by funding from the USFWS (including a postdoctoral contract to S. Haas and P. Johnson), the National Science Foundation (DEB-0841758, DEB-1149308), the National Institutes of Health (R01GM109499), and the David and Lucile Packard Foundation.

ORCID

Sarah E. Haas  <http://orcid.org/0000-0001-8728-9684>

REFERENCES

- Adams, M. J., Miller, D. A., Muths, E., Corn, P. S., Campbell, E. H., Bailey, L. L., ... Walls, S. C. (2013). Trends in amphibian occupancy in the United States. *PLoS ONE*, 8. <https://doi.org/10.1371/journal.pone.0064347>
- Ankley, G. T., Degitz, S. J., Diamond, S. A., & Tietge, J. E. (2004). Assessment of environmental stressors potentially responsible for malformations in North American anuran amphibians. *Ecotoxicology and Environmental Safety*, 58, 7–16.
- Bacon, J. P., Fort, C. E., Todhunter, B., Mathis, M., & Fort, D. J. (2013). Effects of multiple chemical, physical, and biological stressors on the incidence and types of abnormalities observed in Bermuda's cane toads (*Rhinella marina*). *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 320, 218–237.
- Bacon, J. P., Linzey, D., Rogers, R., & Fort, D. J. (2006). Deformities in cane toad (*Bufo marinus*) populations in Bermuda: Part I. Frequencies and distribution of abnormalities. *Applied Herpetology*, 3, 39–65.
- Ballengée, B., & Sessions, S. K. (2009). Explanation for missing limbs in deformed amphibians. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 312, 770–779.
- Barton, K. (2015). MuMIn: multi-model inference. R package version 1.15.1. Retrieved from <https://cran.r-project.org/web/packages/MuMIn/index.html>
- Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-8. Retrieved from <https://cran.r-project.org/web/packages/lme4/index.html>
- Bini, L. M., Diniz-Filho, J. A. F., Rangel, T. F. L. V. B., Akre, T. S. B., Albaladejo, R. G., Albuquerque, F. S., ... Hawkins, B. A. (2009). Coefficient shifts in geographical ecology: An empirical evaluation of spatial and non-spatial regression. *Ecography*, 32, 193–204.
- Bowerman, J., Johnson, P. T. J., & Bowerman, T. (2010). Sublethal predators and their injured prey: Linking aquatic predators and severe limb abnormalities in amphibians. *Ecology*, 91, 242–251.
- Bridges, C., Little, E., Gardiner, D., Petty, J., & Huckins, J. (2004). Assessing the toxicity and teratogenicity of pond water in North-Central Minnesota to amphibians. *Environmental Science and Pollution Research*, 11, 233–239.
- Burkhardt, J. G., Ankley, G., Bell, H., Carpenter, H., Fort, D., Gardiner, D., ... Lucier, G. (2000). Strategies for assessing the implications of malformed frogs for environmental health. *Environmental Health Perspectives*, 108, 83–90.
- Burnham, K. P., & Anderson, D. R. (2002). *Model selection and multimodel inference: a practical information-theoretic approach*. New York, NY: Springer.
- Burnham, K. P., Anderson, D. R., & Huyvaert, K. P. (2011). AIC model selection and multimodel inference in behavioral ecology: Some background, observations, and comparisons. *Behavioral Ecology and Sociobiology*, 65, 23–35.
- Bush, A. O., Lafferty, K. D., Lotz, J. M., & Shostak, A. W. (1997). Parasitology meets ecology on its own terms: Margolis et al. revisited. *The Journal of Parasitology*, 83, 575–583.
- Cohen, J. M., Civitello, D. J., Brace, A. J., Feichtinger, E. M., Ortega, C. N., Richardson, J. C., ... Rohr, J. R. (2016). Spatial scale modulates the strength of ecological processes driving disease distributions. *Proceedings of the National Academy of Sciences*, 113, E3359–E3364.
- Elston, D. A., Moss, R., Boulinier, T., Arrowsmith, C., & Lambin, X. (2001). Analysis of aggregation, a worked example: Numbers of ticks on red grouse chicks. *Parasitology*, 122, 563–569.
- Flyaks, N., & Borkin, L. (2004). Morphological abnormalities and heavy metal concentrations in anurans of contaminated areas, eastern Ukraine. *Applied Herpetology*, 1, 229–264.
- Gelman, A., & Hill, J. (2007). Understanding and summarizing the fitted models. In R. M. Alvarez, N. L. Beck, & L. L. Wu (Eds.), *Data analysis using regression and multilevel/hierarchical models* (pp. 457–486). New York, NY: Cambridge University Press.
- Goodman, B., & Johnson, P. T. J. (2011). Disease and the extended phenotype: Parasites control host performance and survival through induced changes in body plan. *PLoS ONE*, 6. <https://doi.org/10.1371/journal.pone.0020193>
- Gould, W. R., Patla, D. A., Daley, R., Corn, P. S., Hossack, B. R., Bennetts, R., & Peterson, C. R. (2012). Estimating occupancy in large

- landscapes: Evaluation of amphibian monitoring in the Greater Yellowstone Ecosystem. *Wetlands*, 32, 379–389.
- Gronberg, J., & Spahr, N. (2012). County-level estimates of nitrogen and phosphorus from commercial fertilizer for the Conterminous United States, 1987–2006: U.S. Geological Survey Scientific Investigations Report 2012-5207, 20 p.
- Gurushankara, H., Krishnamurthy, S., & Vasudev, V. (2007). Morphological abnormalities in natural populations of common frogs inhabiting agroecosystems of central Western Ghats. *Applied Herpetology*, 4, 39–45.
- Harrison, X. A. (2014). Using observation-level random effects to model overdispersion in count data in ecology and evolution. *PeerJ*, 2. <https://doi.org/10.7717/peerj.616>
- Harrison, X. A. (2015). A comparison of observation-level random effect and beta-binomial models for modelling overdispersion in binomial data in ecology and evolution. *PeerJ*, 3. <https://doi.org/10.7717/peerj.1114>
- Hayden, M., Reeves, M., Holyoak, M., Perdue, M., King, A., & Tobin, S. (2015). Thrice as easy to catch! Copper and temperature modulate predator-prey interactions in larval dragonflies and anurans. *Ecosphere*, 6. <https://doi.org/10.1890/ES14-00461.1>
- Hayes, T. B., Falso, P., Gallipeau, S., & Stice, M. (2010). The cause of global amphibian declines: A developmental endocrinologist's perspective. *Journal of Experimental Zoology*, 313, 921–933.
- Hoffmann, M., Hilton-Taylor, C., Angulo, A., Bohm, M., Brooks, T. M., Butchart, S. H. M., ... Stuart, S. N. (2010). The impact of conservation on the status of the world's vertebrates. *Science*, 330, 1503–1509.
- Homer, C., Huang, C., Yang, L., Wylie, B., & Coan, M. (2004). Development of a 2001 national land cover database for the United States. *Photogrammetric Engineering & Remote Sensing*, 12, 829–840.
- Hopkins, W., Congdon, J., & Ray, J. (2000). Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. *Environmental Toxicology and Chemistry*, 19, 862–868.
- Hoppe, D. (2005). Malformed frogs in Minnesota: History and interspecific differences. In M. J. Lannoo (Ed.), *Amphibian declines: The conservation status of United States species* (pp. 103–108). Berkeley, CA: University of California Press.
- Jayawardena, U. A., Rohr, J. R., Navaratne, A. N., Amerasinghe, P. H., & Rajakaruna, R. S. (2016). Combined effects of pesticides and trematode infections on hourglass tree frog *Polypedates cruciger*. *EcoHealth*, 13, 111–122.
- Johnson, P. T. J., & Bowerman, J. (2010). Do predators cause frog deformities? The need for an eco-epidemiological approach. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 314, 515–518.
- Johnson, P. T. J., & Chase, J. M. (2004). Parasites in the food web: Linking amphibian malformations and aquatic eutrophication. *Ecology Letters*, 7, 521–526.
- Johnson, P. T. J., Chase, J. M., Dosch, K. L., Hartson, R. B., Gross, J. A., Larson, D. J., ... Carpenter, S. R. (2007). Aquatic eutrophication promotes pathogenic infection in amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 15781–15786.
- Johnson, P. T. J., Dobson, A., Lafferty, K. D., Marcogliese, D. J., Memmot, J., Orlofske, S. A., ... Thielges, D. W. (2010). When parasites become prey: Ecological and epidemiological significance of eating parasites. *Trends in Ecology & Evolution*, 25, 362–371.
- Johnson, P. T. J., & Hartson, R. (2009). All hosts are not equal: Explaining differential patterns of malformations in an amphibian community. *Journal of Animal Ecology*, 78, 191–201.
- Johnson, P. T. J., & Lunde, K. B. (2005). Parasite infection and limb malformations: A growing problem in amphibian conservation. In M. J. Lannoo (Ed.), *Amphibian declines: The conservation status of United States species* (pp. 124–138). Berkeley, CA: University of California Press.
- Johnson, P. T. J., Lunde, K. B., Ritchie, E. G., & Launer, A. (1999). The effect of trematode infection on amphibian limb development and survivorship. *Science*, 284, 802–804.
- Johnson, P. T. J., Lunde, K. B., Thurman, E. M., Ritchie, E. G., Wray, S. N., Sutherland, D. R., ... Blaustein, A. R. (2002). Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. *Ecological Monographs*, 72, 151–168.
- Johnson, P. T. J., & McKenzie, V. J. (2009). Effects of environmental change on helminth infections in amphibians: Exploring the emergence of *Ribeiroia* and *Echinostoma* infections in North America. In B. Fried & R. Toldeo (Eds.), *Biology of echinostomes: From the molecule to the community* (pp. 249–280). New York, NY: Springer.
- Johnson, P. T. J., Preston, D. L., Hoverman, J. T., & Richgels, K. L. D. (2013). Biodiversity decreases disease through predictable changes in host community competence. *Nature*, 494, 230–234.
- Johnson, P. T. J., Reeves, M. K., Krest, S., & Pinkney, A. E. (2010). A decade of deformities: Advances in our understanding of amphibian malformations and their implications. In D. Sparling, G. Linder, C. Bishop, & S. Krest (Eds.), *Ecotoxicology of amphibians and reptiles* (pp. 511–536). Pensacola, FL: SETAC Press.
- Johnson, P. T. J., Rohr, J. R., Hoverman, J. T., Kellermanns, E., Bowerman, J., & Lunde, K. B. (2012). Living fast and dying of infection: Host life history drives interspecific variation in infection and disease risk. *Ecology Letters*, 15, 235–242.
- Johnson, P. T. J., Sutherland, D. R., Kinsella, J., & Lunde, K. B. (2004). Review of the trematode genus *Ribeiroia* (Psilostomidae): Ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. *Advances in Parasitology*, 57, 191–253.
- Kaiser, J. (1997). Deformed frogs leap into spotlight at health workshop. *Science*, 278, 2051–2052.
- Kiesecker, J. M. (2002). Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? *Proceedings of the National Academy of Sciences of the United States of America*, 99, 9900–9904.
- Koutsias, N., Martínez-Fernández, J., & Allgöwer, B. (2010). Do factors causing wildfires vary in space? Evidence from geographically weighted regression. *GIScience & Remote Sensing*, 47, 221–240.
- Lannoo, M. J. (2008). *Malformed frogs: The collapse of aquatic ecosystems*. Berkeley, CA: University of California Press.
- Lannoo, M. J., Sutherland, D. R., Jones, P., Rosenberry, D., Klaver, R. W., Hoppe, D. M., ... Kapfer, J. M. (2003). Multiple causes for the malformed frog phenomenon. In G. L. Linder, S. Krest, D. Sparling, & E. E. Little (Eds.), *Multiple stressor effects in relation to declining amphibian populations* (pp. 233–262). West Conshohocken, PA: American Society for Testing and Materials International.
- Laurentino, T. G., Pais, M. P., & Rosa, G. M. (2016). From a local observation to a European-wide phenomenon: Amphibian deformities at Serra da Estrela Natural Park, Portugal. *Basic and Applied Herpetology*, 30, 7–23.
- Levin, S. A. (1992). The problem of pattern and scale in ecology. *Ecology*, 73, 1943–1967.
- Lunde, K., & Johnson, P. (2012). A practical guide for the study of amphibian malformations and their causes. *Journal of Herpetology*, 46, 429–441.
- Lunde, K., Resh, V., & Johnson, P. T. J. (2012). Using an ecosystem-level manipulation to understand host-parasite interactions and how they vary with study venue. *Ecosphere*, 3. <https://doi.org/10.1890/ES12-00001.1>
- Mahaney, P. (1994). Effects of fresh-water petroleum contamination on amphibian hatching and metamorphosis. *Environmental Toxicology and Chemistry*, 13, 259–265.

- Marco, A., & Blaustein, A. R. (1999). The effects of nitrite on behavior and metamorphosis in cascades frogs (*Rana cascadae*). *Environmental Toxicology and Chemistry*, 18, 946–949.
- Marino, J. A., & Werner, E. E. (2013). Synergistic effects of predators and trematode parasites on larval green frog (*Rana clamitans*) survival. *Ecology*, 94, 2697–2708.
- McCallum, M. L., & Trauth, S. E. (2003). A forty-three year museum study of northern cricket frog (*Acris crepitans*) abnormalities in Arkansas: Upward trends and distributions. *Journal of Wildlife Diseases*, 39, 522–528.
- Ouellet, M. (2000). Amphibian deformities: Current state of knowledge. In D. Sparling, G. Linder, & C. Bishop (Eds.), *Ecotoxicology of amphibians and reptiles* (pp. 617–646). Pensacola, FL: SETAC Press.
- Ouellet, M., Bonin, J., Rodrigue, J., DesGranges, J., & Lair, S. (1997). Hindlimb deformities (ectomelia, entrodactyly) in free living anurans from agricultural habitats. *Journal of Wildlife Diseases*, 33, 95–104.
- Pochini, K. M., & Hoverman, J. T. (2017). Reciprocal effects of pesticides and pathogens on amphibian hosts: The importance of exposure order and timing. *Environmental Pollution*, 221, 359–366.
- R Development Core Team (2014). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <http://www.r-project.org>
- Ramirez, P. Jr, & Mosley, S. (2015). Oil and gas wells and pipelines on U.S. Wildlife Refuges: Challenges for managers. *PLoS ONE*, 10. <https://doi.org/10.1371/journal.pone.0124085>
- Reeves, M. K., Doppl, C. L., Zimmer, H., Tjeerdema, R. S., & Trust, K. A. (2008). Road proximity increases risk of skeletal abnormalities in wood frogs from national wildlife refuges in Alaska. *Environmental Health Perspectives*, 116, 1009–1014.
- Reeves, M. K., Jensen, P., Dolph, C. L., Holyoak, M., & Trust, K. A. (2010). Multiple stressors and the cause of amphibian abnormalities. *Ecological Monographs*, 80, 423–440.
- Reeves, M. K., Medley, K. A., Pinkney, A. E., Holyoak, M., Johnson, P. T. J., & Lannoo, M. J. (2013). Localized hotspots drive continental geography of abnormal amphibians on U.S. wildlife refuges. *PLoS ONE*, 8. <https://doi.org/10.1371/journal.pone.0077467>
- Reeves, M. K., Perdue, M., Blakemore, G. D., Rinella, D. J., & Holyoak, M. (2011). Twice as easy to catch? A toxicant and a predator cue cause additive reductions in larval amphibian activity. *Ecosphere*, 2. <https://doi.org/10.1890/ES11-00046.1>
- Roberts, C., & Dickinson, T. (2012). *Ribeiroia ondatrae* causes limb abnormalities in a Canadian amphibian community. *Canadian Journal of Zoology*, 90, 808–814.
- Rohr, J. R., Civitello, D. J., Crumrine, P. W., Halstead, N. T., Miller, A. D., Schotthoefer, A. M., ... Beasley, V. R. (2015). Predator diversity, intraguild predation, and indirect effects drive parasite transmission. *Proceedings of the National Academy of Sciences*, 112, 3008–3013.
- Rohr, J. R., Raffel, T. R., Sessions, S. K., & Hudson, P. J. (2008). Understanding the net effects of pesticides on amphibian trematode infections. *Ecological Applications*, 18, 1743–1753.
- Rohr, J. R., Schotthoefer, A. M., Raffel, T. R., Carrick, H. J., Halstead, N., Hoverman, J. T., ... Beasley, V. R. (2008). Agrochemicals increase trematode infections in a declining amphibian species. *Nature*, 455, 1235–1239.
- Sessions, S. K., & Ballengée, B. (2010). Explanations for deformed frogs: Plenty of research left to do (a response to Skelly and Benard). *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 314, 341–346.
- Sessions, S. K., Franssen, R. A., & Horner, V. L. (1999). Morphological clues from multilegged frogs: Are retinoids to blame? *Science*, 284, 800–802.
- Skelly, D. K., & Benard, M. F. (2010). Mystery unsolved: Missing limbs in deformed amphibians. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 314, 179–181.
- Skelly, D. K., Bolden, S. R., Freidenburg, L. K., Freidenfelds, N. A., & Levey, R. (2007). *Ribeiroia* infection is not responsible for Vermont amphibian deformities. *EcoHealth*, 4, 156–163.
- Smalling, K., Reeves, R., Muths, E., Vandever, M., Battaglin, W., Hladik, M., & Pierce, C. (2015). Pesticide concentrations in frog tissue and wetland habitats in a landscape dominated by agriculture. *Science of the Total Environment*, 502, 80–90.
- Souder, W. (2000). *A plague of frogs: Unraveling an environmental mystery*. Minneapolis, MD: University of Minnesota Press.
- Stopper, G. F., Hecker, R. A., Franssen, R. A., & Sessions, K. A. (2002). How trematodes cause limb deformities in amphibians. *Journal of Experimental Zoology*, 294, 252–263.
- Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science*, 306, 1783–1786.
- Taylor, B., Skelly, D., Demarchis, L. K., Slade, M. D., Galusha, D., & Rabinowitz, P. M. (2005). Proximity to pollution sources and risk of amphibian limb malformation. *Environmental Health Perspectives*, 113, 1497–1501.
- Thelin, G., & Stone, W. (2013). Estimation of annual agricultural pesticide use for counties of the conterminous United States, 1992–2009: U.S. Geological Survey Scientific Investigations Report 2013-5009, 54 p.
- Thiemann, G., & Wassersug, R. (2000). Patterns and consequences of behavioural responses to predators and parasites in *Rana*. *Biological Journal of the Linnean Society*, 71, 513–528.
- USFWS (1999). Standard operating procedures for abnormal amphibian monitoring. Retrieved from <https://doi.org/10.5061/dryad.dc25r>
- USGS (2017). Hydrologic unit maps. Retrieved from <https://water.usgs.gov/GIS/huc.html>
- Venables, W. N., & Ripley, B. D. (2002). *Modern applied statistics with S*. New York, NY: Springer-Verlag.
- Vidic, R., Brantley, S., Vandenbossche, J., Yoxtheimer, D., & Abad, J. (2013). Impact of shale gas development on regional water quality. *Science*, 340. <https://doi.org/10.1126/science.1235009>
- Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 11466–11473.
- Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). GLMM applied on the spatial distribution of koalas in a fragmented landscape. In M. Gail, K. Krickeberg, J. M. Samet, A. Tsiatis, & W. Wong (Eds.), *Mixed effects models and extensions in ecology with R* (pp. 469–492). New York, NY: Springer.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Haas SE, Reeves MK, Pinkney AE, Johnson PTJ. Continental-extent patterns in amphibian malformations linked to parasites, chemical contaminants, and their interactions. *Glob Change Biol*. 2018;24:e275–e288. <https://doi.org/10.1111/gcb.13908>