Effects of atrazine and climate change on amphibian larval development and growth

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Jennifer Heartsong Olker

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Dedication

This thesis is dedicated to Willow, one of the next generation of frog-catchers.

Abstract

The distribution and population persistence of many North American amphibians depends on environmental factors operating at multiple spatial scales. Anthropogenic and naturally occurring stressors, including contaminants, predators, and pond-drying, have been shown to affect amphibian growth, development, and health. The herbicide atrazine (2-chloro-4-ethylamino-6-isoproyl-amino-s-triazine) is one of the most widely used pesticides in the U.S., but in some amphibian species has been shown to reduce size and health at metamorphosis and alter gonadal function, presumably through endocrine disruption. Environmental changes predicted by climate models could exacerbate these atrazine impacts, as well as have direct effects on amphibian development and population persistence through accelerated pond-drying and habitat loss or modification. The objectives of this project were to: 1) quantify native anuran developmental responses to the combined effects of atrazine exposure and accelerated pond-drying rates; and 2) quantify potential effects of these and other environmental stressors on amphibian occurrence and health in the Prairie Pothole Region.

Amphibian growth, development, and physiological state (skeletal/eye malformations and gonadal development) were assessed in northern leopard frog (*Rana pipiens*) and wood frog (*Rana sylvatica*) in experimental exposures and field surveys in the U.S. Prairie Pothole Region across a range of environmentally relevant atrazine concentrations (0.1, 20, and 200 µg/L) and in combination with climate change and other environmental factors suspected to affect amphibian larval development. Atrazine exposure during larval development significantly decreased survival and growth, and delayed development in both *R. pipiens* and *R. sylvatica* but only at the highest

concentration (200 µg/L). Presence, abundance, and severity of testicular oocytes (TOs) did not appear to be related to atrazine exposure in the experimental or field specimens; however, TO prevalence differed greatly between species (>40% in *R. pipiens* and <5% in *R. sylvatica*). In the field surveys, amphibian growth, development, and physiological state (skeletal/eye malformations and gonadal development) responded differently to individual environmental variables as well as a composite index combining environmental variables from the wetland-, local-, and landscape scales.

This research showed that atrazine exposure during amphibian larval development had sub-lethal impacts on growth and development, which could, in turn, reduce annual recruitment and survival of juveniles (and thus negatively impact populations). No relationship was found between atrazine exposure and TO presence, prevalence, or severity, suggesting that: 1) interpretation of TO occurrence or prevalence in field-collected specimens should be critically examined; 2) caution should be exercised when considering the occurrence or prevalence of TOs in ranid species as indicators of endocrine disruption or environmental condition; 3) mechanisms influencing TO development in ranid species are complex and atrazine does not appear to have an endocrine disrupting effect on gonads of two ranid species; and 4) additional research is needed to understand the reproductive or population-level impacts of TOs and to address species differences in TO prevalence and severity. From this research, amphibian breeding (presence or success) is recommended as a better indicator of local population status (and recruitment) than species presence or calling. Skeletal malformation occurrence and prevalence may be good indicators of human influences, and along with breeding success, may be effective measures of environmental conditions.

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Executive Summary

Introduction

Amphibian populations are declining worldwide, with species becoming extinct or threatened on almost every continent in both disturbed and pristine areas (Stuart et al. 2004). In addition to the major threat of habitat loss, amphibian populations are impacted by both natural and anthropogenic factors, including introduced species, pollution, climate change, over-exploitation, and disease (Alford & Richards 1999; Collins & Storfer 2003; Halliday 2008). Chemical contamination is a major concern for amphibian populations and is exceptionally challenging to evaluate due to complex interactions and far-reaching impacts, such as environmental levels of some wide-spread chemicals such as atrazine (Collins & Storfer 2003).

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is one of the most widely used pesticides in the U.S., but in some amphibian species has been shown to reduce size and health at metamorphosis and alter gonadal function. Atrazine in Midwest wetlands shows high spatial and temporal variability; however, concentrations of 10.9-172.2 µg/L in streams (Scribner et al. 2000; Battaglin et al. 2003) and 33.8 - >200 µg/L in wetlands (Murphy et al. 2006a; Papoulias et al. 2013; Schoff et al. *In review*) have recently been recorded. While atrazine at these concentrations is not lethal to model amphibians (Giddings 2005; Solomon et al. 2008) sub-lethal effects of atrazine exposure (as low as 0.1 µg/L) have been shown in both amphibians and fish (Rohr & McCoy 2010). Effects on amphibian growth, development, and reproductive development may vary across species and by study (Solomon et al. 2008; Rohr & McCoy

2010), but reductions in size at metamorphosis (Coady et al. 2004; Rohr & Crumrine 2005; Hayes et al. 2006a), immune system functioning (Brodkin et al. 2007; Christin et al. 2013), and potential alterations of gonadal function due to endocrine disruption (Hayes et al. 2003; Murphy et al. 2006a; McDaniel et al. 2008) are commonly reported. Many of these non-lethal effects, however, are not well understood, with discrepancies across studies, species, and regions (Solomon et al. 2008; Rohr & McCoy 2010). By altering amphibian growth and development atrazine may contribute to amphibian declines through reducing survival and reproductive capabilities. Connecting the proximate causes (death and/or decreased recruitment) to the ultimate causes such as environmental pollutants has been challenging because of the cryptic nature of many amphibians, natural population fluctuations, and the multitude of interactions among these causes (Hayes et al. 2010b). In natural breeding habitat, there is a plethora of additional factors that have been shown or predicted to impact amphibian growth, development, and gonadal development, including other xenobiotics, nutrients, predators, food availability, parasites, and climate change. Therefore, effective understanding of the role of agrochemicals in amphibian population declines and ecosystem impacts responses must include studies at multiple scales, combining experimental and observational studies that incorporate stressor interactions (Boone et al. 2005; Mann et al. 2009).

Climate change is a broad factor that should be incorporated into all predictions of organismal or population-level responses to chemicals/contaminants, as changes in temperature and precipitation directly impact amphibian populations (habitat loss and degradation such as potential reduced water quality) and amphibian development

(through predicted accelerated drying of ponds and wetlands), and may also have indirect impacts through interactions with pesticides and other stressors (Zaga et al. 1998; Boone & Bridges 1999; Pounds 2001; Rohr et al. 2004). The non-lethal interactions of atrazine exposure with additional stressors, such as accelerated pond-drying due to reduced precipitation or changes in precipitation seasonality, on growth, development, and gonadal formation have not yet been investigated. This study attempts to address broadly the combined effects of climate (i.e., accelerated pond drying) and land use (i.e., corn-applied atrazine) on the health and distribution of native anurans.

Objectives

The objectives of this project were to:

1) Quantify native anuran developmental responses to the combined effects of atrazine exposure and accelerated pond-drying rates.
2) Quantify potential effects of the above and other environmental stressors on amphibian occurrence and health in the Prairie Pothole Region.

Methods and Results

This project assessed amphibian growth, development, and physiological state (skeletal/eye malformations and gonadal development) in native species in experimental exposures and field surveys across a range of environmentally relevant atrazine concentrations, and in combination with climate change and other environmental factors suspected of affecting amphibian larval development. This research integrated ecotoxicology, developmental biology, and landscape ecology to identify the driving factors and interactions that result in higher prevalence of reduced growth, altered

development, and gonadal and skeletal abnormalities in amphibians native to North America.

The specific aims were to: 1) assess the dose-response effect of atrazine and interaction with accelerated pond-drying on survival, size, development, and gonadal development in native frog species; 2) assess whether atrazine exposure responses and stressor interactions observed in experimental exposure models were associated with gonadal development of wild frogs; and 3) assess influence of multiple stressors on amphibian occurrence and health responses (e.g. malformations or gonadal anomalies as measures of physiological state) to environmental variables in natural wetland habitats.

Experimental exposures

Outdoor experimental mesocosms were used to test the dose-response to atrazine and the interaction of atrazine with accelerated pond-drying on survival, size, development, and gonadal development in two native Midwest frog species (wood frog, *Rana sylvatica* and northern leopard frog, *Rana pipiens*). Three environmentally relevant atrazine concentrations (0.1, 20, 200 µg/L) and two pond-drying rates (normal and accelerated drawdown) were used in a fully-crossed randomized block design in separate exposure experiments (from hatch to metamorphosis) for each species.

Survival, size, and somatic development. Atrazine exposure at the highest concentration (200 µg/L) during larval development significantly decreased survival and growth, and delayed development in both *R. pipiens* and *R. sylvatica,* but had no significant effects at 0.1 or 20 µg/L, compared to control (no atrazine). Survival to the end of each experiment was very high across all treatments, at 94.2% (212/225) for *R.*

pipiens, and 87.8% (253/288) for *R. sylvatica*; however, atrazine concentration had a significant negative effect on *R. sylvatica* survival with lowest survival and highest proportion of individuals dying during metamorphosis in the highest atrazine exposure (200 μg/L). This atrazine concentration also significantly altered development. In *R. sylvatica*, initiation and completion of metamorphosis was delayed and metamorphic size was reduced. In *R. pipiens*, significantly fewer tadpoles initiated and completed metamorphosis; however development time and metamorphic size did not differ in those that reached metamorphosis. Drawdown rate did not independently affect growth or development. By altering amphibian growth and development, atrazine may contribute to amphibian declines through reduced survival and reproductive capabilities.

Gonadal development. Presence of oocytes in testicular tissue (testicular oocytes, TOs) has been used as an endpoint in assays for endocrine-disrupting substances in amphibian and fish. The lack of definitive protocols and variety of histopathological techniques used to evaluate TOs make it challenging to compare studies and species. First, we evaluated sampling techniques for assessing TO presence, abundance (total and number normalized by gonad size), and severity (compared with a validation set) in wild metamorphic *Rana pipiens* (northern leopard frog). We determined that counting the total number of TOs was unnecessary because it correlated highly with more efficient measures, and was inappropriate without consideration of variable body and gonad size. Subsampling 10-25% of all sections accurately predicted the overall mean TOs per section and a sample size of 10 males per site was sufficient to determine TO presence

and relative severity, suggesting that both of these time- and cost-saving methodologies are effective for evaluating prevalence of amphibian TOs.

Next, we evaluated TO presence, abundance, and severity across a range of atrazine exposure in reared and wild metamorphic frogs from two native species, *R. pipiens* and *R. sylvatica*. In addition to the experimentally exposed tadpoles in the outdoor mesocosms (described above), wild metamorphic *R. pipiens* were collected from eleven wetlands in the Prairie Pothole Region that contained atrazine at concentrations ranging from below the nominal detection limit (0.011 µg/L) to 0.855 µg/L. Atrazine exposure during larval development did not appear to be related to the presence, abundance, or severity of testicular oocytes (TOs) in the experimental or field specimens; however, TO prevalence differed greatly between species (>40% in *R. pipiens* and <5% in *R. sylvatica*). Drawdown rate did not independently affect presence or severity of TOs. Given the species differences in TO prevalence and lack of a definitive response to atrazine, these results suggest that atrazine may not be a strong endocrine disruptor, as has been suggested in the literature (Hayes et al. 2003, 2010a).

Field surveys and collections

In a larger study assessing impacts of multiple stressors on amphibians, wild populations of *R. pipiens* were surveyed in 149 seasonal and semi-permanent wetlands across the U.S. Prairie Pothole Region (PPR) east of Montana for the presence of adults and eggs, and larval and metamorphic frogs were sampled; additional surveys were conducted for skeletal malformations and gonadal anomalies (n = 13 and 9 wetlands, respectively). Potential stressors and habitat variables (e.g., land use, water quality,

predators) were measured at the wetland, local, and landscape scales around natural breeding wetlands. Principle Components Analysis (PCA) was used to characterize the major environmental gradients across the PPR. The relationship of nine amphibian response metrics (presence, calling, breeding, and physiological state) with environmental variables from three spatial scales was evaluated using an information theoretic approach to identify influential factors and evaluate how these relationships might vary by scale. Three major gradients were observed across the study area: developed land (urban, agriculture, and road density), geographic setting (which includes human population density, stream density, elevation, and wetland surface area), and wetland density. There were considerable differences across amphibian metrics in response to the environmental gradients and individual variables at each scale (wetland, local, and landscape). Five amphibian metrics (presence, calling, breeding, skeletal malformations, severe gonadal anomalies) differed across geographic setting. Breeding was positively related to wetland density and several within-wetland variables (water depth and specific conductivity). Skeletal malformations were positively related to urban development. Gonadal anomalies were found in all wetlands but there were few significant relationships with the environmental variables measured (including atrazine). From this research, amphibian breeding (presence or success) is recommended as a better indicator of local population status (and recruitment) than species presence or calling. Skeletal malformation occurrence and prevalence may be good indicators, and along with breeding presence/absence, may be an effective measure of environmental conditions.

Discussion and Conclusions

This research showed that chronic atrazine exposure during larval development had sub-lethal impacts on growth and development, which could, in turn, impact amphibian populations through reduced annual recruitment and, potentially, survival of juveniles. Atrazine exposure affected survival, growth, and development at the highest concentration (200 µg/L), which exceeds the expected environmental concentrations under current application rates, estimated at ~100 µg/L (Rohr & McCoy 2010); however, concentrations up to 10 mg/L have been measured in runoff and surface waters near corn fields after precipitation events (Klaassen & Kadoum 1979; Hatfield et al. 1996; Edwards et al. 1997; Du Preez et al. 2005). Additionally, continued conversion of grassland to crop agriculture increases the amount of pesticides applied annually, and the current trend and predictions of increased corn plantings (Wright & Wimberly 2013) suggest that the responses found in this experiment may become more common in natural breeding wetlands.

In the methods evaluation, severity scoring proved to be an efficient and effective method to evaluate specimens. While further evidence of the impact of TOs on reproductive outcomes is needed, scoring severity and normalizing the number of TOs by gonadal size are likely more biologically relevant methods than counting the total number of TOs. Evaluating 10 males per site using the recommended specimen-level assessment methods was sufficient to detect wetlands with TOs as well as to estimate TO prevalence and severity.

The results from the exposure experiment and field collections indicate: 1) a high TO prevalence in wild and lab-raised *R. pipiens*, 2) no relationship between atrazine

concentration and gonadal endpoints, and 3) differences in TO occurrence in two ranid species reared under controlled conditions. We recommend that TO occurrence or prevalence in field-collected specimens be critically examined and that caution be exercised when considering the TO occurrence/prevalence in ranid species as indicators of endocrine disruption or environmental condition. This research suggests that mechanisms influencing TO development in ranid species are complex and that atrazine may not be a strong endocrine disruptor, as has been suggested in the literature (Hayes et al. 2003, 2010a). Additionally, these results highlight the need for additional research on the reproductive and population-level impacts of TOs.

Further analysis of the field surveys and assessment of physiological state of metamorphic *R. pipiens* found considerable differences across amphibian metrics in response to the environmental gradients and independent predictors at each scale (wetland, local, and landscape). From this research, amphibian breeding (presence or success) is recommended as a better indicator of local population status (and recruitment) than species presence or calling. Breeding was related to geographic location (or more likely in certain ecoregions) as well as overall wetlands density, and several individual variables measured within the wetland and at the local scale. This metric requires the presence of females and adequate or desirable breeding habitat, which is not included in presence alone or calling. Measures of physiological state may also be good indicators; however, these metrics are only possible if the species is present and breeding is successful, as many of these metrics typically include only metamorphic individuals from a known breeding location. Additionally, measures of physiological state respond

differently to environmental changes, such as the differences between malformations and gonadal anomalies found in the PPR, and more research is needed to understand if each of these endpoints are responding to or indicating habitat quality or environmental condition.

Presence, breeding, and health of metamorphic *R. pipiens* are essential components of a sustainable population; however, long-term population monitoring, including breeding success, is the only way to determine population trends and identify areas at risk of local extirpation that may lead to greater population discontinuity and decline. Clearly, to understand habitat and landscape characteristics that support amphibians and inform adaptive management plans for predicted impacts of climate change, amphibian populations and aquatic ecosystems need to be monitored across large geographic scales.

Overview and General Introduction

Overview

This research assessed amphibian growth, development, and gonadal development in native species in experimental exposures and field surveys across a range of environmentally relevant atrazine concentrations and in combination with climate change and other environmental factors suspected to affect amphibian larval development.

The objectives of this project were to:

1) quantify native anuran developmental responses to the combined effects of atrazine exposure and accelerated pond-drying rates.
2) quantify potential effects of these and other environmental stressors on amphibian occurrence and health in the Prairie Pothole Region.

The resulting products from this research are four chapters, written as stand-alone manuscripts:

Chapter 1. Effects of atrazine and accelerated pond-drying rate on amphibian larval development and growth

Chapter 2. An assessment of testicular oocytes in field-captured *Rana pipiens*

Chapter 3. Effects of atrazine on testicular oocyte presence and severity in two North American amphibian species

Chapter 4. Landscape factors influencing amphibian larval development, growth, and gonadal development

The following endpoints were measured, as metrics of individuals or at wetlands that could affect amphibian population persistence (Table 3, Figures 1 & 2):

1) Altered gonadal morphology

- Methods for assessment (individuals and wetlands) – *Chapter 2*
- Prevalence, abundance, and severity of anomalies (testicular oocytes) assessed:
    - With exposure to atrazine and accelerated pond-drying in mesocosms - *Chapter 3*
    - Across atrazine concentrations and a range of environmental stressors in field collections - *Chapters 2,4*
- Species differences in prevalence of anomalies (testicular oocytes) – *Chapter 3*

2) Size at metamorphosis and time to metamorphosis (larval developmental period) assessed:

- With exposure to atrazine and accelerated pond-drying in mesocosms (2 species) - *Chapter 1*

3) Survival to metamorphosis assessed:

- With exposure to atrazine and accelerated pond-drying in mesocosms (2 species) - *Chapter 1*

4) Breeding success was assessed with evidence of breeding (eggs, larvae, metamorphs):

- Across atrazine concentrations and a range of environmental stressors in field collections - *Chapter 4*

5) Malformations in metamorphic frogs:

- Across atrazine concentrations and a range of environmental stressors in field collections - *Chapter 4*

We hypothesized that there would be a measureable dose-response to environmentally relevant atrazine concentrations with: 1) increased incidence of gonadal anomalies; 2) decreased size at metamorphosis; and 3) non-monotonically altered developmental rate, with either shorter or longer time to metamorphosis. When combined with the additional stressor of accelerated drawdown, we predicted that these negative developmental responses would be exacerbated. We hypothesized that the incorporation of additional stressors present in the natural breeding wetlands would increase the strength of models explaining amphibian health (gonadal anomalies and malformations) as well as allow for development of occurrence and breeding models.

General Introduction

This introduction provides a review of the literature and background information used for developing the objectives, hypothesis, and methodology for this research. Much, but not all, of the below material is included in the Introductions and Discussions of the Chapters 1-4.

Amphibian population declines

The current rate of amphibian extinctions and population declines is considered a 6th mass extinction (Collins & Crump 2009) in global history. Amphibians are declining worldwide and appear to be more threatened than birds or mammals, with 33% of Anura (frogs and toads) and 46% of Caudata (salamanders and newts) species classified as

either threatened or extinct (Stuart et al. 2004; IUCN 2008). Historically abundant or 'common' species also have also been declining over the last 50 years (Houlahan & Findlay 2004; Wake & Vredenburg 2008; Adams et al. 2013). Amphibian declines are attributed to numerous factors, but there is no clear pattern across species or regions. In addition to widespread habitat loss, amphibian populations are impacted by both natural and anthropogenic factors, including competition with introduced species, pollution, climate change, over-exploitation, and disease (Alford & Richards 1999; Collins & Storfer 2003; Halliday 2008).

Amphibians as Indicators

Amphibians are an excellent model for assessing environmental impacts on aquatic organisms because they are exposed to a variety of stressors, including natural environmental variations, point and non-point source nutrients and pesticides, invasive species, and climate change (Westerman et al. 2003). Developing amphibians are particularly sensitive to stressors because of their permeable skin and dependence on water. Stressors that impact larval development can directly affect annual population recruitment and adult fitness, making this a vital component in understanding population and community effects (Duellman & Trueb 1994; Semlitsch 2000).

The scope of current amphibian declines may reflect the extent of worldwide environmental degradation; amphibians are considered indicators of environmental condition, with population persistence and health/condition used as models for assessing environmental impacts on aquatic organisms and ecosystems (Vitt et al. 1990; Westerman et al. 2003; Welsh Jr & Hodgson 2008). The biphasic life history of many

North American species makes them dependent on both aquatic and terrestrial habitats, and therefore subject to widely-varying stressors. For example, because they undergo aquatic embryonic, larval, and metamorphic stages, amphibians are good indicators of chemical contamination in water, while retention of permeable skin throughout all life stages makes them vulnerable to terrestrial contaminants (Westerman et al. 2003; Bernanke & Köhler 2009; Quaranta et al. 2009). Sensitivities to local or wetland conditions include physiological or behavioral responses to hydroperiod (Pechmann et al. 1989; Snodgrass et al. 2000), predators (Hecnar & M'Closkey 1997; Adams 1999), introduced species (Kiesecker et al. 2001a; Knapp et al. 2007), disease (Carey et al. 1999; Skerratt et al. 2007), and chemicals (Mann et al. 2009). At the landscape level, amphibian presence and diversity are sensitive to habitat fragmentation (Kolozsvary & Swihart 1999; Knutson et al. 2000; Willson & Dorcas 2003), roads (Reeves et al. 2008; Bouchard et al. 2009), urbanization (Pillsbury & Miller 2008; Hartel et al. 2009; Guzy et al. 2012), and lack of non-breeding habitat for foraging and hibernation (Smith & Keinath 2007)

Additionally, amphibians have been used as indicators of biological diversity, based on their major role in some ecosystems. Amphibians make up a large component of the biomass in ecosystems, such as northern hardwood forests of the US (Burton & Likens 1975), tropical forests of Puerto Rico (Stewart & Woodbright 1996), southeastern U.S. wetlands (Gibbons et al. 2006), and Midwest U.S. wetlands (Connelly et al. 2008). Amphibians consume algae and insects, transfer energy between aquatic and terrestrial ecosystems, and act as a food source for many other organisms (Merrell 1977; Whiles et al. 2006; Altig et al. 2007; Connelly et al. 2008). Loss or decline of amphibians could

substantially impact aquatic systems as well as the surrounding terrestrial systems, as energy flow through wetlands would be reduced or redirected to other organisms, algal growth could expand, and the annual pulse of high energy food sources to terrestrial systems in the form of metamorphic amphibians would decline or disappear.

Amphibian developmental plasticity

A variety of invertebrate, fish, amphibian, and plant species exhibit phenotypic plasticity in development and life history. Research has shown that this plasticity is adaptive in many species, meaning that the phenotype observed maximized their fitness given the environmental conditions (Stearns 1989), and adaptations generally occur at or above the species level (Gotthard & Nylin 1995). Amphibian developmental plasticity is a well-developed area of research with many lines of inquiry continuing to be studied, focusing primarily on the length of the larval period (time to metamorphosis) and size at metamorphosis (Newman 1992).

It is unclear how many amphibian species display developmental plasticity; however, it has been observed in many and is especially common in those breeding in ephemeral/seasonal water bodies or in environments with highly unpredictable wet seasons, such as deserts (Newman 1992; Denver 1997). Amphibians display developmental plasticity with larval period dependent on both biotic (competition, density, food) and abiotic (temperature, pond drying, salinity) factors (Denver 1997). This plasticity allows larval amphibians to metamorphose (turn into terrestrial, adult form) at varying speeds depending on the environment. By cuing development to external

signals, larvae are able to maximize potential benefit from increased growth and minimize the risk of mortality while in their aquatic form.

Most North American amphibians depend on both aquatic and terrestrial habitat to successfully complete their life cycle (Stebbins & Cohen 1995). Most amphibian eggs are laid in or over water, depositing the larvae into an aquatic environment to develop. Larvae develop in this aquatic environment until transformation into the adult form through metamorphosis. Larval periods for amphibians vary greatly across species as well as across environmental conditions with larval periods lasting from less than 10 (*Scaphiopus couchii*) to more than 1000 days (*Rana fuscigula, Rana catesbeiana*) (Denver 1997). The length of the larval period has a rigid upper limit based on the 'ancestral pond' and a lower limit that is based on growth and developmental rates along with the critical minimum size for metamorphosis (Denver 1997). Amphibian larvae have developed through evolutionary history to best utilize their environment to balance opposing pressures of growth and differentiation (development). Development times should thus be based on the length of inundation in the original 'ancestral pond', with modifications (or plasticity) based on the stressors present in this pond as it also evolved. The extent of these modifications to development rate is constrained by physiological possibilities and biochemical processes. These limits are not well understood for most species; generally, only observational data is used to determine maximum and minimum larval periods.

For some species, the length of larval period is highly variable depending on the environmental conditions, suggesting the presence of adaptive developmental plasticity.

For example, bullfrog (*Rana catebeiana*) and green frogs (*Rana clamitans*) have a minimum development time of approximately 150 days with the maximum duration two to ten times longer. This highly variable length of larval period is based on these species' ability to overwinter as tadpoles for one or more years if the conditions are right and could be dependent on their developmental stage when the water temperatures begin to cool (Werner 1986). On the other end of the development time spectrum are toads such as American toad (*Bufo americanus*) and Great Plains spadefoot toad (*Spea bombifrons*) which can develop in less than 8 days with maximum larval periods up to three times longer. These quick-developing species commonly breed in temporary water and are assumed to have evolved developmental plasticity that allows them to accelerate development when their aquatic habitat begins to dry (Denver 1997).

Physiological and environmental cues for growth and development.

Physiologically, the balance between growth and development is thought to be dependent on a neurohormonal stress response to the environmental conditions (Denver 1997). Growth is accelerated (and metamorphosis inhibited) by prolactin and growth hormones, which results in a larger size at metamorphosis, and presumably a larger, more fit adult. Metamorphosis is accelerated (and growth inhibited) by thyroid hormones and corticosteroids, which allows individuals to escape drying conditions or other risk of mortality. The activation of hormones that promote or inhibit each pathway is dependent on sensory cues, such as proximity to pond surface, depth of water or swimming area, and temperature (Denver 1997, 2009; Denver et al. 1998). If temperature is the only cue, observed variation in larval period would be a consequence of environmental effects

speeding development rate through increased hormone production and action (Denver 1997); however, in experiments controlled for temperature, development accelerated as a continual function of water level decrease (Denver et al. 1998). Environmental factors have been shown to inhibit growth during premetamorphosis, but stimulate development during prometamorphosis, including crowding, resource limitation, habitat desiccation, and predation (Denver 1997). This switch in response to environmental factors depending on developmental stage provides support for dependence of the lower limit on a critical minimum size for metamorphosis (Denver 1997), and allows one or more individuals to complete metamorphosis and escape the aquatic environment while inhibiting development in the remaining tadpoles.

Atrazine and amphibian development

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is one of the most widely used pesticides in the U.S., with the majority applied to corn and sorghum in the Midwest (USEPA 2003). Atrazine has moderate water solubility and a slow degradation rate under certain conditions, with a half-life water of 41-237 days in water (Giddings 2005). Atrazine is eventually transported to wetlands and lakes through runoff and aerial deposition, with detectable levels found far from application. For example, atrazine was detected in 87% of lake samples across Minnesota in 2007 even in nonagricultural northern regions (MDA 2008). The concentration of atrazine in the aquatic environments has high spatial and temporal variability; however, concentrations up to 10.9-172.2 µg/L in streams (Scribner et al. 2000; Battaglin et al. 2003) and 33.8 - >200 µg/L in wetlands (Murphy et al. 2006a; Papoulias et al. 2013; Schoff et al. *In*

review) have been measured in the Midwest United States. Therefore, organisms in aquatic ecosystems such as lakes and wetlands could be chronically exposed to atrazine concentrations that exceed the drinking water limit of 3 μg/L as well as the 10-20 μg/L level that EPA identifies for the risk of ecological effects (USEPA 2003). Typical atrazine applications of 1 kg/ha are applied to crops pre-emergence to control broadleaf weeds, generally in mid-April to mid-May for corn in the Midwest (USEPA 2003). Because the typical timing of atrazine application coincides temporally with breeding for many U.S. Midwest amphibian species (Fischer et al. 1999; Knutson et al. 2004; Naugle et al. 2005), tadpoles and larvae may be exposed throughout development. The atrazine concentrations measured in breeding wetlands are currently believed to be non-lethal to amphibians (Giddings 2005; Solomon et al. 2008); however sub-lethal effects of atrazine exposure (as low as 0.1 μg/L) have been shown in fish and amphibians (Rohr & McCoy 2010).

The effects of atrazine on amphibian growth, development, and reproductive development vary across species and studies, as highlighted in several recent reviews (Table 1, Solomon et al. 2008; Rohr & McCoy 2010). Ecologically relevant atrazine concentrations (0.1 - 25 μg/L) have been shown to have indirect and sublethal effects, including altering length of larval period (with evidence of both delayed and accelerated development), size at metamorphosis, behavior, immune function, and gonadal morphology and function. There are some consistencies in developmental effects of atrazine exposure, with reduced growth rate and altered developmental rate (length of larval period); however, discrepancies across studies demonstrate potential species

differences in development and sensitivity to atrazine. Larval exposure resulted in smaller size at or near metamorphosis in most of the species included in a recent meta-analysis (Rohr & McCoy 2010). Atrazine exposure slowed development in some species (Boone & James 2003; Coady et al. 2004; Rohr & Crumrine 2005; Freeman et al. 2005), while development was accelerated or not affected in other species (Hayes et al. 2002; Rohr et al. 2004; Orton et al. 2006; Williams & Semlitsch 2010). Such nonmontonic developmental responses were expected given the complex system controlling metamorphosis and potential to disrupt physiological responses to environmental cues (Wilbur & Collins 1973).

The endocrine disruption potential of atrazine exposure has received much attention because of the potential impacts on fecundity and population persistence. Low concentrations of atrazine (< 1 μg/L) have been shown to cause variable reproductive responses across amphibian species and studies (Solomon et al. 2008); despite these differences in response, a recent meta-analysis has shown developmental and reproductive impacts of atrazine exposure across fish and amphibian studies (Rohr & McCoy 2010). At ecologically relevant concentrations, atrazine exposure induced abnormal gonad development in laboratory experiments, including: 1) gonadal dysgenesis (ambiguous or abnormal development in progress, size, or structure), 2) discontinuous (segmented) gonads, 3) mixed gonadal tissue (intersex), and 4) testicular oocytes (Hayes et al. 2002, 2003, 2006a, 2006b; Carr et al. 2003). The effect of atrazine on gonadal development remains controversial, as other studies have not been able to

replicate these reproductive effects (Coady et al. 2004; Jooste et al. 2005; Orton et al. 2006; Oka et al. 2008).

The presence of oocytes in testicular tissue, hereafter called testicular oocytes or TOs, is often used as an endpoint in assays for endocrine-disrupting substances in amphibians (Hecker et al. 2006) and fish (Johnson et al. 2010a). TOs have been documented in frogs after lab exposures to known and suspected endocrine disrupting chemicals (EDCs) (MacKenzie et al. 2003; Hayes et al. 2003; Coady et al. 2004; Jooste et al. 2005), as well as in wild populations across a wide range of habitats (Hayes et al. 2003; McDaniel et al. 2008; Du Preez et al. 2009; Papoulias et al. 2013). TOs have also been used as biomarkers for environmental contamination in field surveys of frogs (Hayes et al. 2003; Skelly et al. 2010) and fish (Hinck et al. 2009; Blazer et al. 2012), with agricultural run-off and wastewater treatment plants usually implicated as likely sources of endocrine disrupting compounds.

In amphibians, sex is determined genetically; however there are many epigenetic factors that can override the genotypic sex determination (Hayes 1998; Eggert 2004). For example, *R. sylvatica* reared at high temperature can be induced to develop as phenotypic males (Witschi 1929), regardless of genetic sex. Amphibian gonadal differentiation is also sensitive to exogenous steroid hormones and full sex reversal or intersex can be induced by estrogens (Hayes 1998; Hogan et al. 2008; Nakamura 2009), especially if exposure occurs during early sensitive developmental periods (Ogielska 2009). Induction of aromatase has been suggested as the mechanism by which atrazine exposure disrupts endocrine function resulting in gonadal anomalies (Hayes et al. 2002). Aromatase

converts androgens such as testosterone to estrogens, and external signaling for aromatase induction could alter the balance of sex hormones within the gonad, potentially causing feminization of gonads including inducing gonia cells to differentiate into oogonia within the testis (Nakamura 2009). Induced expression of the aromatase gene (CYP19) by atrazine has been documented in other vertebrates (Crain et al. 1997; Sanderson et al. 2000), but has not yet in amphibians.

Climate change and amphibian development in the Prairie Pothole Region

Climate change is considered a major threat to amphibians in areas otherwise protected from habitat destruction and contaminants (Collins & Storfer 2003), directly impacting populations through habitat loss and degradation, and affecting development by accelerating pond and wetland drying. Additionally, breeding phenology and distributions of species have been altered due to climatic changes (Beebee 1995; Carroll et al. 2009), and female survival and fecundity may be reduced due to warmer winters (Reading 2007). Furthermore, changing climatic conditions may also have indirect impacts through interactions with pesticides and other stressors.

The Prairie Pothole Region (PPR) forms the northeastern edge of the Great Plains, encompassing a large area of diverse wetlands that represent crucial aquatic resources for flood control and aquatic and terrestrial production (van der Valk 1988). This area contains over 3 million wetland basins, including a variety of wetland types from semi-permanent wetlands, which remain wet throughout most years, to seasonal and temporary wetlands, which hold water for one to several months (Stewart & Kantrud 1971; Winter

& Rosenberry 1995; Murkin et al. 2000). Well known as the 'duck factory' because of the high waterfowl productivity (Hoekman et al. 2002), these wetlands also historically support amphibian communities capable of thriving with the variable water availability (van der Valk 1988; Oldfield & Moriarty 1994; Balas et al. 2012).

Because most of the PPR is subject to mixed use agriculture and open grazing, wetlands are routinely exposed to a variety of anthropogenic stressors such as pesticides, nutrients, and domestic animal pathogens (Tiner 1984, 2003). In addition to effects from these stressors, it is anticipated that aquatic ecosystems in this region will be severely impacted by climate change, particularly due to increasing temperature and reduced precipitation (Johnson et al. 2005, 2010b). Climate models indicate that air temperatures are expected to increase at least 2°C in this region by the end of the century, with variable changes in seasonality and amount of precipitation (IPCC 2007; Galatowitsch et al. 2009). Such changes could negatively impact PPR wetlands, many of which are entirely dependent on precipitation to maintain water levels (Winter & Rosenberry 1995; Winter 2000). Duration of water retention and vegetation cycling are both predicted to change, forcing semi-permanent wetlands, which are characterized as remaining wet in a majority of years, to becoming more seasonal, characterized by annual drying. Likewise, current seasonal wetlands will become more ephemeral or temporary, and temporary wetlands, which usually contain water for only a brief period, will rarely hold water (Johnson et al. 2010b). These predicted changes would directly impact water availability and aquatic habitat for developing amphibian larvae.

Altered wetland hydrology could greatly impact amphibian populations through reducing habitat availability, accelerating developmental rate, and reducing immune function. Many amphibians can accelerate larval development when faced with threat of pond drying, allowing survival despite naturally variable water availability. This adaptive developmental plasticity has been observed in many species and is especially common in those breeding in ephemeral water bodies or in environments with highly unpredictable wet seasons (Newman 1992; Denver 1997). By cuing development to external biotic and abiotic signals, larvae can maximize potential benefit from increased growth and minimize risk of mortality while in aquatic form.

Risk of mortality due to pond desiccation is a serious threat to many amphibians that breed in temporary ponds. Length of larval period and size at metamorphosis are both expected to decrease with increasing desiccation rate, based on experimental and observational data as well as biochemical processes, specifically hormone development and processing (Newman 1992). By accelerating development, larvae ensure their survival to the juvenile stage. Forced rapid development, however, produces smaller juveniles, which corresponds with lower winter survival, higher predation rates, and reduced future reproductive success. Accelerating development results in smaller size at metamorphosis for most species studied (Table 2, Newman 1992; Álvarez & Nicieza 2002; Altwegg & Reyer 2003; Gervasi & Foufopoulos 2008), although there is an interaction with food availability, crowding, and other stressors (Álvarez & Nicieza 2002). Smaller size at metamorphosis has been associated with reduced survival through increased predation as well as reduced winter survival and adult breeding capacity

(Duellman & Trueb 1994). Shorter larval periods reduced immune capacity in *Rana sylvatica* (Gervasi & Foufopoulos 2008), and size at metamorphosis reduced survival and growth rate in *Rana lessonae* and *Rana esculenta* (Altwegg & Reyer 2003). Additionally, size at metamorphosis affected energy reserves and jumping ability in *Discoglossus galganoi* (Álvarez & Nicieza 2002).

In addition to pond desiccation, accelerated development and altered morphology have been observed in response to predators, reduced food availability, or toxicants, with potential interactions among these stressors as well (Boone & James 2003; Relyea 2005a, 2006; Boone et al. 2007). Length of larval period and size at metamorphosis was reduced by exposure to pesticides, including atrazine (Table 1, Hayes et al. 2006a; Boone et al. 2007). How developmental endpoints respond to multiple stressors and stressor interactions remains largely unknown. Based on other multiple stressor exposure experiments, it is predicted that effects of atrazine will be intensified by the additional developmental stress due to the increased temperatures, reduced precipitation, and rapid pond drying that are predicted with climate change (Rohr et al. 2008; Mann et al. 2009). The range of amphibian developmental and health responses to pesticide exposure and pond drying highlights the need to incorporate potential interactions and covariates into both experimental and observational research.

Study species

Field surveys of R. pipiens *in the PPR.* Considered a 'common' species throughout the Midwest, *R. pipiens* have been declining across their range since the 1970s and appear to be susceptible to local extinctions due to habitat loss and

anthropogenic stressors (Smith & Keinath 2007). This species is associated with open grassland areas that contain a variety of wetland and lake types which are utilized by different life stages. Adults emerge from overwintering habitat (typically permanent wetlands or lakes), migrating to seasonal and semi-permanent wetlands to breed in early to mid-spring to lay 2000-65000 eggs per female. Tadpoles complete development and transform into metamorphic frogs in 90 to 120 days, and disperse from natal wetlands en masse (Merrell 1977; Smith & Keinath 2007). Adults and juveniles will forage in the uplands surrounding their summer aquatic habitat, presumably on a daily basis, returning to the water when not foraging (Merrell 1977). In the fall, metamorphic *R. pipiens* will disperse from their natal pond and migrate 1-2 km to overwintering locations, along with adults and juveniles (Merrell 1977). This biphasic life history of many anurans in the Midwest U.S. makes them susceptible to stressors (or dependent on environmental conditions) at multiple scales (Figure 1). Within the wetland, *R. pipiens* are dependent on the habitat and water quality for breeding and successful larval development to metamorphosis, including holding water until metamorphosis is complete and limiting exposure to (or having protection from) predators, parasites, and damaging chemicals. In the immediate surrounding upland, land use impacts accessibility of wetlands to breeding adults and successful dispersal of young-of-the-year metamorphic frogs, pesticide and nutrient runoff into the wetland, and food availability for foraging adult, juvenile, and metamorphic *R. pipiens*. At the landscape scale, *R. pipiens* are impacted by land uses such as intensive agriculture and urban and road development that historically reduce

wetland density and create barriers or challenges to amphibian migration between overwintering and breeding habitats.

Exposure experiments with native anurans. The native amphibian species, *R. pipiens*, has been used as a benchmark for many ecotoxicology and developmental studies, including responses to atrazine (Hayes et al. 2003, 2006a; McDaniel et al. 2008; Rohr et al. 2008). Use of this species allows for direct comparisons to experimental exposures and field surveys across a large geographical extent; however, *R. pipiens* may have a greater or different sensitivity to endocrine disruption when compared to other laboratory or native species (MacKenzie et al. 2003; Solomon et al. 2008). Additionally, there are several challenges to using *R. pipens* in mesocom experiments, including: 1) difficulty in locating eggs that are not potentially contaminated with atrazine due to the overlap in species range with the agricultural regions; 2) longer larval development can reduce survival and induce overwintering tadpoles, especially in northern extent of range; and 3) smaller metamorphic size when compared to field specimens (Olker, unpublished data).

Including an additional native species, *Rana sylvatica* (wood frog, a common northern species associated with forested wetlands) increases understanding of the variation in amphibian development and species-specific responses to atrazine. Based on its occurrence primarily in the cold regions of North America, *R. sylvatica* is expected to be sensitive to the increased temperatures predicted with climate change, resulting in possible range shifts. This congener of the commonly studied *R. pipiens* breeds early, develops quickly, survives well, and replicates field observations for size and age at

metamorphosis (Olker, unpublished data). For many of the above reasons, *R. sylvatica* are becoming a more common species for ecotoxicological and developmental experiments (Kiesecker et al. 2001b; MacKenzie et al. 2003; Rohr & Crumrine 2005; Gervasi & Foufopoulos 2008). Using a combination of two native species for the experimental exposure addresses variability across species and allows predictions of wild population effects across a large spatial extent. The geographical distributions of *R. pipiens* and *R. sylvatica* combined cover more than half of North America (NPWRC 1997).

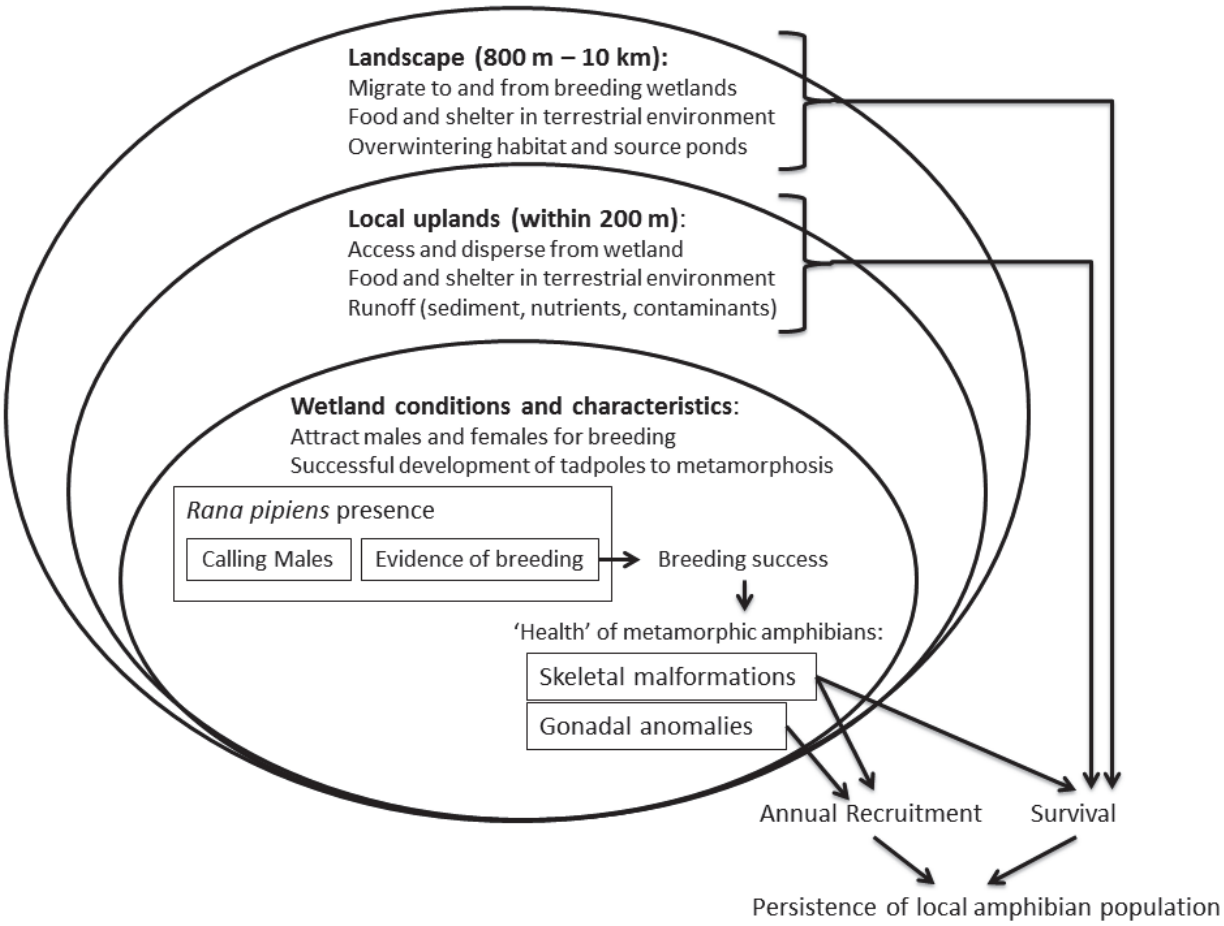

Figure 1. Diagram of conditions, characteristics, and stressors at multiple scales that impact amphibian metrics (in boxes) that could be used as indicators of wetland quality or environmental condition, and predict persistence of local amphibian populations.

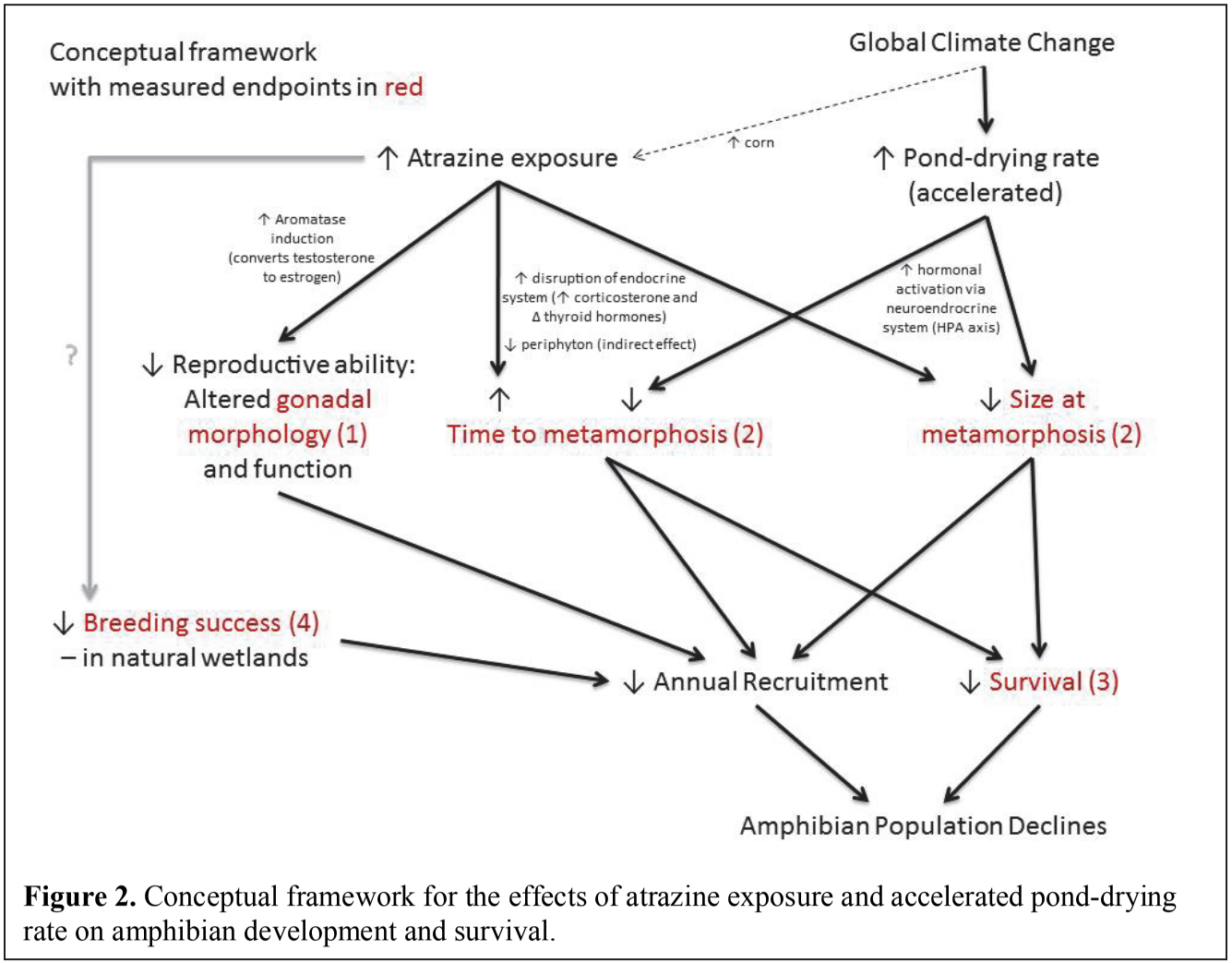

Figure 2. Conceptual framework for the effects of atrazine exposure and accelerated pond-drying rate on amphibian development and survival.

Table 1. Effect of atrazine on amphibian growth, development and gonadal development after chronic exposure during larval stages, including relevant references for laboratory and field exposures.

Group	Endpoint	Relationship with atrazine	Effect conc (µg/L)	Species	Life stage	Exposure duration	Setting	Ref
Growth	Size at or near metamorphosis	**none**	25	*Xenopus laevis*	larval	78	lab	(Carr et al. 2003)
		none		*Rana pipiens*	larval	until metamorphosis	mesocosm	(Langlois et al. 2010)
		-	0.1	*Rana pipiens*	larval	until metamorphosis	lab	(Hayes et al. 2006a)
		-	10	*Rana clamitans*	larval	273 days	lab	(Coady et al. 2004)
		- [a]	25	*Rana sylvatica*	larval	30 days	mesocosm	(Rohr & Crumrine 2005)
		-	5-40 mg/L	*Rhinella arenarum*	embryonic, larval	24 hours - 45 days	lab	(Svartz et al. 2012)
		-[b]	0.26 – 8.3	*Rana pipiens*	larval	Unknown	field	(Christin et al. 2013)
		none		*Limnodynastes tasmaniensis*	larval	Gosner stage 28 - 42	lab	(Spolyarich et al. 2010)
Development	Age at or time to metamorphosis	-	10	*Rana clamitans*	larval	273 days	lab	(Coady et al. 2004)
		-	25	*Rana sylvatica*	larval	30 days	mesocosm	(Rohr & Crumrine 2005)
		none		*Rana pipiens*	larval	until metamorphosis	lab	(Rohr & Crumrine 2005)
		none		*Rana pipiens*	larval	until metamorphosis	mesocosm	(Langlois et al. 2010)
		+	5-40 mg/L	*Rhinella arenarum*	embryonic, larval	24 hours - 45 days	lab	(Svartz et al. 2012)
		none		*Limnodynastes tasmaniensis*	larval	Gosner stage 28 - 42	lab	(Spolyarich et al. 2010)

Table 1, continued.

Gonadal morphology	Dysgenesis (delayed development)	+	0.1, 25	*Rana pipiens*	larval	until metamorphosis	lab	(Hayes et al. 2003)
		? [c]	0.8?	*Rana pipiens*	larval	unknown	field	(Hayes et al. 2003)
		none[d]		*Limnodynastes tasmaniensis*	larval	Gosner stage 28 - 42	lab	(Spolyarich et al. 2010)
	Discontinuous (segmented) gonads	+	25	*Xenopus laevis*	larval	78	lab	(Carr et al. 2003)
		none[d]		*Limnodynastes tasmaniensis*	larval	Gosner stage 28 - 42	lab	(Spolyarich et al. 2010)
	Mixed gonadal tissue (intersex)	+	25	*Xenopus laevis*	larval	78	lab	(Carr et al. 2003)
	Testicular oocytes (testicular ovarian follicles, testicular oogenesis)	+	0.1, 25	*Rana pipiens*	larval	until metamorphosis	lab	(Hayes et al. 2003)
		+**?** [e]	0.2?	*Rana pipiens*	larval	unknown	field	(Hayes et al. 2003)
		+ [f]	ND-3.14	*Rana pipiens*	larval	unknown	field	(McDaniel et al. 2008)
		none		*Rana pipiens*	larval	until metamorphosis	mesocosm	(Langlois et al. 2010)
		none		*Rana blairi* (and *R. pipiens*)	larval	Unknown	field	(Papoulias et al. 2013)
		none[d]		*Limnodynastes tasmaniensis*	larval	Gosner stage 28 - 42	lab	(Spolyarich et al. 2010)
Gonadal function	Testosterone	**none** [g]	ND-3.13	*Rana pipiens*	larval	unknown	field	(McDaniel et al. 2008)
		+**?** [h]		*Bufo marinus*	adult	unknown	field	(McCoy et al. 2008)
	Estrogen receptor alpha mRNA (brain)	+	0.1, 1.8	*Rana pipiens*	larval	until metamorphosis	mesocosm	(Langlois et al. 2010)

[a] negative effect when snails present in atrazine treatment; [b] lower body indices in Ag-collected compared to 'reference'; [c] all at 1 'contaminated' site in Nebraska with 0.8 µg/L atrazine; [d] 1 specimen in 3 µg/L treatment ;[e] all but 1 site had 5-92% TOs, highest level at 'uncontaminated' site in Wyoming (with 0.2 µg/L atrazine); [f] Ag vs Ref (Ag and non-Ag); prop. TO not directly correlated with atz conc, but with mixtures (atrazine + nitrate) and total # of pesticides; [g] measured in juvenile males; [h] decreased in Ag-collected males, no atrazine conc. reported.

Table 2. Select studies on amphibian developmental plasticity.

Species	Stress	Setting	Result	Ref
Rana temporaria	Desiccation, food levels	lab	temporary pond population had genetically shorter development time; developmental thresholds not different between populations; L-shaped reaction norms	(Lind et al. 2008)
Rana temporaria	Desiccation	lab	interpopulation differences	(Laurila et al. 2002)
Rana temporaria	Desiccation	lab	northern populations develop faster than southern populations with less flexibility (constrained by trade-offs)	(Merilä et al. 2004)
Scaphiopus hammondi	Desiccation	lab	develop accelerated as water level decreased (continuum of response); reversible; not related to temperature, instead reduced swimming volume and proximity to water surface	(Denver et al. 1998)
Rana sylvatica	Desiccation	lab	shorter development time; weaker immune system	(Gervasi & Foufopoulos 2008)
Discoglossus galganoi	Temperature, food type	lab	larval period varied with temp; size at metamorphosis function of food quality and temperature; temperature affected post-metamorphic energy reserves and locomotion (jumping)	(Álvarez & Nicieza 2002)
Rana temporaria	Predators	mesocosm	little evidence for cost of plasticity; behavior and morphology	(Steiner & van Buskirk 2008)
Rana lessonae, Rana esculenta	Competition, predators, desiccation	mesocosm	large metamorphs had greater survival and larger at maturity; late metamorphs had lower survival and grew slower	(Altwegg & Reyer 2003)
Rana sylvatica	Predators	mesocosm	inducible traits have genetic basis	(Relyea 2005b)
Rana pipiens	Pesticide mixtures	lab	pesticide mixtures more detrimental than single pesticides; metamorphosis delayed, size reduced; immunosuppression increased	(Laurila et al. 2002)
Bufo americanus, Rana sphenocephala Ambystoma maculatum	pesticides, fertilizer, predators	mesocosm	stressor interactions; metamorphosis and size altered (reduced or increased depending on treatment)	(Boone et al. 2007)

Table 3. Endpoints to assess atrazine impacts and accelerated pond-drying on amphibian growth, development, and gonadal development in exposure experiments (Chapters I and III) and field surveys (Chapter IV), with potential population-level impacts.

	Predicted Responses			
Endpoint	**Atrazine**	**Accel. Pond-drying**	**Potential impacts on individuals**	**Potential impacts on populations**
Survival* to metamorphosis	↓ survival	↓ survival	death, cannot reproduce	reduced recruitment; genetic diversity lost
Size* (length and weight at metamorphosis)	Smaller	smaller	increased predation risk and juvenile mortality; older reproductive age; reduced fecundity	reduced recruitment and population size; overall lower reproductive rates in population
Development* **(**time to forelimb emergence and metamorphosis)	slower or faster development	faster development	increased or decreased exposure to contaminants and aquatic predators; increased risk of desiccation	reduced recruitment, reduced population size
Gonadal development (sex ratio*, anomalies, testicular oocyte presence/abundance)	more females ↑ abnormalities ↑ TOs	none, potentially less developed gonads	reduced reproductive potential	overall lower reproductive rates in population, reduced population size

*assessed only in exposure experiments

Chapter 1. Effects of atrazine and accelerated pond-drying rate on amphibian larval development and growth

Introduction

The upper Midwest United States supports a wide variety of land uses on a diverse landscape that often contains abundant wetlands that can support aquatic communities (van der Valk 1988; Fischer et al. 1999; Balas et al. 2012). Aquatic ecosystems in this region are exposed to numerous anthropogenic stressors originating from agriculture and development, including fragmentation, sedimentation, and chemical and nutrient contamination (Neely & Baker 1989; Carpenter et al. 1998; Gleason et al. 2011). Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine), a triazine herbicide with moderate water solubility, is used primarily on corn, which is the predominant crop in much of this region. Atrazine applications coincide temporally with breeding and larval development of many native Midwest amphibian species (Fischer et al. 1999; Knutson et al. 2004; Naugle et al. 2005) that lay eggs in water. Their larvae develop in aquatic environments that may contain chemicals such as atrazine originating from runoff and aerial drift. Atrazine in Midwest wetlands show high spatial and temporal variability; however, concentrations of 10.9-172.2 μg/L in streams (Scribner et al. 2000; Battaglin et al. 2003) and 33.8 - >200 μg/L in wetlands (Murphy et al. 2006a; Papoulias et al. 2013, Schoff et al. *In review*) have recently been recorded. While atrazine at these concentrations is not lethal to model amphibians (Giddings 2005; Solomon et al. 2008) sub-lethal effects of atrazine exposure (as low as 0.1 μg/L) have been shown in both amphibians and fish (Rohr & McCoy 2010). Effects on amphibian growth, development, and reproductive development may vary across species and by study,

(Solomon et al. 2008; Rohr & McCoy 2010), but reductions in size and health at metamorphosis (Coady et al. 2004; Rohr & Crumrine 2005; Hayes et al. 2006a) and alterations of gonadal function due to endocrine disruption (Hayes et al. 2003; Murphy et al. 2006a; McDaniel et al. 2008) are commonly reported. In addition, by altering amphibian growth and development, atrazine may contribute to amphibian declines through reducing survival and reproductive capabilities (Appendix A Table S1).

Climate change is anticipated to be a major threat to amphibians, even in areas otherwise protected from habitat destruction and contaminants (Collins & Storfer 2003). In degraded areas, changing climatic conditions is expected to exacerbate the effects of chemicals on populations by accelerating pond and wetland drying. Aquatic ecosystems in the upper Midwest United States are anticipated to be severely impacted by climate change, particularly due to increasing temperature and reduced precipitation (Johnson et al. 2005). Climate models indicate that air temperatures are expected to increase at least 2°C in this region by the end of the century, with variable changes in seasonality and amount of precipitation (IPCC 2007; Galatowitsch et al. 2009). Such changes could negatively impact wetlands in the Prairie Pothole Region (PPR), many of which are entirely dependent on precipitation to maintain water levels (Winter & Rosenberry 1995; Winter 2000). Duration of water retention and vegetation cycling are both predicted to change, forcing semi-permanent wetlands, which are characterized as remaining wet in a majority of years, to becoming more seasonal, characterized by annual drying. Likewise, current seasonal wetlands will become more ephemeral or temporary, and temporary

wetlands, which usually contain water for only a brief period, will rarely hold water (Johnson et al. 2010b).

Understanding how chemical and physical stressors impact growth, condition, and survival of developing amphibians will allow estimates of effects on annual recruitment, which are necessary to address population level impacts (Semlitsch 2000; Hayes et al. 2010b). Our objective was to assess the dose-response effect of the herbicide atrazine in combination with accelerated pond-drying rate, which is a predicted response of wetlands to climate change models (Johnson et al. 2010b), on growth and development in two North American amphibian species. We used experimental mesocosms to test the effects of accelerated pond-drying in combination with larval exposure to environmentally relevant atrazine concentrations on survival, growth, and development of two North American frog species, northern leopard frog (*Rana pipiens*) and wood frog (*Rana sylvatica*). How amphibian development and growth responds to multiple stressors, including pesticides and climate change, remains largely unknown. Based on other multiple stressor exposure experiments (Rohr et al. 2008; Mann et al. 2009), we hypothesized that effects of atrazine would be intensified by the additional developmental stress associated with climate change, such as increased temperatures, reduced precipitation, and rapid pond drying.

Methods

In this experimental exposure study, we used two frog species common in the upper Midwest U.S.; *Rana pipiens*, which is associated with grassland wetlands, and *Rana sylvatica*, which is a northern species associated with forested wetlands. In addition

to comparing independent species responses, this protocol allows predictions of wild populations over varying habitats covering more than half of North America (NPWRC 1997).

This study consisted of two experiments, one for each species, conducted in different years with slight differences in methodology, as described below. Each experiment was analyzed separately. In both experiments, we used a fully-crossed randomized block design with outdoor aquatic mesocosms to test the individual and interactive effects of atrazine concentration and drawdown rate, with a static, non-renewal exposure (Appendix A Table S2). Mesocosms were constructed from 37 gallon (140 L) low-density polyethylene (LPDE) stock tanks with mesh screen covers filled with 100 L of conditioned water prepared according to published recommendations (Rowe & Dunson 1994; Boone & James 2005; Semlitsch & Boone 2009) from an uncontaminated source.

Developing amphibian larvae were exposed from hatch through metamorphosis to combinations of two pond-drying rates (normal and accelerated, hereafter called drawdown rates) and three ecologically relevant atrazine concentrations (0.1, 20, 200 µg/L). Water was removed from mesocosms at two rates: normal, in which depth was decreased by 2 cm or 8% of initial levels per week) or accelerated, depth decreased by 4 cm (16%) per week. The difference between 'normal' and 'accelerated' desiccation rates was comparable to laboratory experiments in which *R. sylvatica* development responded differently to moderate and slow desiccation rates (0.5-1 L per day versus 0.5 L every other day from 10 L tanks, Gervasi & Foufopoulos 2008). Drawdown began when 50%

of observed tadpoles in the experiment reached the stage considered responsive to stimuli for metamorphosis (Gosner stage 37, Denver 1997; Glennemeier & Denver 2002), and continued until the accelerated drawdown mesocosms reached a depth of 5 cm (after 5 weeks), where they were then maintained. Excess water from rain was removed as needed. Atrazine concentrations were verified with enzyme-linked immunosorbent assay (ELISA) with a nominal minimum detection limit of 0.05 µg/L (Atrazine kit, Abraxis LLC, Warminster, PA). All mesocosms were assayed at the start and end of each experiment.

Experiment 1 was conducted in 2006 with *R. pipiens* and included six treatments (three atrazine concentrations X two drawdown rates), plus control (normal drawdown only) and acetone (solvent) control for both normal and accelerated drawdown, with five replicate mesocosms each starting with five tadpoles (Appendix A Table S2). *R. pipiens* were collected as eggs on May 11, 2006 from Douglas County, WI, hatched in outdoor aquaria, and distributed to pre-dosed mesocosms when they reached free-swimming, Gosner stage 26, 15 days after collection.

Experiment 2 was conducted in 2009 with *R. sylvatica* and included six treatments (three atrazine concentrations X two drawdown rates), plus the control for both normal and accelerated drawdown, with six replicate mesocosms each starting with six tadpoles (Appendix A Table S2). No solvent control was necessary because atrazine was dissolved in deionized water. Because collected eggs had low hatching rates, free-swimming *R. sylvatica* tadpoles (Gosner stage 26, 22 days after fertilization) were

collected directly from a wetland in St. Louis County, MN, on May 16, 2009 and distributed to pre-dosed mesocosms.

Endpoints

We tracked tadpole survival weekly throughout the experiment (with daily checks after forelimb emergence), length and weight at the completion of metamorphosis (Gosner stage 46), and development with time to forelimb emergence as evidence of metamorphic climax and tail resorption as completion of metamorphosis (Table 1). Additionally, body condition index (BCI) was used to evaluate the relationship between weight and snout-to-vent length relative to treatment (Karraker & Welsh 2006; Janin et al. 2011; MacCracken & Stebbings 2012). Each experiment ended when all specimens had completed metamorphosis (July 31, 2009 for *R. sylvatica*) or until ice formed on the mesocosms (November 3, 2006 for *R. pipiens*).

To monitor potential indirect effects of treatments, we measured water temperature, chlorophyll-A, and periphyton growth. In both experiments, general water quality (pH, temperature, dissolved oxygen, conductivity) was assessed weekly in all mesocosms (YSI model 63, YSI Incorporated, Yellow Springs, OH). Water temperature was also measured continuously at 30 minute intervals with HOBO dataloggers in a subset of non-atrazine exposed mesocosms.

In experiment 2 (*R. sylvatica*, 2009), primary production as relative *in vivo* chlorophyll-A was measured in all mesocosms on five dates spanning the exposure period using a handheld fluorimeter (AquaFluor, Turner Designs, Sunnyvale, CA). On four of the five sampling dates, we also quantitatively measured chlorophyll-A in a subset

of mesocosms (8-11 per sampling date) with acetone extraction (Stevenson et al. 1996). Periphyton growth was measured in each mesocosm using two artificial substrates. Pre-scored 23 cm long acrylic rods (0.635 cm diameter) were deployed upright at the center and 5 cm by 10 cm sections of LDPE were hung on the wall of each mesocosm. Artificial substrates were scraped and processed for periphyton dry weight after 44 days (June 11-July 23) (Goldsborough et al. 1986; Stevenson et al. 1996).

Statistical analyses

We analyzed developmental endpoints at the specimen- and mesocosm-levels (Table 2). Specimen-level endpoints (e.g., snout-to-vent length, days to reach metamorphosis) were analyzed with mixed models that included fixed effects (atrazine, drawdown rate, interaction of atrazine and drawdown rate) and random effects (mesocosm, block, atrazine concentration*block and drawdown rate*block). Block effect was not significant ($\alpha = 0.05$), and therefore was excluded from analyses, keeping mesocosm as a random effect to account for correlation across specimens from the same mesocosm. Mesocosm-level endpoints (e.g., average weight, variation of weight, proportion to reach metamorphosis, survival) with analysis of variance (GLM model) with fixed effects (atrazine, drawdown rate, interaction of atrazine, and drawdown rate) and block as a random effect. Block effect was not significant ($\alpha = 0.05$) and therefore was excluded from ANOVA models. Significant fixed effects were identified based on $\alpha = 0.05$. The most conservative test would include the Bonferroni adjustment for multiple tests (which would reduce the cut-off value for significance within each set of analyses to α/# of tests); however, this adjustment was not applied because the response variables

were not independent (survival, growth, and development were correlated; see Appendix A Tables S3 & S4). Additionally, the Bonferroni adjustment was not applied because it greatly reduces power and increases the probability of false negatives (β), which is considered more costly than false positives in ecotoxicological research (Thursby et al. 1997). For significant fixed or interaction effects, pairwise comparisons were made with the Tukey-Kramer post-hoc test which includes an adjustment for multiple comparisons within each model ($\alpha = 0.05$). The effect of treatment on the relationship of weight to snout-to-vent length and weight to time to complete metamorphosis was evaluated with analysis of covariance (ANCOVA), assuming equal slopes. Treatment effects on water temperature, chlorophyll-A, and periphyton growth were evaluated with analysis of variance (GLM model) with fixed effects (atrazine, drawdown rate, interaction of atrazine and drawdown rate) and block as random effects. Endpoints were transformed with natural log as needed to normalize data and all analyses were completed with SAS 9.1 (SAS Institute, 2002-2010).

Results

For each experiment, atrazine concentrations were verified at the start to be within acceptable ranges, with mean concentrations for the 0.1, 20, and 200 µg/L treatments of 0.23, 21.9 and 206.8 µg/L (*R. sylvatica*) and 0.20, 22.3 and 197.9 µg/L (*R. pipiens*), respectively. Atrazine concentrations in the control and solvent control mesocosms were below the nominal minimum detectable limit of 0.05 µg/L.

Survival until the end of each experiment was very high across all treatments, at 94.2% (212/225) for *R. pipiens*, and 87.8% (253/288) for *R. sylvatica* (Table 1, Figures 1

C and D, Appendix A Figures S2 and S4). In both species, 6-8% of deaths occurred in the relatively brief period between Gosner stage 42 (initiation of metamorphosis) and Gosner stage 45 (completion of metamorphosis), and over half of all deaths occurred in the high atrazine concentration treatment. In the *R. sylvatica* experiment, atrazine concentration had a significant negative effect on survival ($F_{3,44} = 5.71$, $p = 0.0022$), with lowest survival among individuals exposed to the highest atrazine concentration (200 µg/L). The proportion of *R. sylvatica* dying during metamorphosis was significantly higher in the high atrazine concentration (Table 2, $F_{3,44} = 5.14$, $p = 0.0039$).

While overall survival was high in both species, developmental rate varied, with only 47.6% of *R. pipiens* completing metamorphosis during the experiment compared to 87.8% of *R. sylvatica* (Table 1). The proportion of *R. pipiens* larvae reaching metamorphic climax (forelimb emergence, Gosner stage 42) and completing metamorphosis (Gosner stage 45) was significantly different across atrazine treatments (Table 2, Figure 1B, Appendix A Figure S4, $\alpha=0.05$), with fewer reaching and completing metamorphosis in the high atrazine concentration treatment. Additionally, the developmental stage attained by the end of the experiment or collection (whichever came first) was significantly lower in high atrazine exposed *R. pipiens* (Table 2, $F_{3,148} = 4.65$, $p = 0.0039$).

Eighty-eight percent of *R. sylvatica* individuals completed metamorphosis (Table 1, Figure 1A); however development and growth were negatively correlated with atrazine concentration, evidenced by longer larval period (days to reach Gosner stage 42 and 45) and smaller metamorphic frogs in the high atrazine concentration treatments (Figure 2,

Table 2, α=0.05). Body condition was also negatively correlated with atrazine concentration, with the highest body condition index in control-exposure frogs and reduced BCI in frogs exposed to 0.1, 20, and 200 µg/L atrazine concentrations (Figure 3A, Table 2, $F_{3,215} = 4.88$, p= 0.0026). This result was also seen in the ANCOVA analysis, where there was a significant atrazine treatment effect on the relationship between weight at metamorphosis and snout-to-vent length, with the regression line for the non-atrazine exposed treatment significantly higher (intercept greater, with assumed equal slopes) than the three atrazine treatments (Figure 3A, R^2=0.68, p<0.001). We found no overall relationship between weight and number of days to complete metamorphosis (Gosner stage 45); however, there was a negative trend in the highest atrazine concentration with longer larval periods resulting in smaller frogs at metamorphosis (Figure 3B).

Although there was no independent effect of drawdown rate, there was an interaction effect between drawndown rate and atrazine concentration on the proportion of *R. pipiens* reaching Gosner stage 42 (p=0.048) and overall progression of development (p=0.034). In the accelerated drawdown treatment, development was slower in non-atrazine and high (200 µg/L) atrazine exposed, and faster in the intermediate (0.1 and 20 µg/L) atrazine exposed tadpoles. These significant interactions could have been driven by the slow development in the non-atrazine, accelerated drawdown mesocosms, which was the opposite of the hypothesized acceleration of development with accelerated drawdown. This resulted in only 13 of the 25 original tadpoles completing metamorphosis in the non-atrazine, accelerated drawdown treatment, compared to 19 of

the original 25 frogs completing metamorphosis in the non-atrazine, normal drawdown treatment

Water temperature was not significantly different in the normal and accelerated drawdown treatments for either experiment (α=0.05, Appendix A Table S7), likely because mesocosms were half-buried to simulate natural wetlands and prevent water temperature spikes with expected summertime air temperature fluctuations. Water temperature varied between the two experiments, with higher average temperature (18°C) in Experiment 1 (*R. pipiens*, 2006) than in Experiment 2 (17°C, *R. sylvatica*, 2009). Primary productivity (measured in Experiment 1, *R. sylvatica*, 2009) did not appear to differ by atrazine concentration or drawdown rate. Chlorophyll-A ranged from <0.4 to 73.2 µg/L during the experimental exposure, with one control mesocosm as a clear outlier with very high levels of chlorophyll-A (Appendix A Table S8). However, growth and development analyses that included or excluded data from this high-productivity mesocosm did not differ in results or significance level.

Relative *in vivo* chlorophyll-A differed by atrazine concentration and by sampling date, but the only significant effect was on the earliest date (June 11), when the 20 µg/L treatment had higher concentrations than all other treatments (ANOVA, α=0.05). Periphyton growth in the atrazine treated mesocosms did not differ from the control, however there was a significant difference between the 0.1 and 20 µg/L treatments (ANOVA, p=0.03), with the 20 µg/L treatment containing the highest and 0.1 µg/L containing the lowest amounts of periphyton growth (Appendix A Table S9).

Discussion

Exposure to 200 μg/L atrazine delayed development in both native frog species, but the consequences of that delay may differ, depending on the life histories of each species. For instance, since *R. sylvatica* breed and develop in temporary wetlands that typically dry out sometime during the summer, they may have a greater risk of desiccation and death if metamorphosis is delayed. It is not surprising that many amphibian species, including *R. sylvatica*, can adjust their developmental rates to some degree in response to changing hydrologic conditions (Newman 1992; Denver 1997). The ability to accelerate development may be adaptive in that it enhances the probability of survival to metamorphosis and a successful transition to a less water-dependent, terrestrial stage, but it usually comes at the cost of decreased size, which can reduce overwintering survival and recruitment (Duellman & Trueb 1994; Altwegg & Reyer 2003). By modifying developmental rates based on external biotic and abiotic cues, larvae can maximize potential benefit from increased growth when aquatic conditions are good, and minimize risk of mortality when they are poor. In our experiments, nearly all control and atrazine-exposed *R. sylvatica* completed metamorphosis, but frogs exposed to high atrazine concentrations took longer to develop and, contrary to the typical response to slower development, were smaller than the non-atrazine exposed metamorphic *R. sylvatica*.

This atypical response of slower development and smaller size observed in *R. sylvatica* experiments suggests that tadpoles exposed to 200 μg/L atrazine were under considerable stress and unable to develop at the same rate as those not exposed to atrazine. Longer development usually results in larger frogs at metamorphosis, while

accelerated development produces smaller organisms (Newman 1992; Álvarez & Nicieza 2002; Altwegg & Reyer 2003; Gervasi & Foufopoulos 2008). Interactions between developmental rate, metamorphic size, food availability, crowding, and other stressors have been noted (Álvarez & Nicieza 2002). Smaller size at metamorphosis has been associated with increased predation risk as well as reduced winter survival, growth rate, and adult breeding capacity (Duellman & Trueb 1994; Altwegg & Reyer 2003). Additionally, size at metamorphosis affected energy reserves and jumping ability in Iberian painted frogs (*Discoglossus galganoi*) (Álvarez & Nicieza 2002), which could impact juvenile survival.

Immune system stress may contribute to the significantly higher mortality rates observed during late developmental stages, particularly in the 200 μg/L atrazine treatment. During metamorphosis amphibians undergo complex reorganization and restructuring of tissues and organs as they transform from exclusively aquatic larvae to adults that are often more terrestrial. The shift from larval to adult immune systems includes a dramatic reduction in lymphocytes, stimulated by rising corticosteroid hormone levels late in development (Rollins-Smith 1998; Denver 2009). The decrease in lymphocytes, which can be up to 80%, may correspond to an increased vulnerability to pathogens in metamorphic amphibians. Field observations of mass death of metamorphic frogs (Carey et al. 1999) and increased mortality seen in our late-stage larvae support this notion of a window of vulnerability that may be especially evident when exposed to multiple stressors. In addition to this critical period during metamorphic transition,

reduced immune capacity has been found in *R. sylvatica* with experimentally shortened larval periods (Gervasi & Foufopoulos 2008).

In *R. pipiens*, exposure to 200 µg/L atrazine delayed development, as was seen with *R. sylvatica*; however, because of longer developmental period of this species, the delay resulted in fewer individuals completing metamorphosis by the end of the experiment (which was terminated at the end of the summer). *R. pipiens* typically breeds in long-lasting seasonal or semi-permanent wetlands and can overwinter as tadpoles in northern latitudes (Merrell 1977), which eliminates the absolute necessity to complete metamorphosis during one season. Differences in life history between the two species may explain their different responses to chemical stressors. Based on the trends and the results from *R. sylvatica*, we speculate that growth in *R. pipiens* was also affected by atrazine concentration, but that the effect was not detected due to the low number of *R. pipiens* completing metamorphosis (small sample size).

Growth and developmental responses to atrazine in both species could have been due to endocrine disruption and/or indirect effects resulting from disruptions in food supplies. Exposure to sublethal atrazine concentrations (10 µg/L – 40 mg/L) have been shown to alter time to metamorphosis in *Rana clamitans* (Coady et al. 2004), *Rhinella arenarum* (Svartz et al. 2012), and *Ambystoma tigrinum* (Larson et al. 1998). Physiologically, the balance between growth and development is thought to depend on neurohormonal stress responses to environmental conditions. Metamorphosis is accelerated and growth inhibited by thyroid hormones and corticosteroids (Denver 1997) and atrazine may affect development by disrupting the endocrine activity controlling

these hormones (Larson et al. 1998; Denver 2009). Chronic larval exposure to atrazine could induce corticosterone and thyroxine production by modifying an environmental cue (Larson et al. 1998), or could trigger an environmental stress response that releases corticotropin-releasing factor, which controls activity of thyroid and interrenal glands (Denver 2009). However, metamorphosis is only possible after the larva reaches a minimum size (Wilbur & Collins 1973), which depends on the availability of sufficient high-quality food.

Atrazine has been shown to affect growth and development by altering tadpole food supply (Boone & James 2003; Rohr & Crumrine 2005). Although we did not detect a significant effect of atrazine on periphyton or chlorophyll-A, food sources could have been reduced by the herbicide in the first few days of the experiment, during critical early larval development, and then recovered before sampling. This rapid recovery, as well as potential changes in food quality due to shifts to diatom-based communities, has been documented in studies of periphyton exposed to atrazine (Hamilton et al. 1987).

The lack of developmental response to accelerated drawdown rates was most likely because the critical volume of water per tadpole was not reached during the drawdown period. These experiments started with a lower tadpole density (16-20 L water per tadpole) than current recommendations of 10 L per tadpole (Boone & James 2005; Boone et al. 2007). By the end of the experiments, the accelerated drawdown mesocosms contained approximately 16 L each, or 4-16 L per remaining tadpole(s), which may have been sufficient water volume to prevent hormone production necessary for accelerated development. Tadpole growth and development are regulated by hormonal signals that

are cued by environmental conditions, such as proximity to the pond surface, depth of swimming area, and temperature (Denver 1997; Relyea 2005). Therefore, a second possible explanation for the absence of a developmental response to drawdown rates in our experiments is the lack of significant difference in water temperature between the normal and accelerated drawdown treatments. However, in experiments that controlled for temperature, development accelerated as a continual function of water level decrease (Denver et al. 1998). A third possibility is that the accelerated drawdown rate was not fast enough to induce early metamorphosis. However, the drawdown rates were determined based on rates shown to accelerate development in amphibian species (Denver et al. 1998; Gervasi & Foufopoulos 2008) and field observations of water loss during amphibian larval development in seasonal and semi-permanent wetlands in the Prairie Pothole Region (unpublished data).

Although no independent drawdown rate effect was detected in our mesocosm experiments, the developmental delay caused by atrazine could cause amphibians to be more susceptible to other environmental stresses. Slower development in natural settings means that organisms may be exposed to predators, competitors, and/or aquatic toxicants for longer periods, and may increase the window of vulnerability to pathogens associated with metamorphic immune suppression. In addition, a longer developmental period increases the risk of being caught in a drying pond, or the likelihood of failure to complete development (metamorphosis) during one growing season. For species like *R. sylvatica*, such a delay would mean death, while *R. pipiens* may be able to overwinter as tadpoles; however, they would then face increased susceptibility to a variety of other

challenges such as freezing, anoxic conditions, and predation, in addition to longer exposure to contaminants in the aquatic environment.

This research showed that chronic atrazine exposure during larval development had sub-lethal effects on growth and development. These effects, in turn, could impact amphibian populations through reduced annual recruitment and, potentially, survival of juveniles. Annual recruitment and adult survival are the two components necessary to maintain populations, and thus should be the endpoints used to understand how pesticides impact populations (Hayes et al. 2010b). Effects of atrazine on survival, growth, and development in both *R. pipiens* and *R. sylvatica* were detected only at the highest concentration (200 µg/L), with few differences at 0.1 or 20 µg/L, compared with no atrazine. Although 200 µg/L atrazine exceeds the expected environmental concentrations under current application rates (expected ~100 µg/L) (Rohr & McCoy 2010), concentrations up to 10 mg/L have been measured in runoff and surface waters near corn fields after precipitation events (Klaassen & Kadoum 1979; Hatfield et al. 1996; Edwards et al. 1997; Du Preez et al. 2005). Additionally, continued conversion of grassland to crop agriculture results in increased amounts of pesticides applied annually, and the current trend of increased corn plantings (Wright & Wimberly 2013) suggests that the responses found in this experiment may become more common in natural breeding wetlands.

Clearly, the amphibian larval environment affects survival to metamorphosis and immediately post-metamorphosis, and there can be carry-over and even permanent effects on growth, survival, locomotion, reproductive capacity, and immune functioning (Duellman & Trueb 1994; Denver 2009). Therefore, we conclude that altered amphibian

growth and development due to chronic larval exposure to atrazine may contribute to amphibian declines through reduced survival and reproductive capabilities, and that the likelihood of these negative effects will increase with the projected changes in land use and climate.

Table 1. Mesocosm experiment outcomes for *Rana pipiens* and *Rana sylvatica*. Overall percentage (and number out of total) for survival, death during metamorphosis, and reaching/completing metamorphosis. Mean ± standard deviation (range) for size, days for development, and stage attained.

Endpoint	Response variable	Northern leopard frog (*R. pipiens*)	Wood frog (*R. sylvatica*)
Survival	% survival to end of experiment	94.2% (212/225)	87.8% (253/288)
	% died during metamorphosis (Gosner stage 42-46)	6% (7/116)	8% (22/275)
Size	Snout to vent length at metamorphosis	24.7 mm ± 1.8 (21 - 29)	17.9 mm ± 1.4 (13 - 21.5)
	Weight at metamorphosis	0.99 g ± 0.26 (0.44 – 1.66)	0.39 g ± 0.10 (0.17 – 0.79)
	Body condition index (BCI = W/SVL)	0.042 ± 0.008 (0.026 – 0.059)	0.022 ± 0.004 (0.012 – 0.046)
Development	% to reach metamorphic climax (forelimb emergence, Gosner stage 42)	51.5% (116/225)	95.5% (275/288)
	% to complete metamorphosis (Gosner stage 45)	47.6% (107/225)	87.8% (253/288)
	Days to reach metamorphic climax (forelimb emergence, Gosner stage 42)	105 days ± 14.9 (<67[a] – 154)	52 days ± 4.5 (42 – 72)
	Days to complete metamorphosis (Gosner stage 45)	112 days ± 16.4 (67 – 162)	56 days ± 4.4 (47 – 76)
	Gosner stage attained by end of experiment (or collection at metamorphosis)	41.6 ± 3.7 (36 – 46)	45.8 ± 0.6 (42 – 46)

[a] earliest forelimb emergence missed in *R. pipiens* (seen at Gosner stage 45 on day 67)

Table 2. Statistical results of specimen (mixed models) and mesocosm (ANOVA) outcomes of growth, development, and survival in *Rana pipiens* and *Rana sylvatica* after chronic larval exposure to atrazine (0, 0.1, 20, 200 µg/L) and two rates of pond drawdown (normal, accelerated). NS = no significant effects.

	Rana pipiens		***Rana sylvatica***	
Specimen-level endpoints	**significant factor(s)[b] F_{df}, p**	**diff from control[c]**	**significant factor(s)[b] F_{df}, p**	**diff from control[c]**
BCI = Weight/ Snout-to-vent length	NS		Atrazine $F_{3,215} = 4.88$, p= 0.0026	0.1, 20, 200
snout-to-vent length, mm[a]	NS		NS	
weight, g[a]	NS		Atrazine $F_{3,215} = 3.76$, p= 0.0117	20, 200
days to reach Gosner stage 45[a]	NS		Atrazine $F_{3,227} = 5.72$, p= 0.0009	200
Gosner stage attained by end of experiment (or collection at metamorphosis)	Atrazine $F_{3,148} = 4.65$, p= 0.0039	200	NS	
Mesocosm-level endpoints				
proportion survived to end of experiment[a]	NS		Atrazine $F_{3,44} = 5.71$, p= 0.0022	200
proportion to reach Gosner stage 42[a]	Atrazine $F_{3,36} = 4.64$, p= 0.0076	200	NS	
proportion died during metamorphosis, Gosner stage 41-45[a]	NS		Atrazine $F_{3,44} = 5.14$, p= 0.0039	200
proportion to reach Gosner stage 45[a]	Atrazine $F_{3,36} = 3.10$, p= 0.0387	200	NS	
variance of days to reach Gosner stage 45[a]	NS		NS	

[a] Natural log transformation; [b] Significant fixed factors based on at $\alpha = 0.05$. The most conservative test would include the Bonferroni adjustment for multiple tests (with 10 models for each experiment, the cut-off value for significance would be $\alpha/10 = 0.05/10 = 0.005$); however, this adjustment was not applied because response variables were not independent (survival, growth and development are correlated) and this adjustment greatly increases the probability of false negatives (β), and, thus, greatly reduces power.; [c] Pairwise comparisons for significant factors with the Tukey's post hoc test, with levels identified as different from control based on $p < 0.05$ with Tukey-Kramer adjustment for multiple comparisons.

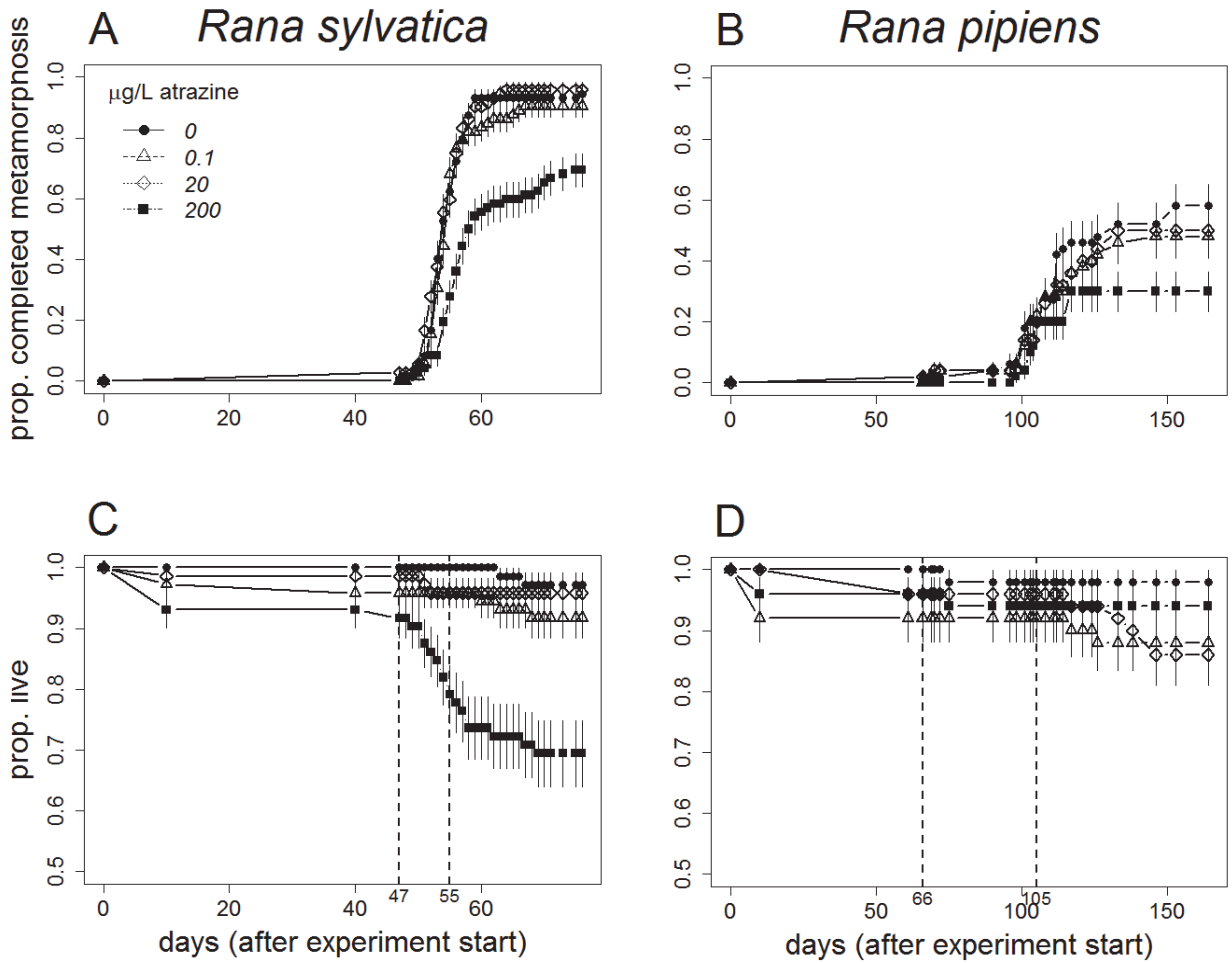

Figure 1. Metamorphosis (A, B) and survival (C, D) curves by treatment with standard error bars for *Rana sylvatica* (A, C) and *Rana pipiens* (B, D). Filled circle for 0 µg/L, open triangle for 0.1 µg/L, open diamond for 20 µg/L, filled square for 200 µg/L. Panels C and D have dashed lines for the earliest and average number of days to metamorphosis.

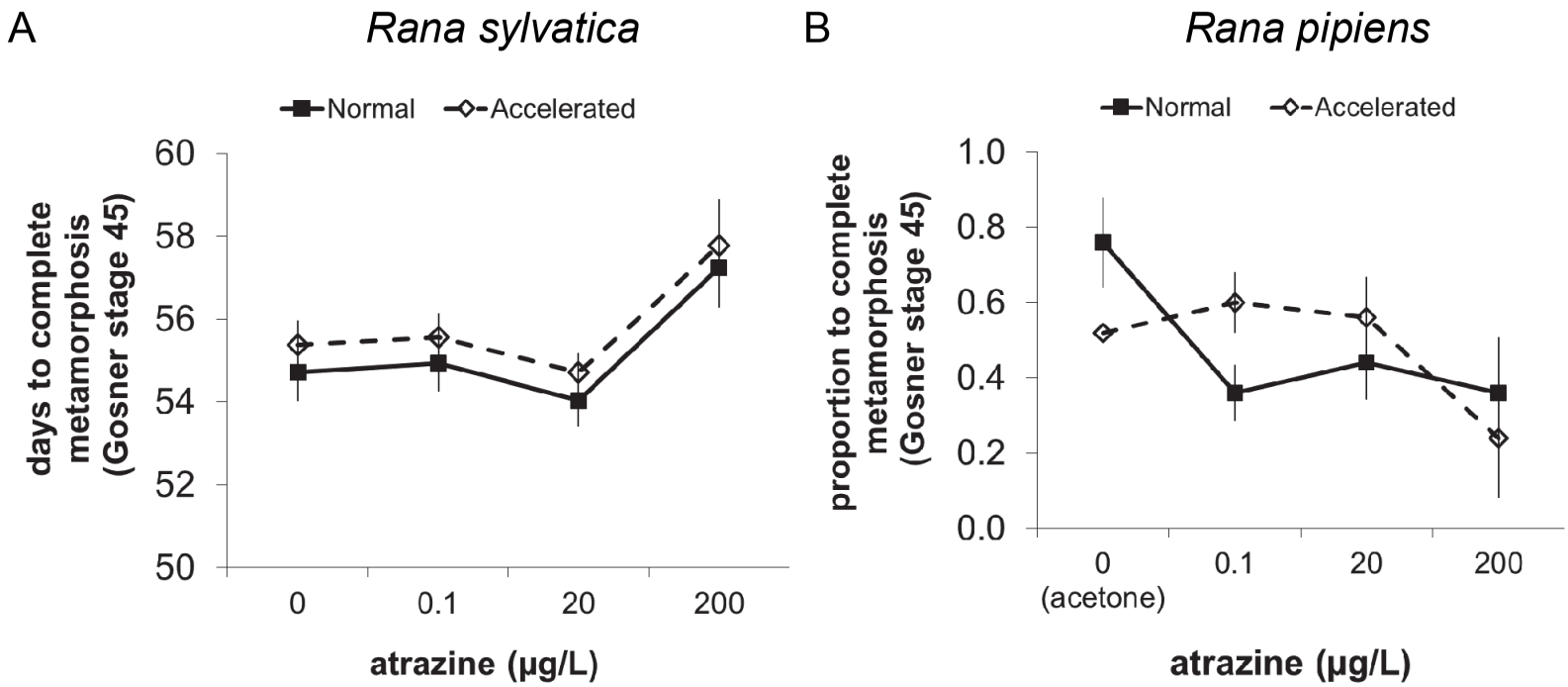

Figure 2. Development was delayed in the high atrazine treatment for both frog species. A. In *Rana sylvatica*, number of days to complete metamorphosis, Gosner stage 45 (mean with SE), was significiantly higher in the high atrazine concentration treatment compared to control $p \leq 0.01$ (ANOVA).The number of days to reach metamorphic climax (forelimb emergence, Gosner stage 42) had the same pattern ($p<0.05$, data not shown). B. In *Rana pipiens*, the proportion of specimens completing metamorphosis, Gosner stage 45 (mean with SE) was signficantly lower in the high atrazine concentration treatment compared to control $p \leq 0.05$ (ANOVA), while the % reaching metamorphic climax (forelimb emergence, Gosner stage 42) had same pattern ($p<0.01$, data not shown).

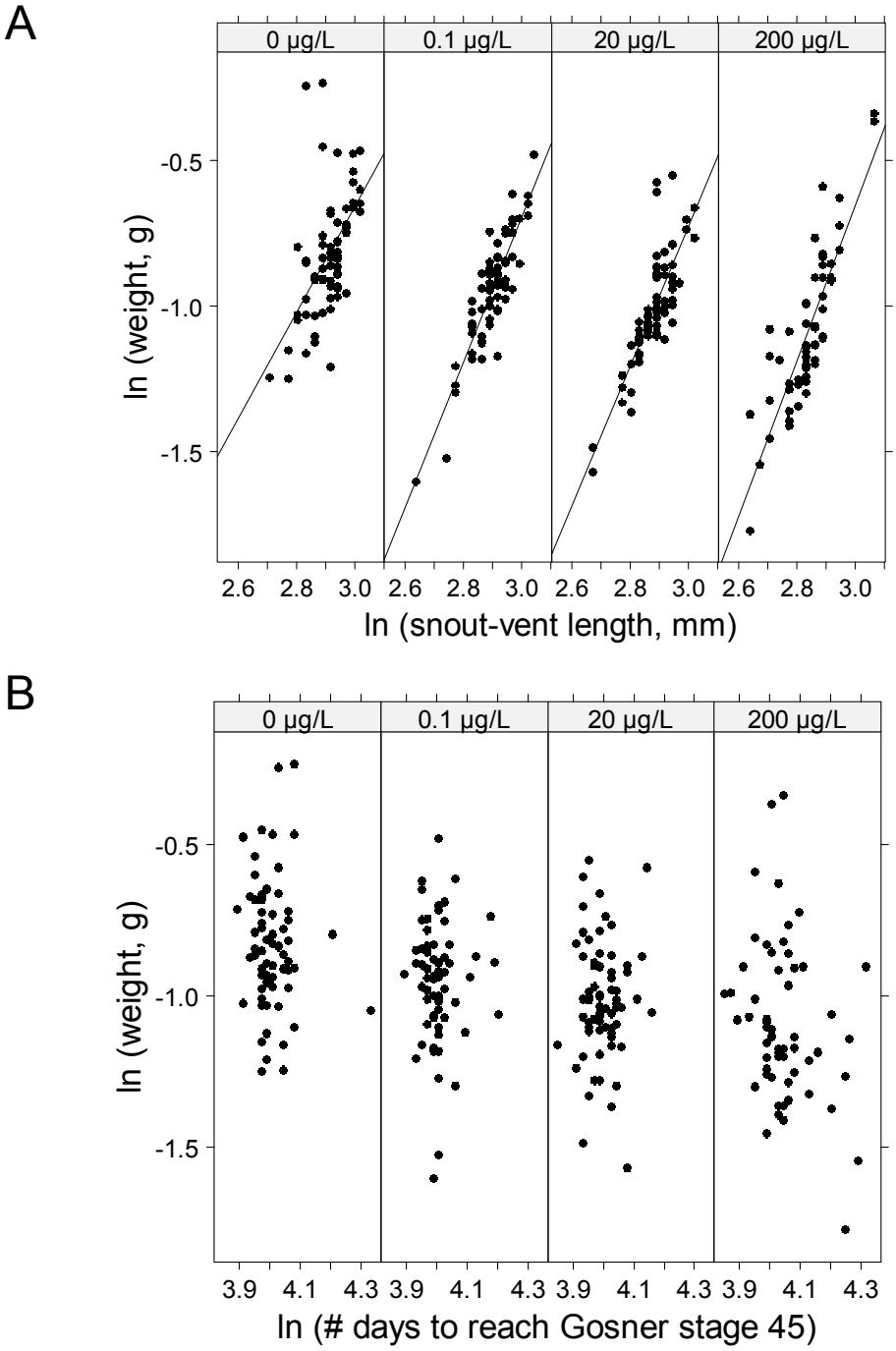

Figure 3. Relationship of *Rana sylvatica* weight at metamorphosis with (A) snout-to-vent length and (B) number of days to complete metamorphosis (Gosner stage 45) with simple linear regression best fit line by atrazine concentration. Panel A linear regression significant ($p<0.001$); control (0 µg/L) significantly higher in weight and length (ANCOVA, $p<0.001$). Panel B linear regressions not significant ($\alpha=0.05$); control (0 µg/L) weight significantly higher than all atrazine treatments (ANCOVA, $p<0.001$).

Chapter 2. An assessment of testicular ooctyes in field-captured *Rana pipiens*

Introduction

Endocrine disrupting chemicals (EDCs) in aquatic ecosystems create environmental problems of great concern for wildlife and potentially for human reproductive health (WHO 2003). A multitude of chemicals are known or expected to have endocrine-disrupting effects, and a variety of biomarkers, including gonadal anomalies, altered hormones, and induction of gender-specific proteins (Houtman et al. 2011) have been described. The presence of oocytes in testicular tissue, hereafter called testicular oocytes or TOs, has captured the attention of the public and is often used as an endpoint in assays for endocrine-disrupting substances in fish (Johnson et al. 2010a) and amphibians (Hecker et al. 2006).

While TOs are commonly used as a gonadal pathology endpoint in multiple species, definitive protocols do not exist for assessing TOs, which makes comparisons between studies and species challenging. TOs have been documented in frogs after lab exposures to known and suspected EDCs (MacKenzie et al. 2003; Hayes et al. 2003; Coady et al. 2004; Jooste et al. 2005), as well as in wild populations across a wide range of habitats (Hayes et al. 2003; McDaniel et al. 2008; Du Preez et al. 2009; Papoulias et al. 2013). TOs have also been used as biomarkers for environmental contamination in field surveys of fish (Hinck et al. 2009; Blazer et al. 2012) and frogs (Hayes et al. 2003; Skelly et al. 2010), with agricultural run-off and wastewater treatments plants usually implicated as EDC sources. However, even though TOs are commonly assessed in EDC studies, the field lacks consistent terminology and standardized criteria in gonadal evaluations.

Hecker et al.(2006) addressed terminology issues and provided general terminology recommendations for fish and amphibians. For instance, TOs are frequently referred to as intersex, but are sometimes also called testi-ova or ovatestis, with slightly different descriptions for each term (Hecker et al. 2006). In addition to the general terminology recommendations provided by Hecker et al. (2006), there are currently recommended methodologies specific to several fishes, including smallmouth bass (Blazer et al. 2007) and flounder (Bateman et al. 2004). Developing standardized methods for evaluating reproductive anomalies including TOs with biologically relevant metrics is essential to make progress researching environmental EDCs.

The objectives of this study were to evaluate TO distribution in wild metamorphic *Rana pipiens* (northern leopard frog), to determine appropriate sampling technique, and to develop recommendations for assessing TO presence, abundance, and severity in individuals and within natural breeding locations. Specifically, we tested the hypothesis that TOs were uniformly distributed throughout *R. pipiens* gonads and identified the best methods to assess or score TOs for individual specimens and TO prevalence in natal wetlands.

Methods

Study wetlands and frog collections

Metamorphic *R. pipiens* were collected from 11 wetlands in the Prairie Pothole Region (PPR) across a range of potential exposure to anthropogenic stressors from the highly agricultural and developed region of north-central Iowa to the grassland-dominated region of central North Dakota as described in detail in Schoff et al. (*In*

review). Briefly, metamorphic *R. pipiens* were collected by dip net from seasonal and semi-permanent prairie wetlands with one wetland in IA, five wetlands in SD, three wetlands in MN, and 2 wetlands in ND. These wetlands were targeted for frog collections from 149 wetlands surveyed across the U.S. portion of the PPR east of Montana based on evidence of *R. pipiens* breeding and range of potential exposure to EDCs, including agricultural chemicals.

We collected 531 *R. pipiens* at or near metamorphosis (Gosner stages 44-46, Gosner 1960) between July 6 and August 3, 2005, before they left their natal pond, targeting collection of at least 50 individuals per wetland. We targeted *R. pipiens* because it is widespread, associated with grassland wetlands throughout much of North America, and has been used as a benchmark North American amphibian species for ecotoxicology and developmental studies, including endocrine disruption (Hayes et al. 2003, 2006a; Orton et al. 2006; McDaniel et al. 2008; Rohr et al. 2008; Van Schmidt et al. 2012).

Frogs were measured (snout to vent and snout to tail, if present), staged (Gosner 1960), euthanized with overdose of tricane methoanesulfonate (MS-222), and preserved in Bouin's solution (Sigma, St. Louis, MO) for 48 hours and then stored in 70% ethanol until dissection. Sex was determined based on gross gonadal morphology at dissection, with 266 identified as males (gonads from one male were unusable due to damage). The gonad-kidney complex was removed for histological analysis, with excess fat body and portions of the kidney trimmed to fit cassettes. Tissue was processed using standard methods through a series of xylenes and alcohols and finally embedded in paraffin. Both gonads were longitudinally step-sectioned in their entirety with 5 μm sections and 50 μm

steps in order to intersect any TO in the gonad only once. Slides were stained with hematoxylin and eosin and viewed with an Olympus BH-2 compound microscope to identify and count TOs.

TO assessments

Abundance and severity of TOs were evaluated in the 265 males collected across all 11 wetlands. TOs were defined using the following criteria: 1) oocytes within male specimens (based on gross gonadal morphology) with some normal testicular tissue, and 2) containing intact nucleus with nucleoli. All sections of each specimen were evaluated for presence and number of TOs. For each specimen, a representative section from the mid-portion of the gonad was selected for determination of severity and establishing relative gonadal size. This section was identified as the largest section within two sections (~100 μm) of the median section.

The general location of TOs were categorized by their presence in sections, categorized as occurring in mid-gonad versus outer edges (either ventral or dorsal from middle), and evaluated in more detail for the representative section, where TO presence was documented in 8 regions of the gonad. The gonad was divided three ways (anterior versus posterior, cortical versus medullar, and outer versus medial), which overlapped to create 8 regions (Appendix B Figure S1). With this method, each region could be characterized by all three division methods; for example, the anterior portion consisted of four regions (1-4), with one anterior/cortical/medial (1), one anterior/cortical/outer (2), one anterior/medullar/medial (3), and one anterior/medullar/outer (4). At the specimen-level, TOs were assessed by total number of TOs per gonad, average number of TOs per

section, number of TOs normalized by relative size of gonad, and severity score (see below) of the representative section. Relative gonadal size was measured as areal extent (mm^2) of representative section with ImageJ (Rasband 2014, http://imagej.nih.gov/ij/) and digital pictures of representative sections taken on an Olympus BH-2 compound microscope on 4X magnification with an Olympus SP-350 digital camera and Cam2Com Picture Software (http://sabsik.com/Cam2Com/).

Severity scores were developed based on a validation set of *R. pipiens* gonads from a mesocosm exposure experiment (Olker 2014a) similar to those created for several fish species (Bateman et al. 2004; Blazer et al. 2007). To define severity scores, testicular sections were prepared as outlined above for 49 male *R. pipiens* exposed to presumptive estrogenic compounds. TO prevalence was evaluated in all testicular sections to establish range (0-165 per specimen, 0-27 per section), and images representing a continuum of TO prevalence were selected. We verified that scoring a representative median section was comparable to scoring all sections within a specimen (data not shown). Scores ranged from zero (no TOs) to 5 (severely impacted) (Figure 1) and were based on examination of the entirety of one representative gonadal section. A score of 0 indicated that no testicular oocytes were observed. A score of 1 indicated one TO, and 2 indicated two TOs in the representative section. Scores above 2 indicated multiple TOs in the section, with scores of 3 and 4 representing moderate and severe and score of 5 indicating that portions of the gonadal section are dominated by oocytes.

Statistical Procedures

Specimen-level analyses. To assess the location of TOs in the gonad we included only Gosner stage 46 males with TOs (n=86, from 11 wetlands). We tested the null hypothesis that TOs were uniformly distributed with a paired t-test per specimen to compare the number of TOs in sections from the left versus the right gonad, and sections from the middle (80%) versus the outer (20%). Across all specimens, we also compared total number of TOs in left versus right gonad with a t-test, and anterior v. posterior/cortex v. medulla with a χ^2-test.

To determine the best method to assess or score TOs, we compared methods to summarize the presence and abundance of TOs per specimen (Table 1 and Appendix B Table S1, total, mean per section, total normalized for gonad section area, total and mean per section in subsets of sections, number in representative section, and severity score of representative sections) with all stages (n=264, 1 specimen excluded due to damage). Total TOs per specimen, mean per section, total number normalized by relative gonadal size, and number in representative section were compared using correlation coefficients. For each specimen, subsets of sections were summarized for TO presence, abundance, and mean number per section to determine whether collecting or evaluating fewer sections would change the assessment outcome. Subsets containing 25% (every 4th section) and 10% (every 10th section) from the middle 80% of the testes were compared to the mean number of TOs per section with correlation coefficients and linear regression (using mean from multiple subsets). Severity scores were then compared based on

differences in total TOs, average TOs/section, and total TOs normalized by gonad section area with 1-way analysis of variance (ANOVA) of square-root transformed data.

Wetland-level analyses. To determine the best method for evaluating TOs at the wetland-level, we used all males at all stages (Gosner stage 44-46, Gosner 1960), to mimic realistic field collection (n=261 with 4 specimens excluded due to damage or different collection date). TO prevalence was compared across the methods described above to evaluate specimens. To identify the number of males needed to detect the presence and estimate severity of TOs we first used a probabilistic approach to identify target sample size based on population prevalence. Assuming TO prevalence to be 15, 30, or 50% (as detected in these wetlands), collecting 10 males will result in an 80, 97 or 99% chance, respectively, of collecting at least one with TOs (using the probability of finding no TOs equals (1-prevalence)^sample size). We used these results to sub-sample six wetlands (those with at least 10 males in original collection) to calculate average and variation in TO prevalence and severity (see Appendix B Table S2).

All statistical analyses were completed with SAS 9.1 (SAS Institute 2002-2008) with transformations to normalize variables when necessary.

Results

Gonadal distribution of TOs

TOs were found throughout the gonad, with higher probability of presence in the middle 80% of sections, but with no other trend in location within the gonad. The outer 20% of the longitudinal sections (10% ventral, 10% dorsal) had significantly fewer TOs than the middle 80%, with mean total of 1.4 ± 0.34 S.E. compared to 14.4 ±2.6 S.E.

(Appendix B Table S3, $p<0.0001$ for paired t-tests overall, all but three specimen t-tests: significant at $\alpha=0.05$). There were no differences in total number of TOs, mean TOs/section, or severity between left and right gonads within individuals (t-tests, $\alpha=0.05$) or across all specimens (paired t-tests, $\alpha=0.05$). Within the representative (~median) section TOs were evenly distributed, with no difference between anterior and posterior or cortex and medulla (χ^2 test with even distribution for expected values, $\alpha=0.05$, Appendix B Table S4).

TO presence, abundance, and severity

All methods for assessing TOs in specimens were significantly correlated ($p<0.0001$), with Pearson correlation coefficients ranging from 0.60 to 1.0 (Table 1). Total number of TOs was correlated with other methods of evaluating TOs with coefficients of 0.62 and greater, with very high correlations (≥ 0.97) with total number of TOs detected in subsets of 80, 25, and 10% of sections. Similarly, mean TOs/section had very high correlations (≥ 0.98) with mean TOs/section detected in subsets of 80, 25, and 10% of sections. The total number of TOs in the middle 80% has a nearly 1:1 relationship with the total number of TOs (linear regression, slope = 0.9, R2=0.99, $p<0.001$), while the other subsets and representative section have significantly positive linear relationships with slopes less than 1 (Appendix B Figure S2, $p<0.001$).

Specimen TO presence/absence did not change with assessment method, even when subsets of sections were evaluated. If only 10% of sections were evaluated, TO presence was detected in 93% (106/114) of specimens containing TOs and the average number of TOs per section for most specimens did not deviate by more than two TOs (a

conservative estimate of biological relevance). With sub-sampling 10% of the sections, the average number of TOs per section deviated less than two for 97% (111/114) of TO-containing specimens, based on an average of 10 sub-sampling events. When all sub-sampling events are compared separately, the average number of TOs/section deviated less than two for 73% (83/114) of TO-containing specimens. As expected, the number of deviations decreased when more sections were evaluated. Sub-sampling 25% of sections resulted in the average number of TOs deviating no more than two in 98% of specimens, for an average across 4 replications, and in 92% of specimens when all replicates were evaluated separately.

The number of TOs (total, average, and normalized by area) were significantly different across severity scores (Figure 2, ANOVA, $p<0.001$, average number of TOs not shown), ranging from 0-21 (mean 0.6) total TOs in the 0 category, and 34-169 (mean 107.2) total TOs in the 4 category. Only scores of 1 and 2 were not significantly different from one another (Tukey's post-hoc test, $\alpha=0.05$); however, a severity score of 5 was only assigned to one specimen which had 138 TOs total (average of 5.1 per section and 93/mm^2 gonadal section).

Wetland-level. For field collections, TO prevalence changed very little across methods to evaluate specimens (Figure 3). Wetland-level TO prevalence did not decrease greatly when evaluated with severity score or fewer sections per specimen (80, 25, or 10%). Wetland-level TO prevalence was also unchanged when 10 males were sub-sampled (Figure 3, 10 replicate sub-samples not significantly different from original prevalence, t-test by wetland with $\alpha=0.05$).

Discussion

This study demonstrates that the even distribution of TOs throughout the middle of *R. pipiens* gonads allows sub-sampling of sections without suffering a loss of information on TO presence or relative abundance. The very high correlations across sampling methods provides justification for efficient sub-sampling rather than counting each TO in serial samples of entire gonads, as recommended by various authors (Hecker et al. 2006). Evaluating fewer sections proved to be an effective method for detecting TOs and quantifying relative abundance. This sampling method assesses relative abundance, severity, and regions of gonad impacted, which is more efficient and cost-effective and may be more biologically relevant than quantifying the total number of TOs per gonad.

The location of TOs within gonads may provide insight on the pathway and timing of TO development. If male primordial germ cells receive signals to become oocytes before migration to the genital ridge early in gonadal differentiation, we might expect TOs to follow normal oogonia patterns of development in which gonial cells remain in the cortex where they divide and grow (Ogielska 2009). However, the frequency of TOs was the same in inner and outer regions of gonadal sections, which suggests that TOs derive from gonial cells that have already migrated into the medulla, following normal spermatogonia patterns. From this observation, we could assume that signaling initiating formation of TOs may occur during migration of gonial cells into the medulla, a developmental stage that has previously been identified as a 'sensitive-window' for steroid exposure in *Xenopus laevis* (Villalpando & Merchant-Larios 1990).

Gonad differentiation in many anurans is thought to follow the differentiated pattern in which testes and ovaries develop directly from undifferentiated tissue, with no intermediate mixed-sex or all-ovary stage (Ogielska 2009); however, in some species there is evidence that TOs are a normal, transient part of gonadal development (Storrs-Méndez & Semlitsch 2010). In normal testicular development the elongated undifferentiated gonad becomes progressively shortened and bean-shaped, with the anterior portion developing into the testis and posterior portion being lost. If TOs are normal, transient cells in frog testes, they could be more common in the regions that are lost during later gonadal development such as the posterior region. However, this idea was not supported by evaluations of TO location in these metamorphic *R. pipiens*, as TOs were not more frequently found in the posterior regions. Additionally, no substantial differences in TO prevalence or abundance were observed across developmental stages, as described in Schoff et al.(*In review*).

Recommendations

Based on this work, we recommend that the presence of frog TOs can be assessed using severity scoring of 2-4 longitudinal sections from the middle of gonads from 10 males per wetland. Our recommendations are to: 1) embed gonadal tissue to accommodate sectioning longitudinally, 2) face the block to approximately the middle of the gonad, 3) mount and stain four step-sections (50 µm between steps), and 4) score the worst-case severity observed in the mounted sections for each specimen. For TO prevalence and abundance, 2-8 sections were sufficient for specimen evaluation. For many specimens every 10^{th} section (1-6 sections) was sufficient for TO evaluation,

however for 7% (8/114) of the specimens this method missed TOs, and for up to 27% (31/114, across all replicate sub-samples) it underrepresented the average number of TOs. We recommend collection of four step-sections from the middle of the gonad as the most efficient method to obtain multiple sections for mounting, staining, and scoring.

The severity scoring method proved to be an efficient and effective method to evaluate specimens. The time to score one section was a tenth or less of that needed to count all TOs in all sections, and the number of TOs (total and normalized by gonadal section area) was significantly different across the range of scores. While serial sectioning or 50 μm step-sections through entire gonad are clearly not necessary, severity scores based on one representative section missed detecting TO presence in 18% (21/114) of TO-containing specimens and were not able to distinguish specimens with total number of TOs in the 10-20 range (Figure 2, scores of 1 and 2). Therefore, we recommend evaluating the four sections collected from the middle of the gonad and scoring the worst-case severity per specimen. Severity scores can then be statistically evaluated with the Rao-Scott Cochran-Armitage by Slices (RSCABS) test that includes severity of individual specimens and frequency of severity scores for experimental designs that have replicate subjects within each tank, aquarium, or mesocosm (Green et al. 2014).

While further evidence of the impact of TOs on reproductive outcomes is needed, scoring severity or normalizing the number of TOs by gonadal size are likely more biologically relevant methods than simply counting the total number of TOs. Also, given the variation in body size (snout-to-vent length) and gonadal size, the number of TOs

might not be as important as the proportion of the gonad impacted or severity score. When the number of TOs (total or average) is used as an endpoint, it should be normalized across specimens based on a common measure of gonad size such as weight, area, or areal extent of maximum section (a representative section from the middle of the gonad was used here).

While common, merely reporting the presence/absence of TOs in a specimen or at a site can obscure variation in severity. For instance, in this study, potential biologically relevant differences would not be captured by presence/absence figures when the number of TOs per affected specimen ranged from 1-223 and prevalence by wetland ranged from 16-100% (max of 94% if excluding wetlands with only one male). Wetlands with higher TO prevalence tended to have higher average severity scores; however some of the most affected specimens (severity scores of 3 and above) were found at wetlands with low prevalence. This was also seen in wild roach (*Rutilus rutilus*) where the specimen with the highest TO severity (referred to as intersex index) was found at a site with low TO prevalence (Bjerregaard et al. 2006). While severity scoring has been used for TO evaluation in fish (Bateman et al. 2004; Bjerregaard et al. 2006; Blazer et al. 2007) and is common in other histopathological endpoints (Wolf et al. 2010; Green et al. 2014), it has not been regularly applied to TO evaluation in frogs. In the one exception that we are aware of, TO severity was evaluated for wild *R. pipiens* and *R. clamitans* (green frog) juveniles and adults based on number and distribution of TOs in the testes (McDaniel et al. 2008).

Evaluating 10 males per wetland using the recommended specimen-level assessment methods was sufficient to detect wetlands with TOs as well as to estimate TO prevalence and severity. This is a much lower number than targeted in our research plan (n=50) as well as numerous reported field collections designed to evaluate TOs (Murphy et al. 2006a; Du Preez et al. 2009; Papoulias et al. 2013); however, it is very similar to current recommendations for TO assessment in smallmouth bass (Blazer et al. 2007).

Notably, for our two wetlands with high TO prevalence (>75%), specimen-level assessment did not change the TO prevalence, which remained >76%, even when assessed with only the representative section and with sub-samples of 10 males (Figure 3). Maximum severity at these wetlands was 4 and 5 (n=56 and 18 males, respectively), and remained high even with smaller sample sizes (maximum severity score of 3 or greater for all sub-samples of 10 specimens). For the two wetlands with the lowest TO prevalence (<17%), the specimen- and wetland-level assessment methods also resulted in comparable TO prevalence (10 and 16%, Figure 3); however, the maximum severity scores varied more than one score from the original 2 and 4 (n=51 and 61 males, respectively) in 30-50% of the subsamples of 10 males. Although TO prevalence was more variable for wetlands with intermediate prevalence or small sample sizes (Figure 3), differences in TO prevalence across specimen-level and wetland-level assessment methods were generally less than 14% different from original prevalence, with the greatest difference at wetland #12 (53% observed versus 27% based on severity score of a representative section). These results suggest that fewer specimens need be collected and fewer sections per specimen need to be assessed to collect accurate, biologically

relevant data, which saves time and money. For field studies, these sampling methods also result in a much smaller collection of frogs than current standard procedures, which may reduce the impact of this research of frog populations.

We hope these recommendations will provide guidance on sample size, sectioning protocol, and assessment methods to standardize future evaluation of TOs in frogs with methods that are effective, repeatable, and biologically relevant. Standardized methods are essential for comparison across studies and experiments, as well as further evaluation of the impact of TOs on reproductive impacts.

Table 1. Pearson correlation coefficients of specimen testicular oocyte (TO) evaluation methods for wild-caught metamorphic (Gosner stage 44-46) *Rana pipiens* from 12 wetlands in the Prairie Pothole Region. All methods had statistically significant positive correlations ($p<0.0001$). Data shows TO-containing specimens (114/265); analysis of all specimens or only Gosner stage 46 had nearly identical results (data not shown).

		All sections			***Middle 80%***		***Sub-sample: 25%[a]***		***Sub-sample: 10%[b]***		***Severity: 1 section[c]***	***Representative section[c]***	
		#TOs	**Mean TOs**	**#TOs/ area**	**Total TOs**	**Mean TOs**	**Total TOs**	**Mean TOs**	**Total TOs**	**Mean TOs**	**Max Sev**	**#TOs**	**#TOs/ area**
All sections	**# TOs (total)**	1.00											
	Mean TOs	0.89	1.00										
	#TOs/ area	0.87	0.77	1.00									
Middle 80%	**# TOs**	0.99	0.88	0.85	1.00								
	Mean TOs	0.89	0.99	0.77	0.89	1.00							
Sub-sample: 25%	**# TOs**	0.99	0.86	0.85	0.99	0.87	1.00						
	Mean TOs	0.89	0.99	0.76	0.88	0.99	0.88	1.00					
Sub-sample: 10%	**# TOs**	0.97	0.93	0.88	0.97	0.94	0.96	0.93	1.00				
	Mean TOs	0.87	0.98	0.75	0.87	0.99	0.84	0.97	0.93	1.00			
Severity: 1 section	**Max Sev**	0.62	0.60	0.62	0.63	0.62	0.61	0.60	0.65	0.61	1.00		
Repre-sentative section	**# TOs**	0.84	0.80	0.76	0.83	0.81	0.83	0.80	0.86	0.79	0.70	1.00	
	#TOs/ area	0.67	0.68	0.76	0.67	0.70	0.65	0.67	0.74	0.70	0.68	0.90	1.00

[a] based on mean of 4 sub-samples each consisting of 25% of sections from the middle 80%, [b] based on mean of 10 sub-samples each consisting of 10% of sections from the middle 80%, [c] representative section was largest section within 100 um of the median section

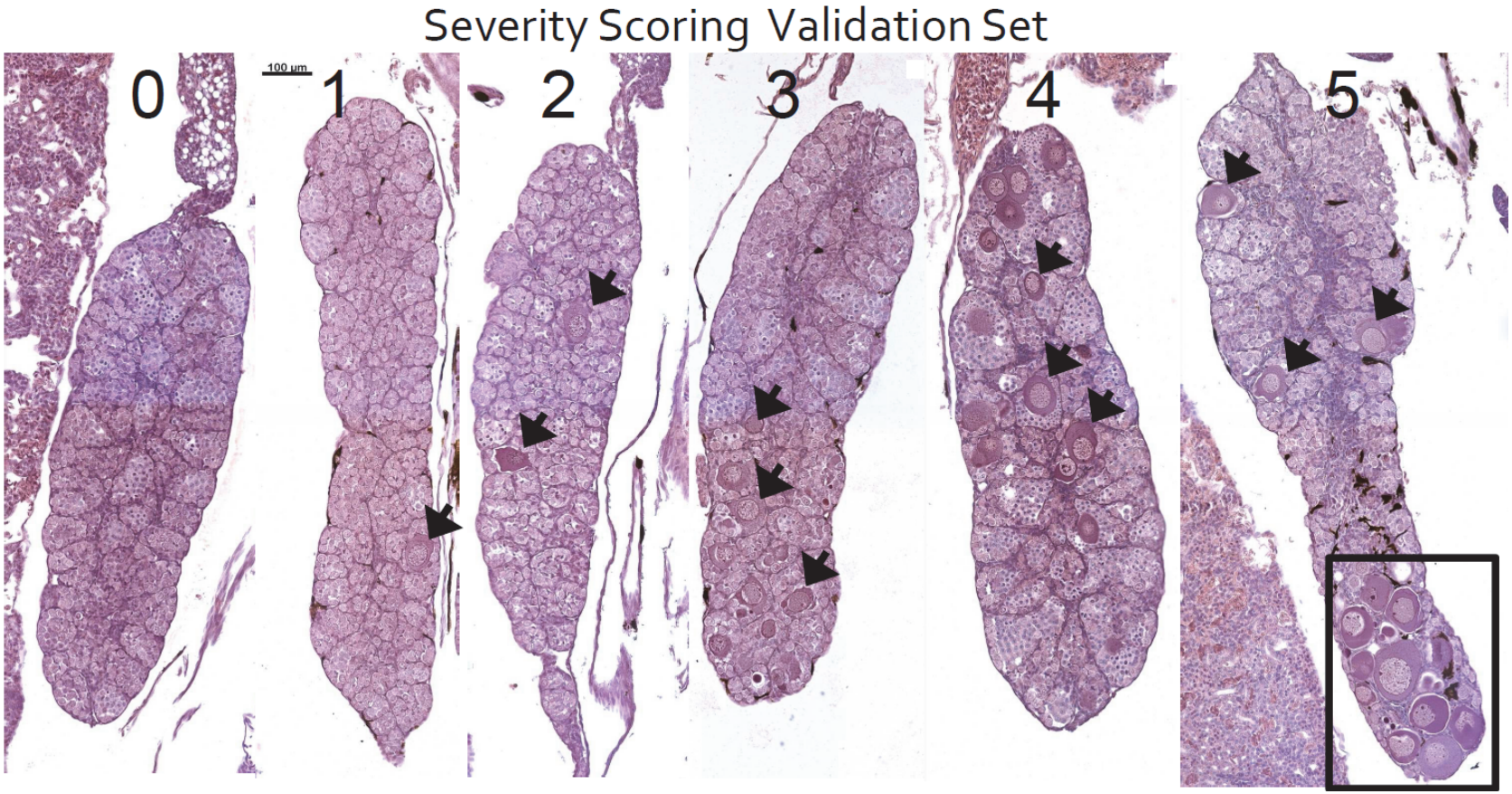

Figure 1. Severity scoring from zero (0) or no testicular oocytes (TOs) to 5 or severely impacted. Validation set examples from mesocosm-raised *Rana pipiens* (Olker 2014a). A score of 1 indicated one TO, and 2 indicated two TOs in the representative section. Scores above 2 indicated multiple TOs in the section, with scores of 3 and 4 representing moderate and severe and score of 5 indicating that portions of the gonadal section are dominated by oocytes. Arrows indicate examples of TOs, box around section of testis dominated by ooctyes.

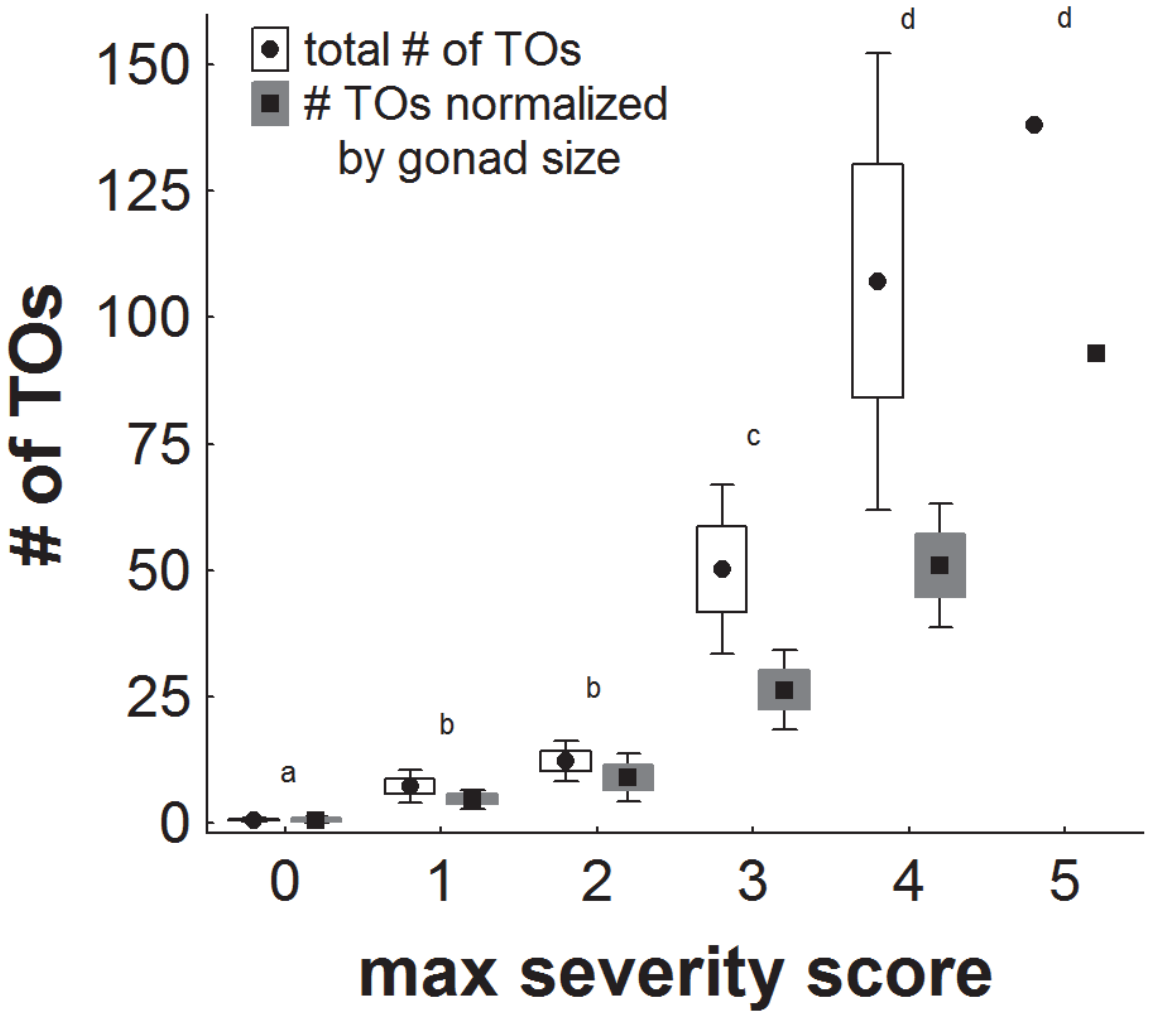

Figure 2. Number of testicular oocytes (TOs), total and normalized by gonad section size, by severity score (max of left and right representative sections); average with 2 SD box and 95% CI whiskers, letters indicate significant differences for both total and normalized TOs ($p<0.001$, ANOVA with square root transformation and Tukey's post hoc test with unequal sample size). Average number of TOs showed same pattern (data not shown, $p<0.001$, ANOVA with square root transformation and Tukey's post hoc test with unequal sample size).

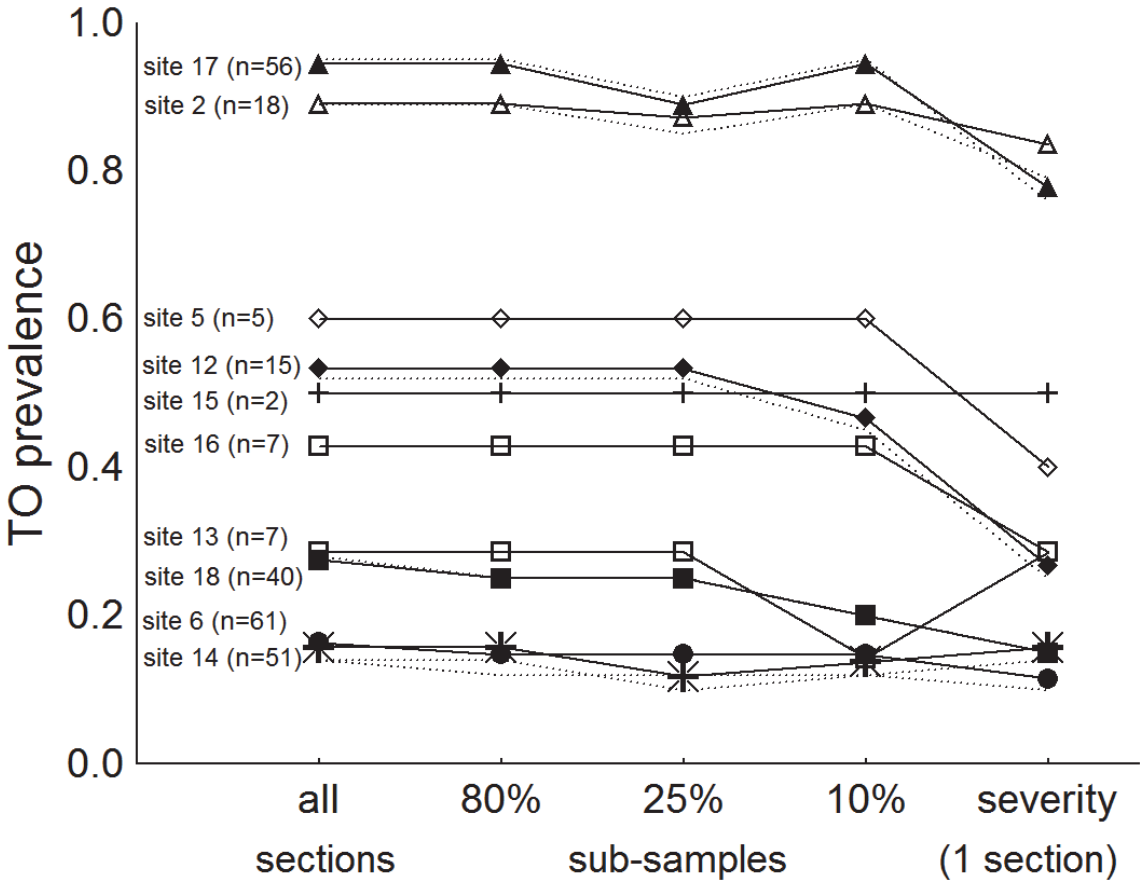

Figure 3. Prevalence of testicular oocytes (TOs) across assessment methods by collection wetland, with solid lines representing TO prevalence based on specimen-level assessment methods and dashed lines representing the mean values from 10 resampling events to collect 10 males for the 6 wetlands with at least 10 males in original sampling event. All stages included (Gosner 44-46) with 4 specimens excluded from wetland-level analysis due to damage or different collection date and 1 wetland excluded due to only 1 male captured (see Appendix B Table S2 for details).

Chapter 3. Effects of atrazine on testicular oocyte presence and severity in two North American amphibian species

Introduction

Chemical exposure and climate change are considered two major causes for amphibian population declines. The effects of both of these factors are exceptionally challenging to quantify due to complex interactions and the potentially far-reaching impacts, such as environmental levels of some wide-spread chemicals such as the herbicide atrazine (Collins & Storfer 2003; IUCN 2008; Mann et al. 2009; Hof et al. 2011). Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is one of the most widely used herbicides in the U.S., with the majority applied to corn and sorghum in the Midwest (USEPA 2003). Atrazine, which degrades fairly slowly with a half-life of 41-237 days in water (Giddings 2005; Jablonowski et al. 2011), is eventually transported to water bodies through runoff and aerial deposition, and detectable levels are often found far from the site of application (Chernyak et al. 1996). For example, atrazine was detected in 87% of water samples from lakes across Minnesota in 2007 even in nonagricultural northern regions (Minnesota Department of Agriculture (MDA) 2008) and in 99% (148/149) of wetlands sampled in the U.S. Prairie Pothole Region (PPR), including those in northwestern North Dakota, which were far from corn crops (Schoff et al. *In review*).

Organisms in lentic ecosystems could be chronically exposed to atrazine due to lack of flow and the long half-life of atrazine (Giddings 2005). Determining actual exposure concentration and duration is challenging; however, there is evidence that atrazine concentrations in agriculture-surrounded lakes and wetlands can exceed the EPA

drinking water standard of 3 µg/L as well as the 10-20 µg/L level associated with risk of ecological effects (USEPA 2003). Atrazine concentration in rainfall in the Midwestern and Northeastern U.S. has been measured at 40 µg/L (Nations & Hallberg 1992; Goolsby et al. 1997). In aquatic ecosystems, this concentration may be diluted by mixing with surface water, increased through added run-off from agricultural fields, or concentrated as water evaporates from the aquatic ecosystem (Knutson et al. 2004). At current application rates, the estimated environmental concentration is ~100 µg/L (Rohr & McCoy 2010), and, in the Midwest U.S., up to 172.2 µg/L has been measured in streams (Scribner et al. 2000; Battaglin et al. 2003) and 48.1µ/L in wetlands (Papoulias et al. 2013; Schoff et al. *In review*).

The primary objective of this study was to determine the effects of exposure to environmentally-relevant atrazine concentrations in combination with accelerated pond-drying (a prediction of climate change) on gonadal anomalies in amphibians. Climate change is a broad factor that must be incorporated into all predictions of organismal or population-level responses to chemicals/contaminants, as changes in temperature and precipitation directly impact amphibian populations (habitat loss and degradation such as potential reduced water quality) and amphibian development (through predicted accelerated drying of ponds and wetlands), but may also have indirect impacts through interactions with pesticides and other stressors (Zaga et al. 1998; Boone & Bridges 1999; Pounds 2001; Rohr et al. 2004). The non-lethal interactions of atrazine exposure with additional stressors, such as accelerated pond-drying, on the growth, development, and gonadal formation have not yet been investigated.

Measured atrazine concentrations from aquatic ecosystems are currently believed to be non-lethal to model amphibian species (Giddings 2005; Solomon et al. 2008). However, sub-lethal effects of atrazine exposure (as low as 0.1 μg/L) have been shown in amphibians (Rohr & McCoy 2010), including altering length of larval period (with evidence of both delayed and accelerated development), size at metamorphosis, behavior, immune function, and gonadal morphology and function. Atrazine exposure consistently reduces growth rate of amphibians and has variable effects on developmental rate (length of larval period). Larval exposure resulted in smaller size at or near metamorphosis for most species included in a recent meta-analysis (Rohr & McCoy 2010). Atrazine exposure slowed development in some species (Boone & James 2003; Coady et al. 2004; Rohr & Crumrine 2005; Freeman et al. 2005; Olker 2014b), while development was accelerated or not affected in other species (Hayes et al. 2002; Rohr et al. 2004; Orton et al. 2006; Williams & Semlitsch 2010). A range of effects on gonadal function and morphology have also been documented in relation to low concentrations of atrazine, including increased estrogen receptors (McCoy et al. 2008), delayed gonadal development (dysgenesis), and mixed gonadal tissue (intersex) or testicular oocytes (Hayes et al. 2002, 2003, 2006a, 2006b; Carr et al. 2003).

In amphibians, sex is determined genetically; however, there are many epigenetic factors that can override the genotypic sex determination (Hayes 1998; Eggert 2004). For example, *R. sylvatica* reared at high temperature can be induced to develop as phenotypic males (Witschi 1929) regardless of genetic sex. Amphibian gonadal differentiation is also sensitive to exogenous steroid hormones and full sex reversal or intersex can be induced

by estrogens (Hayes 1998; Hogan et al. 2008; Nakamura 2009), especially if exposure occurs during early sensitive developmental periods (Ogielska 2009). Induction of aromatase has been suggested as the mechanism by which atrazine exposure disrupts endocrine function resulting in gonadal anomalies (Hayes et al. 2002). Aromatase converts androgens such as testosterone to estrogens, and external signaling for aromatase induction could alter the balance of sex hormones within the gonad, potentially causing feminization of gonads including inducing gonia cells to differentiate into oogonia within the testis (Nakamura 2009). Induced expression of the aromatase gene (CYP19) by atrazine has been documented in other vertebrates (Crain et al. 1997; Sanderson et al. 2000), but has not yet been shown in amphibians.

The presence of oocytes in testicular tissue, hereafter called testicular oocytes or TOs, has captured the attention of the public and is often used as an endpoint in assays for endocrine-disrupting substances in fish (Johnson et al. 2010a) and amphibians (Hecker et al. 2006). This gonadal anomaly has been documented in field-collected frogs (Hayes et al. 2003; McDaniel et al. 2008; Du Preez et al. 2009; Papoulias et al. 2013) as well as in exposure experiments to atrazine and known endocrine disrupting chemicals (MacKenzie et al. 2003; Hayes et al. 2003; Coady et al. 2004; Jooste et al. 2005); however little is known about gonadal development in frogs and potential reproductive impacts of TOs.

Different responses to atrazine have been seen across studies, species, and regions (Solomon et al. 2008; Rohr & McCoy 2010), possibly due to species sensitivities or environmental conditions. Notably, field studies (McDaniel et al. 2008; Rohr et al. 2008)

and exposure experiments (Boone & James 2003; Bridges et al. 2004; Christin et al. 2004; Rohr & Crumrine 2005; Rohr et al. 2008) have suggested that other stressors can act synergistically with atrazine. Interactions of natural stressors, contaminants, and predators have been shown to affect amphibian growth, development, and health (Rohr et al. 2004; Christin et al. 2004; Relyea 2005a, 2006; Boone et al. 2007). Therefore, an understanding of the contribution of agrochemicals to amphibian population declines must include studies at multiple scales, combining controlled exposure experiments and field observations using standard laboratory models as well as native species (Mann et al. 2009).

We used experimental mesocosms to assess the dose-response effect of atrazine and its interaction with accelerated pond-drying on the incidence and severity of gonadal anomalies in two North American anuran species, northern leopard frog (*Rana pipiens*) and wood frog (*Rana sylvatica*). We hypothesized that: 1) exposure to environmentally relevant atrazine concentrations would alter gonadal development in a dose-dependent fashion, resulting in increased incidence and severity of gonadal anomalies, and 2) the addition of a second stressor, accelerated pond-drying, would exacerbate these responses.

Methods

We conducted two mesocosm exposure experiments, the first with *R. pipiens*, from May 26 to November 3, 2006 and the second with *R. sylvatica* from May 16 to July 31, 2009. We compared the results from these two experiments to field-collected specimens to identify any differences in wild versus reared specimens and to provide support for results from mesocosm exposure experiments. The experimental exposure

results were compared to field collections of metamorphic *R. pipiens* from 11 wetlands in the Prairie Pothole Region, in which no relationship was found between atrazine concentrations and TO prevalence and abundance (Schoff et al. *In review*). Because of the large discrepancies in TO prevalence between the two species in the experimental exposures, we also compared the results to larval, metamorphic, and post-metamorphic *R. sylvatica* collected from one seasonal wetland in northern Minnesota.

Exposure experiments were conducted in outdoor aquatic mesocosms using a fully-crossed randomized block design, as described in (Olker 2014b), following published recommendations for mesocosm toxicology experiments with amphibians (Rowe & Dunson 1994; Boone & James 2005; Semlitsch & Boone 2009). Briefly, the individual and interactive effects of atrazine concentration and drawdown rate were tested with chronic exposure of developing amphibian larvae to three atrazine concentrations (0.1, 20, 200 µg/L) and an unexposed control, in a static, non-renewal system using 37 gallon (140 L) low-density polyethylene (LPDE) stock tanks with mesh screen covers, filled with conditioned water (held for14 days in 700 gallon polyethylene tanks with a mixture of plant material, including aquatic detritus and oak leaves) from an uncontaminated source (see below). The atrazine exposure treatments were combined with two pond-drying rates (normal and accelerated, hereafter called drawdown rates), to achieve six treatments plus controls (no atrazine exposure) for both experiments. Experiment 1 (*R. pipiens*, 2006) used three atrazine concentrations X two drawdown rates, plus control (normal drawdown only; see below for rate determination), and solvent control (because atrazine was dissolved in acetone before dosing mesocosms) for both

normal and accelerated drawdown. In Experiment 2 (*R. sylvatica*, 2009) there were three atrazine concentrations X two drawdown rates, plus control (no atrazine) for both normal and accelerated drawdown. For Experiment 2, atrazine was dissolved in deionized water instead of acetone to remove the need for a solvent control treatment. At the start of each experiment each mesocosm contained 100 L of water and five *R. pipiens* or six *R. sylvatica* tadpoles at Gosner stage 26 (free-swimming).

Atrazine concentration was measured using an enzyme-linked immunosobent assay with a nominal detection limit of 0.05 μg/L (Abraxis LLC, Warminster, PA). We verified the absence of atrazine in the source water and the nominal concentrations of atrazine in each treatment with water from each mesocosm assayed at the start the experiments. Additionally, water from each mesocosm was assayed at the end of the each experiment to confirm exposure throughout the duration. Pond-drying rates were designed to mimic observations from natural wetlands, as well as rates known to initiate metamorphosis (Denver et al. 1998; Gervasi & Foufopoulos 2008). 'Normal pond-drying' mimicked the natural water loss rates observed in seasonal and semi-permanent wetlands, with 8% of maximum depth lost per week. Water was removed from the 'accelerated pond-drying' treatment mesocosms twice as fast, with 16% of maximum depth lost per week. Water was removed manually, starting on day 48 (July 12 for *R. pipiens*, July 2 *R. sylvatica*) at weekly intervals or as needed based on precipitation, until the mesocosms were 5 cm deep (10 weeks for normal, 5 weeks for accelerated drawdown treatments).

Developmental stage was recorded and dead or diseased frogs were removed daily. Metamorphic specimens (Gosner stage 45) were transferred into grow-out aquaria with commercially purchased spring water until full tail resorption (Gosner stage 46), which typically took 1-3 days. When metamorphosis was complete, specimens were euthanized with 3 mg/L tricane methoanesulfonate (MS-222), weighed, measured, and preserved for histological analyses, held first in Bouin's solution (Sigma, St. Louis, MO) for 48 hours and then transferred to 70% ethanol or neutral buffered formalin.

Endpoints

Survival to the end of the experiment and the percent of specimens completing metamorphosis were summarized by mesocosm. For each specimen, we also measured weight and snout-to-vent length at metamorphosis and time to reach (Gosner stage 42) and complete (Gosner stage 45) metamorphosis. Sex was determined via gross gonadal morphology. For all males or specimens with undistinguishable or gross anomalies, gonads were removed with kidneys attached for histological analysis. Both gonads and attached kidneys were embedded in paraffin and were step-sectioned (5 μm thick longitudinal sections every 50 μm) in their entirety, stained using standard H&E (hemotoxylin and eosin) methods, and examined using an Olympus BH-2 compound microscope. Potential endocrine disruption was evaluated by the occurrence of gross gonadal anomalies along with the presence and abundance of TOs, defined as ooctyes containing an intact nucleus with nucleoli found in males with some normal testicular tissue. TOs were identified and counted in all sections of all male specimens. Additionally, TO severity was assessed based on a scoring system developed from the

observed range of TO abundance and extent across testes in the metamorphic male *Rana pipiens* (Olker 2014c). TO severity was scored from zero (no TOs) to 5 (severely impacted, with portions of the gonad section dominated by ooctyes). A validation set of photos of each severity was used to score all sections of all male specimens (Appendix C Figure S1). Additional gonadal anomalies or differences were documented at the gross and cellular level. From these assessments we calculated sex ratio and TO prevalence, abundance (average total # per affected specimen), and severity.

Statistical analyses

Survival and percent completing metamorphosis were analyzed with two-way analysis of variance (ANOVA, GLM model) with fixed effects (atrazine, drawdown rate, interaction of atrazine and drawdown rate) and block as a random effect. Block effect was not significant ($p = (\alpha = 0.05)$) and therefore was excluded from ANOVA models. Significant fixed effects were identified based on $\alpha = 0.05$. In a previous mixed model analysis of variance of growth and development data from these experiments, no significant effects of drawdown rate were found (Olker 2014b). Therefore, we used the following recommended approach for toxicological data: snout-to-vent length, weight, and days to complete metamorphosis were analyzed with the non-parametric Jonckheere-Terpstra trend test on replicate medians (Terpstra 1952; Jonckheere 1954; Seshan 2013) and Gosner stage was analyzed with the Rao-Scott Cocharn-Armitage by Slices (RSCABS) test that includes scores (Gonser stage, in this case) of individual specimens and frequency of scores for replicate subjects within a mesocosm (Green et al. 2014).

Sex ratios across treatments were compared using a binomial test (Wilson & Hardy 2002), using the null hypothesis of equal numbers of males and females. The effect of atrazine concentration on TO prevalence was tested with Fisher's Exact Test to compare treatments to control. Abundance of TOs per specimen was analyzed with mixed models analysis of variance that included fixed effects (atrazine, drawdown rate, interaction of atrazine and drawdown rate) and random effects (mesocosm, block, atrazine concentration*block and drawdown rate*block), with the total number of TOs normalized before analysis with the square root transformation. TO severity in representative mid-sections and maximum TO per specimen were analyzed with the RSCABS, as described above. Analyses were completed with SAS 9.2 (SAS Institute 2002-2008), with the exception of the Jonckheer-Terpstra and RSCABS tests which were run in R 3.0.0.

Comparison to wild collections. Previously, we found that TO prevalence in field-collected metamorphic *R. pipiens* was relatively high and was not related to atrazine concentrations in their natal wetlands (Schoff et al. *In review*). Here we re-evaluated these field-collected *R. pipiens* to determine TO severity for comparison to the experimental specimens. Additionally, wild *R. sylvatica* were collected at several developmental stages for comparison to the experimental specimens from one wetland in northern Minnesota. This seasonal wetland was in a forested area within city limits of Duluth, Minnesota and historically has 30-100 breeding pairs of *R. sylvatica*. Of the 75 *R. sylvatica* collected from this wetland, 26 males were included for histological analysis, with two to nine males per developmental stage [nine at Gosner stages 38-40 (tadpole),

two at Gosner stages 42-43 (forelimb emergence), seven at Gosner stages 44-46 (metamorphosis), four one month post-metamorphosis, and five two months post-metamorphosis]. While the limited sampling from one wetland does not provide a comprehensive analysis of susceptibility across developmental stages, it provided verification that the lack of TOs detected in Experiment 1(*R. sylvatica*, 2009) was not due to 'missing' an important developmental stage (as experimental specimens were evaluated at the completion of metamorphosis).

Results

At the start of each exposure experiment, atrazine concentrations were 0.20, 22.3 and 197.9 µg/L (*R. pipiens*) and 0.23, 21.9, and 206.8 µg/L (*R. sylvatica*) for the nominal 0.1, 20, and 200 µg/L treatments, respectively. Survival in both mesocosm experiments was very high, with 87.8% *R. sylvatica* and 94.2% *R. pipiens* surviving to the end of the experiment (Table 1). Atrazine concentration did not affect survival in either species, but greatly reduced the proportion completing metamorphosis and the Gosner stage attained by the end of the experiment in *R. pipiens* (Table 2). This effect of atrazine was not seen in *R. sylvatica*, for which all surviving specimens also completed metamorphosis by the end of the experiment. At the end of the experiment, only 47.6% of the *R. pipiens* had completed metamorphosis, compared to 87.8% of the *R. sylvatica* (Table 1). The effects of atrazine of development were detected in other endpoints in *R. sylvatica*, with delayed development (more days to complete metamorphosis) in the 200 µg/L treatment ($p=0.032$) and smaller size at metamorphosis ($p<0.004$) in the 20 and 200 µg/L treatments (Table 2).

The effect of atrazine on the percent completing metamorphosis resulted in unequal sample sizes for gonadal analysis, as we only included specimens that that completed metamorphosis. Therefore, the gonadal analysis consisted of fewer *R. pipiens* than *R. sylvatica*, and small sample sizes for *R. pipiens* at the highest atrazine concentration (6 males) compared to the 9-16 males from the other atrazine concentrations (Table 3). Sex ratios did not differ from the expected 1:1 ratio for the entire experiment (0.87 F:M and 1.01 F:M for *R. pipiens* and *R. sylvatica*, respectively) or by atrazine treatment (Table 4, α=0.05).

Gonadal anomalies were found in both species across all treatments, including controls (Figure 1). In *R. sylvatica*, 3% (4/131) of the females showed delayed ovary development, with reduced ovary size and delayed cellular development within the ovary (Figure 1D). Ovaries in these specimens consisted of an open lumen (ovarian cavity) surrounded by a cortex layer dominated by primary and secondary oogonia with some diplotene ooctyes. These differed greatly from the typical ovary which is dominated by pre-vitellogenic diplotene oocytes with a few cysts of earlier stages of oogenesis along the periphery, and the central lumen greatly reduced or not obvious (Ogielska 2009). Delayed gonadal development was observed in females found in multiple atrazine treatments (0, 0.1, and 20 µg/L) as well as both normal and accelerated drawdown treatments. In addition, all were within one standard deviation for size and development rate (Olker 2014b). Delayed ovary development was not observed in any experimental *R. pipiens*.

TOs were found in both species, but at very different frequencies. In mesocosm-reared *R. pipiens*, TO prevalence was 71.4%, with prevalence in individual treatments ranging from 33% (3/9) in the 20 µg/L treatment to 81% (13/16) in the acetone (0 µg/L atrazine) treatment, and 83% (5/6) in the control (water only, 0 µg/L atrazine) (Table 3, Figure 2). There was no significant effect of atrazine treatment on TO prevalence (Fisher's Exact Test, p=0.078); however, pairwise comparisons showed that specimens from the 20 µg/L treatment had a significantly lower TO prevalence (33%, 3/9) than those from the acetone control ($p<0.001$). TOs were present in male *R. pipiens* from both the normal and accelerated drawdown mesocosms, with TO prevalence of 67% (12/18) and 72% (18/25), respectively. Drawdown rate and the interaction of drawdown rate with atrazine concentration had no significant effect on TO prevalence (Fisher's Exact Test, p=0.746 and 0.35).

Affected *R. pipiens* males had a mean of 15 TOs per specimen, with a range of 1 – 165 and 3.5 TOs in the representative section from the middle of the gonad (Table 1). There were no significant fixed effects of atrazine, drawdown rate, or interaction of atrazine and drawdown rate on the number of TOs per specimen (mixed model ANOVA, $\alpha = 0.05$). Severity scores ranged from 0-3 for the control, acetone control, and 200 µg/L treatments, while severely impacted specimens (scores of 4-5) were found in the 0.1 µg/L treatment. In *R. pipiens*, TO severity scores were not significantly different between the water control and the acetone control (RSCABS test, p=0.16-0.44) and scores from the atrazine-exposed specimens did not differ from the acetone control based on the

representative gonadal section or the maximum score per specimen (RSCABS test, p = 0.18 – 0.46).

In the *R. sylvatica* exposure experiment, 41 specimens (8 - 11 males from each atrazine treatment) were selected for preliminary histological analysis based on the overlap of the 95% confidence intervals for size (snout-to-vent length) and development rate (days to reach Gosner stage 45). Only one male in this subset contained TOs (Table 3), with a total of 22 TOs (8 in the representative section) and severity score of 3 in the left and 1 in the right testis. This specimen was from the 0 µg/L atrazine, accelerated drawdown treatment, was within one standard deviation for size and development rate (17.5 mm snout-to-vent length, 0.41 g, and reached Gosner stage 45 in 55 days; Olker (2014b)). To be certain that this low TO prevalence was consistent through the entire set of specimens (including the outliers for size and developmental rate) an additional 49 males, including the remaining 28 unexposed (0 µg/L atrazine) specimens and 21 from atrazine exposure treatments (see Table 3 footnotes for details), were evaluated histologically, with no additional TOs found.

In the wild populations of *R. pipiens* from the Prairie Pothole Region, TO prevalence, abundance, and severity was comparable to the experimental specimens, with high TO prevalence and severity scores up to 4 across the range of measured atrazine concentration (Table 1, Figure 3). No TOs were found in the developmental series of wild *R. sylvatica* from the northern Minnesota population, with testes clearly identifiable at Gosner stage 40 at the gross and cellular level.

Discussion

We found no apparent relationship between atrazine concentration and sex ratio, gonadal formation, or presence/abundance/severity of TOs in experimental or field-collected specimens. In contrast, we found that TOs were present in *R. pipiens* from all treatments, including those not exposed to atrazine, and were present in metamorphic *R. pipiens* from all wetlands surveyed, regardless of atrazine concentration. These results match those found in collections of data for *R. pipiens* and the closely related Plains Leopard Frog (*Rana blairi*) in an agricultural region of Nebraska, in which TOs were present at frogs from all wetlands sampled, regardless of atrazine concentration (Papoulias et al. 2013). We previously documented this lack of relationship between atrazine and TOs presence/abundance in field-captured specimens (Schoff et al. *In review*); here we also showed that TO severity was not related to atrazine concentration for wild or experimentally-exposed *R. pipiens*.

Atrazine exposure impacted growth and development in the experimentally exposed tadpoles, with fewer *R. pipiens* completing metamorphosis, and longer larval period and smaller size at metamorphosis in *R. sylvatica* exposed to the highest concentration (200 µg/L; Olker 2014b). These differences reflect the life history of these related species. As a frog that primarily breeds in ephemeral ponds, *R. sylvatica* can adjust developmental rate to a certain degree, to complete metamorphosis before ponds dry up (Newman 1992; Denver 1997). *R. pipiens*, however, have the ability to delay development and overwinter as tadpoles, which is generally observed in the northern reaches of their geographical range (Merrell 1977). These growth and development responses could have been due to direct disruption of endocrine system or hormonal

balances (Denver 1997, 2009; Larson et al. 1998) or indirect alteration of food quantity or quality (Boone & James 2003; Rohr & Crumrine 2005), as discussed in detail in Olker (2014b). The increased mortality during metamorphosis in the *R. sylvatica* in the high exposure (200 µg/L) suggests that atrazine exposure also stresses the immune system. During metamorphosis, frogs have increased vulnerability to pathogens due to the dramatic decrease in lymphocytes as the larval immune system transition into the adult immune system (Rollins-Smith 1998; Denver 2009). The observed effects of atrazine exposure on survival, growth, and development could have carry-over or permanent effects on juvenile and adult growth, survival, and reproductive capacity (Duellman & Trueb 1994; Denver 2009).

In the experimental exposures, drawdown rate did not affect growth, development, or presence/abundance/severity of TOs in either species. Although the experimental drawdown rates were based on rates of water loss that have been documented in accelerating development in amphibians (Denver et al. 1998; Gervasi & Foufopoulos 2008), we suspect the critical volume of water per tadpole was not reached due to the low tadpole loading rate of 16-20 L of water per tadpole, compared to current recommendations of 10 L per tadpole (Boone & James 2005; Boone et al. 2007).

The high TO prevalence and severity found in *R. pipiens* in all atrazine concentrations of this study does not support the hypothesis that atrazine acts as an endocrine disruptor or that atrazine exposure during development induces TOs. These results are similar to studies that did not find reproductive effects of atrazine exposure in amphibians (Coady et al. 2004; Jooste et al. 2005; Orton et al. 2006; Oka et al. 2008).

However, these results are contradictory to other laboratory studies that have shown that low concentrations of atrazine (<1 ug/L) caused abnormal gonad development (Hayes et al. 2003). Impacts of atrazine on gonadal function have been documented in field studies, including reduced testosterone and altered abundance of testicular cell types (McDaniel et al. 2008), in addition to gonadal anomalies such as gonadal dysgenesis (Hayes et al. 2003), mixed gonadal tissue (Reeder et al. 1998; Murphy et al. 2006b) and TOs (Hayes et al. 2003; Murphy et al. 2006b; McDaniel et al. 2008). These differences across studies and species could be due to a combination of several factors or contaminants affecting gonadal development, as many agricultural chemicals are known or suspected endocrine disruptors (Houtman et al. 2011). The potential involvement of multiple contaminants is supported by field studies that have shown a positive correlation between gonadal anomalies and the proportion of agriculture in the landscape (McDaniel et al. 2008; McCoy et al. 2008).

Notably, we found considerable differences in TO prevalence between two native amphibian species. While TOs were found in both species, prevalence was less than 5% in *R. sylvatica* and greater than 40% in *R. pipiens* from both experimental exposures and field collections. These marked dissimilarities in TO prevalence could be due to species' differences in age at metamorphosis, sensitivity to endocrine disruption, or inherent differences in 'normal' TO occurrence.

In anurans, gonad development is thought to coincide with somatic growth and age rather than developmental stage (McCoy et al. 2007). Since *R. sylvatica* complete metamorphosis much more rapidly than *R. pipiens* (56 days v. 112 days in this study,

respectively), their gonads might be expected to be less developed by metamorphosis and TOs might not yet be present. If that were the case, populations of post-metamorphic *R. sylvatica* would be expected to have a higher TO prevalence. However, we found no TOs in wild *R. sylvatica* one or two months post-metamorphosis and the testes in these males were comparable to those at metamorphosis with cysts of primary spermatocytes and evidence of rete testis formation. Additionally, we found that gonads in *R. sylvatica* tadpoles as early as Gosner stage 38 were differentiated and identifiable as testis or ovary. In fact, we found no TOs in any of the developmental stages examined from tadpole (Gosner stage 38) to 2 months post-metamorphosis, indicating that TOs are not common in this species.

The species differences in TO prevalence could also be due to differential sensitivity to endocrine disruption. Using two native species for the experimental exposures provided an interesting opportunity for comparison across species. As a benchmark native amphibian species, *R. pipiens* has been used for many ecotoxicology and developmental studies, including responses to atrazine (Hayes et al. 2003, 2006a; McDaniel et al. 2008; Rohr et al. 2008). Use of this species allowed for direct comparisons to experimental exposures and field surveys across a large geographical extent; however, *R. pipiens* may have a greater or different sensitivity to endocrine disruption when compared to other laboratory or native species (MacKenzie et al. 2003; Solomon et al. 2008). *R. sylvatica* is becoming a more common species for ecotoxicological and developmental experiments (Kiesecker 2002; MacKenzie et al. 2003; Rohr & Crumrine 2005; Gervasi & Foufopoulos 2008). This congener of the

commonly studied *R. pipiens* may provide an alternative native species that contains desirable characteristics for a model species, such as early breeding, rapid development, high survival, and replication of field observations for size and age at metamorphosis (Olker, unpublished data).

R. sylvatica has been shown to be sensitive to sex reversal after estradiol exposure, compared to other amphibian species including *X. laevis* and *R. pipiens* (Solomon et al. 2008). Other research has shown that *R. pipiens* were more susceptible to sex reversal and development of testicular oocytes (or intersex) than *R. sylvatica* when exposed to estrogenic and anti-estrogenic compounds, but the latter species were more susceptible to morphological alterations such as germ cell maturation and oocyte atresia (MacKenzie et al. 2003). However, because we found no differences in TO prevalence across atrazine treatments, it is unlikely that differential sensitivities can explain the species differences we observed in these experiments.

The possibility that intersex (TOs) or mixed sex is part of normal gonadal development (as suggested by Storrs-Méndez & Semlitsch 2010) has not been supported for many anurans, including *R. pipiens* or *R. sylvatica*, which are thought to follow the differentiated pattern for gonadal development directly to testes or ovary from undifferentiated tissue (Ogielska 2009). Our results do not support the hypothesis that intermediate gonadal stages in *R. pipiens* 'normally' contain TOs, because we observed high TO prevalence in experimental specimens across the three month period that specimens were metamorphosing, as well as the field-collected specimens across the Prairie Pothole Region. It is possible, however unlikely, that our collection of

metamorphic *R. pipiens* during this entire period coincided with an intermediate gonadal stage that contained TOs. Clearly, there is a need for greater understanding of normal development in native species in order to assess toxicological effects at environmentally relevant concentrations.

Population differences in the sensitivity and TO prevalence have been found in *X. laevis* in South Africa (Du Preez et al. 2009) and could be another confounding factor in evaluating TOs as indicators of endocrine disruption. However, the fact that we found at least one male with TOs in all natal wetlands sampled across a large geographical area, combined with the similarities in TO prevalence between the experimental and field-collected *R. pipiens*, which originated hundreds of miles away from one another, suggests that this species commonly contains TOs outside of any population differences.

Given that our results indicate 1) a high TO prevalence in wild and lab-raised *R. pipiens*; 2) no relationship between atrazine concentration and gonadal endpoints; and 3) differences in TO occurrence in two ranid species raised under controlled conditions, we suggest that: 1) interpretation of TO occurrence or prevalence in field-collected specimens be critically examined; 2) caution be exercised when considering the occurrence or prevalence of TOs in ranid species as indicators of endocrine disruption or environmental condition; 3) mechanisms influencing TO development in ranid species are complex and atrazine does not appear to have an endocrine disrupting effect on gonads in two ranid species; and 4) additional research is needed to understand the reproductive or population-level impacts of TOs and to address species differences in TO prevalence and severity.

Table 1. Summary of **s**urvival and gonadal endpoints for mesocosm exposure experiments and wild-caught *Rana pipiens* and *Rana sylvatica*.

	Northern leopard frog (*R. pipiens*)		**Wood frog (*R. sylvatica*)**	
Response variables	***Mesocosm***	***Wild* (11 wetlands)**	***Mesocosm***	***Wild* (1 wetlands)**
% **survival** to end of experiment	94.2% (212/225)	NA	87.8% (253/288)	NA
% completed **metamorphosis** (Gosner stage 45)	47.6% (107/225)	NA	87.8% (253/288)	NA
Sex ratio (F:M)	37 F: 50 M (0.87)	266 F: 262M (1.01)	138 F: 137 M (1.01)	38 F: 37 M (1.03)
% females with **less developed ovaries**	0% (0/37)	NA	3% (4/131)	NA
TO prevalence[a]	71.4% (35/49)	43.4% (111/256)	2.4% (1/41)[d]	0% (0/26)[e]
Average TO abundance[b]	3.5 (SD 5.0), 15.0 (SD 31.2)	3.9 (SD 4.2), 37.8 (SD 42.7)	8 22 (1 specimen)	0 0
Range of TO severity[c]	L: 0-5 R: 0-4	L: 0-4 R: 0-5	L: 0-3 R: 0-1	L: 0 R: 0

NA=not applicable

[a] % males with testicular oocytes; [b] avg of # TOs for most impacted section and avg total # TOs per affected specimen; [c] range of severity score for left and right gonad; all sections scored for mesocosm specimens, representative section (median) scored for wild specimens; [d]Subset of 41 specimens selected for histological analysis, controlling for age and size across treatments; all control specimens were analyzed with no additional TOs observed; [e]Subset of wild specimens selected for histological analysis with 2-9 males per developmental stage (tadpole: Gosner stages 38-40; forelimb emergence: Gosner stages 42-43; metamorphosis: Gosner stages 44-46, one and two months post-metamorphosis).

Table 2. Statistical results of effects of atrazine on survival, development, growth, sex ratios, and gonadal endpoints in *Rana pipiens* and *Rana sylvatica* after larval exposure to atrazine (0, 0.1, 20, 200 µg/L), NS = no significant effects (α =0.05).

		Rana pipiens		*Rana sylvatica*	
Endpoints	**Test**	**p-value**	**diff from control[c]**	**p-value**	**diff from control[c]**
% survival to end of experiment[a]	ANOVA	NS		0.0022	200
% completed metamorphosis (Gosner stage 45)[a]	ANOVA	0.0387	200	NS	
Gosner stage attained by end of experiment (or collection at metamorphosis)	RSCABS	0.002-0.009	0.1, 20, 200	NS	
# days to complete metamorphosis[a]	Jonckheere-Terpstra	NS		0.032	200
snout-to-vent length, mm[a]	Jonckheere-Terpstra	NS		0.004	200
weight, g[a]	Jonckheere-Terpstra	NS		0.001-0.002	20, 200
Sex ratio (F:M)	Binomial test	NS		NS	
TO prevalence	Fisher's Exact Test	NS		NS	
TO abundance[b]	Mixed model ANOVA	NS		NS	
TO severity	RSCABS	NS		NS	

[a] Natural log transformation; [b] Square root transformation; [c] Significant at α = 0.05. The most conservative test would include the Bonferroni adjustment for multiple tests (for the above 10 models the cut-off value for significance would be α/10 = 0.05/10 = 0.005); however, this adjustment was not applied because the response variables were not independent (survival, growth and development are correlated) and the Bonferroni adjustment greatly increases the probability of false negatives (β), and thus greatly reduces power.

Table 3. Number of metamorphic male *Rana pipiens* and *Rana sylvatica* with testicular oocytes over the total number used in histological analysis of gonads from experimental mesocosm exposures by atrazine concentration (µg/L) and drawdown rate (normal/accelerated)

		Control (0 µg/L)	Acetone (0 µg/L)	0.1 µg/L	20 µg/L	200 µg/L	total
Rana pipiens	**normal**	5/6	6/8	2/3	1/3	3/4	17/24
	accel	[a]	7/8	7/9	2/6	2/2	18/25
	total	5/6	13/16	9/12	3/9	5/6	35/49
Rana sylvatica	**normal**	0/4	[a]	0/5	0/2	0/4	0/15
	accel	1/7	[a]	0/6	0/6	0/7	1/26
	total	1/11	[a]	0/11	0/8	0/11	1/41[b]

[a] not applicable

[b] An additional 49 males were evaluated for a total of 90 specimens, with no additional testicular oocytes detected [0 µg/L atrazine treatment (17 from normal, 11 from accelerated drawdown), 0.1 µg/L atrazine treatment (1 from normal, 4 from accelerated drawdown), 20 µg/L atrazine treatment (5 from normal, 3 from accelerated drawdown), 200 µg/L atrazine treatment (3 from normal, 5 from accelerated drawdown)]

Table 4. Sex of metamorphic *Rana pipiens* and *Rana sylvatica* from experimental mesocosm exposures by atrazine concentration (µg/L).

		Control (0 µg/L)	Acetone (0 µg/L)	0.1 µg/L	20 µg/L	200 µg/L	total
Rana pipiens	**F**	3	9	9	12	4	37
	M	6	16	12	10	6	50[a]
	total	9	25	21	22	10	87
Rana sylvatica	**F**	26	[b]	36	38	31	131
	M	42	[b]	30	32	29	133[c]
	total	68	[b]	66	70	60	264

[a] 49 males evaluated for gonad anomalies, 1 excluded due to poor preservation
[b] not applicable
[c] 41 males included in initial gonad anomalies analysis, additional 49 males evaluated (total 90 males)

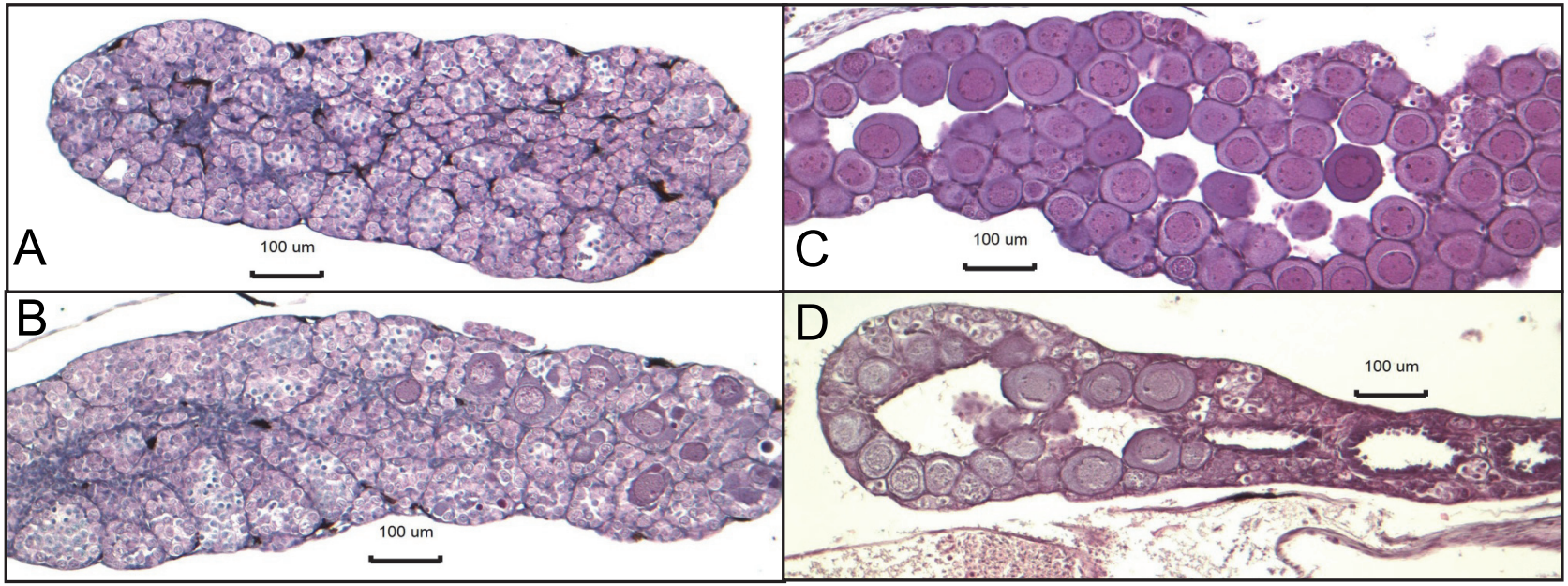

Figure 1. Photomicrographs of longitudinal sections of gonadal tissue from non-atrazine exposed metamorphic *Rana pipiens*: (A) Normal testicular tissue (B) Section containing multiple testicular oocytes; and *Rana sylvatica*: (C) Normal ovarian tissue. (D) Less developed ovary.

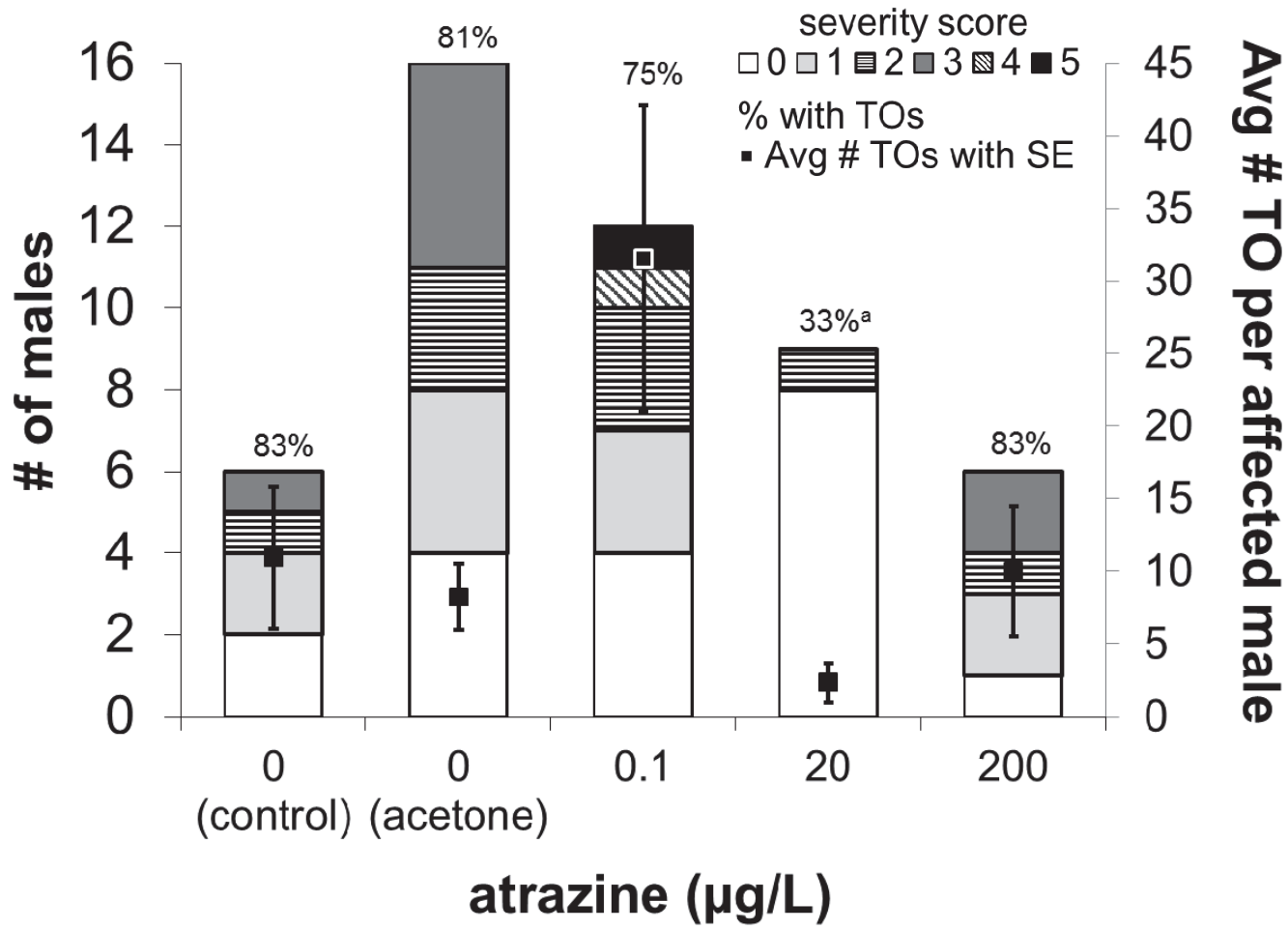

Figure 2. Testicular oocyte prevalence, abundance, and severity in metamorphic *Rana pipiens* across experimental treatments. [a]TO prevalence and severity were significantly lower in 20 µg/L treatment compared to the acetone control (Fisher's Exact Test and Kruskal-Wallis test, $\alpha = 0.05$).

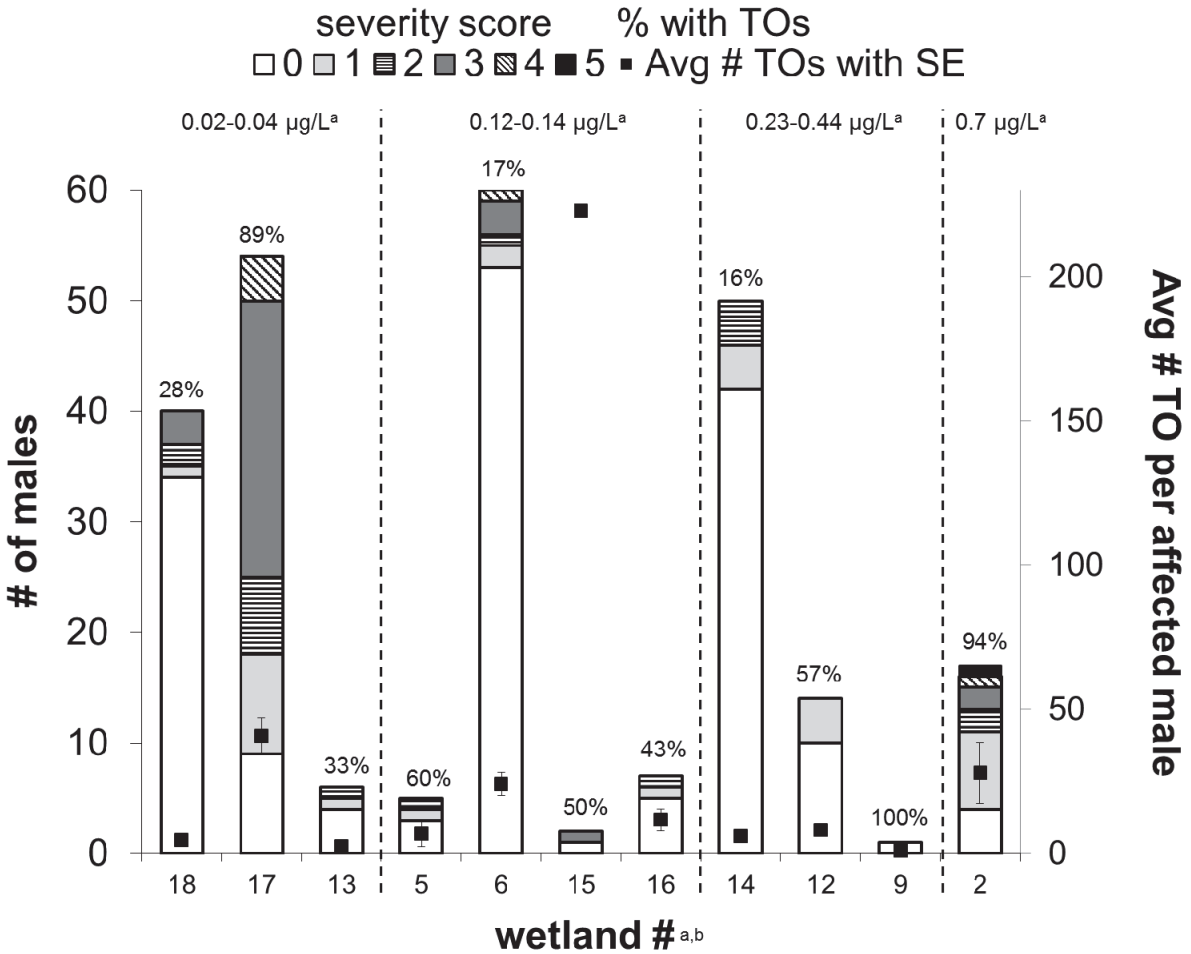

Figure 3. Testicular oocyte prevalence, abundance, and severity in metamorphic *Rana pipiens* across natal wetlands in the Prairie Pothole Region collected in 2005 as described in (Schoff et al. *In review*). [a] ordered by average atrazine concentration from survey 2 and 3; [b] wetland # from (Schoff et al. *In review*)

Chapter 4. Landscape factors influencing amphibian larval development, growth, and gonadal development

Introduction

The current rate of amphibian extinctions and population declines is considered a 6th mass extinction (Collins & Crump 2009) in global history. Amphibians are declining worldwide and appear to be more threatened than birds or mammals, with 33% of Anura (frogs and toads) and 46% of Caudata (salamanders and newts) species classified as either threatened or extinct (Stuart et al. 2004; IUCN 2008). Historically abundant or 'common' species also have also been declining over the last 50 years (Houlahan & Findlay 2004; Wake & Vredenburg 2008; Adams et al. 2013). Amphibian declines are attributed to numerous factors, but there is no clear pattern across species or regions. In addition to widespread habitat loss, amphibian populations are impacted by both natural and anthropogenic factors, including competition with introduced species, pollution, climate change, over-exploitation, and disease (Alford & Richards 1999; Collins & Storfer 2003; Halliday 2008).

The scope of current amphibian declines may reflect the extent of worldwide environmental degradation; amphibians are considered indicators of environmental condition, with population persistence and health/condition used as models for assessing environmental impacts on aquatic organisms and ecosystems (Vitt et al. 1990; Westerman et al. 2003; Welsh Jr & Hodgson 2008). The biphasic life history of many North American species makes them dependent on both aquatic and terrestrial habitats, and therefore subject to widely-varying stressors. For example, because they undergo aquatic embryonic, larval and metamorphic stages, amphibians are good indicators of

chemical contamination in water, while retention of permeable skin throughout all life stages makes them vulnerable to terrestrial contaminants (Westerman et al. 2003; Bernanke & Köhler 2009; Quaranta et al. 2009). Sensitivities to local or wetland conditions include physiological or behavioral responses to hydroperiod (Pechmann et al. 1989; Snodgrass et al. 2000), predators (Hecnar & M'Closkey 1997; Adams 1999), introduced species (Kiesecker et al. 2001a; Knapp et al. 2007), disease (Carey et al. 1999; Skerratt et al. 2007), and chemicals (Mann et al. 2009). At the landscape level, amphibian presence and diversity are sensitive to habitat fragmentation (Kolozsvary & Swihart 1999; Knutson et al. 2000; Willson & Dorcas 2003), roads (Reeves et al. 2008; Bouchard et al. 2009), urbanization (Pillsbury & Miller 2008; Hartel et al. 2009; Guzy et al. 2012), and lack of non-breeding habitat for foraging and hibernation (Smith & Keinath 2007).

Additionally, amphibians have been used as indicators of biological diversity, based on their major role in some ecosystems. Amphibians make up a large component of the biomass in ecosystems, such as northern hardwood forests of the U.S. (Burton & Likens 1975), tropical forests of Puerto Rico (Stewart & Woodbright 1996), southeastern U.S. wetlands (Gibbons et al. 2006), and Midwest US wetlands (Connelly et al. 2008). Amphibians consume algae and insects, transfer energy between aquatic and terrestrial ecosystems, and act as a food source for many other organisms (Merrell 1977; Whiles et al. 2006; Altig et al. 2007; Connelly et al. 2008). Loss or decline of amphibians could substantially impact aquatic systems as well as the surrounding terrestrial systems, as energy flow through wetlands would be reduced or redirected to other organisms, algal

growth could expand, and the annual pulse of high energy food source to terrestrial systems in the form of metamorphic amphibians would decline or disappear.

Amphibian presence and health are potential indicators of wetland 'health' or environmental condition. The relationship between environmental factors and amphibian responses can inform planning and habitat management actions that could prevent further loss of species and negative impacts on aquatic ecosystems. Amphibian species' distribution and population persistence depend on environmental factors operating at multiple spatial scales: wetland habitat, immediate uplands, and surrounding landscape. Assessing the driving factors at multiple scales requires studies across large landscapes. This approach has been used in many regions to identify factors associated with local/regional extirpations (Johnson et al. 2011), identify anuran species that are indicators ecological or wetland condition (Price et al. 2007; Guzy et al. 2012), and correlate species presence and diversity with site-specific to broad scale landscape habitat variables (Findlay & Houlahan 1997; Johnson et al. 2002a; Price et al. 2004; Porej et al. 2004; Otto et al. 2007; Lemckert & Mahony 2010). Large landscape scale approaches have also been used to identify factors associated with metamorphic anuran health, such as body condition and stress hormones (Janin et al. 2011), disease and parasites (Lannoo et al. 2011; Schotthoefer et al. 2011; Hartson et al. 2011), skeletal malformations (Ouellet et al. 1997; Taylor et al. 2005; Piha et al. 2006; Reeves et al. 2008), and gonadal anomalies (Skelly et al. 2010; Papoulias et al. 2013). Given the number of potential metrics and variability in amphibian responses to contaminants and environmental change, amphibian responses to environmental drivers need to be further elucidated.

For amphibian populations to persist, breeding habitat must be accessible and attractive to breeding adults, and must provide appropriate conditions to sustain larval development through metamorphosis (including appropriate hydrology, food, and protection from predators, and be free of lethal/sublethal contaminants). We used an information theoretic approach (Burnham & Anderson 2002) to assess the effects of environmental factors at three spatial scales with metrics quantifying presence, calling, breeding, and developmental endpoints for *Rana pipiens* (Table 1).

Our goals were to:

1) assess the relationship between metrics of presence, calling, breeding, and abnormal development (skeletal malformations and gonadal anomalies) in Northern leopard frog (*Rana pipiens*) and environmental factors across the U.S. Prairie Pothole Region; and 2) evaluate how these relationships vary by spatial scale (within wetland, immediate upland, or landscape).

To address these goals we characterized the environmental gradients across the study area by creating a composite index with variables from multiple scales from within wetland conditions to local land use and landscape features. We then quantified the relationships between the nine amphibian metrics and this composite index (Goal 1). To address the effect of spatial scale (Goal 2), we also quantified the relationships between the nine amphibian metrics and the individual environmental variables measured at three spatial scales (within-wetland, local, and landscape). Our expectations were that *R. pipiens* presence, calling, and breeding would be positively associated with the 'good' environmental conditions, while presence of skeletal malformations and gonadal

anomalies would be positively associated with 'poor' environmental conditions. Based on *R. pipiens* life history requirements, we expected the amphibian metrics to be more strongly associated with a combination of environmental variables from multiple scales (composite index of environmental gradient) than with individual predictor variables from each scale. We further expected that these relationships could potentially serve to identify indicators of environmental conditions for wetland management programs.

Methods

The overall study design included an extensive survey of amphibians at 149 seasonal and semi-permanent wetlands in the U.S. portion of the Prairie Pothole Region of the U.S. Amphibians were sampled from early spring through summer to monitor breeding activity and sample larval and metamorphic frogs. Field measurements were completed to characterize within-wetland and local-scale conditions and features. Spatial data were used to characterize landscape features and conditions at multiple spatial scales. We used Principle Components Analysis to derive a composite index to characterize environmental gradients across the region (hereafter, referred to as an index of environmental gradients). An information theoretic approach was used to quantify relationships between the nine amphibian metrics and environmental characteristics using this index of environmental gradients and individual variables from the three spatial scales (within-wetland, local, and landscape) as predictor variables.

Study Area and Species

Amphibian surveys were conducted at 149 seasonal and semi-permanent wetlands in the U.S. portion of the Prairie Pothole Region east of Montana (Figure 1). The Prairie

Pothole Region forms the northeastern edge of the Great Plains, and contains over 3 million depressional wetland basins that were formed by the late Pleistocene glaciation (Tiner 2003). The wetlands in the region vary from semi-permanent (wet throughout most years) to seasonal and temporary (holding water one to several months) (Stewart & Kantrud 1971; van der Valk 1988; Murkin et al. 2000), and support a variety of wildlife, including waterfowl (Hoekman et al. 2002) and amphibians (van der Valk 1988; Balas et al. 2012). This area encompasses gradients of climate, agriculture, and human development. The dry, seasonal climate that supports this grassland-dominated region varies across ecoregions, with a strong precipitation gradient from east (wetter) to west (drier). Many wetlands in this region are now embedded within a largely agricultural matrix, with a gradient of development from southeast (more urban development and intensive row crop agriculture) to northwest (less development and agriculture).

R. pipiens is considered a 'common' species throughout the US Midwest, but has been declining across its range since the 1970s and appear to be susceptible to local extinctions due to habitat loss and anthropogenic stressors (Smith & Keinath 2007). This species is associated with open grassland areas that contain a variety of wetland and lake types. *R. pipiens* breeds and develops within wetlands, and thus is dependent on habitat quality, including holding water until metamorphosis is completed, and limited exposure to or protection from predators, parasites, and damaging chemicals. In the adjacent upland, certain types of land use can impede or prevent accessibility of wetland to breeding adults and dispersal of young-of-the-year metamorphic frogs, while pesticides and nutrient runoff and food availability can impact foraging adult, juvenile, and

metamorphic frogs. At the landscape scale, *R. pipiens* individuals and populations are impacted by intensive agriculture, urban, and road development that reduce wetland density and establish barriers or challenges to amphibian migration between overwintering and breeding habitats.

Sampling Design

We used a stratified random sampling design based on hydrologic regime (seasonal, semi-permanent), land use (grassland, agriculture), ecoregion, and latitude to select study wetlands. This design encompassed a range of stressors, including wetland-specific characteristics such as pond-drying rate and contaminant run-off from agricultural fields (including the herbicide atrazine, used primarily on corn), and landscape condition (proportion agriculture, urban development, and road density), as described in detail in Schoff et al. (*In review*). To sample across these environmental gradients, we divided the region into 2 X 2 township cells (~12 X 12 miles) and randomly selected cells for wetland sampling within each latitudinal zone of the main five ecoregions that make the U.S. portion of the Prairie Pothole Region. Cells were only included for potential selection if it contained 50 or more wetlands (based on National Wetland Inventory data) and 70% of the cell was within the Prairie Pothole Region boundary. Because calling surveys take place at night, safety and logistical issues dictated that we select clusters of wetlands that were not located within the same complex, but were within easy driving distance of one another during a given sampling event. Four to ten wetlands were selected for surveys within 19 randomly selected cells, based on field verification of standing water, land cover/ land use, and permission from landowners. In

addition to the 124 wetlands in the randomly selected cells, 25 seasonal and semi-permanent wetlands were selected for 'intensive' sampling in and around one cell in the Prairie Couteau ecoregion (Deuel and Brookings counties, South Dakota). This sampling included weekly monitoring of water depth and amphibian activity, and collection of metamorphic *R. pipiens* for malformation assessments.

Survey Methods

Amphibian surveys were conducted four times during the breeding season to assess presence of adults and eggs, and to sample larval and metamorphic amphibians. Presence, breeding, and developmental endpoints were assessed using standard amphibian breeding surveys to detect breeding adults, egg masses, larvae, and emerging metamorphic amphibians (Table 1).

Calling and Daytime Surveys. Amphibian surveys occurred across two years (2004-2005), with 102 wetlands surveyed in 2004 and 108 wetlands surveyed in 2005 (61 wetlands were surveyed in both 2004 and 2005). Breeding adults were detected with nighttime calling surveys conducted during early, mid, and late seasons in accordance with North American Amphibian Monitoring Program (NAAMP) protocol. We used time- and area-constrained daytime visual encounter surveys (VES) to identify adults, larvae, and eggs present, as well as potential predators. During the surveys coinciding with larval development (mid- and late-season), larval *R. pipiens* were sampled with 1 m long dip-net sweeps every 2 minutes during the VES. A final (fourth) visit was conducted to capture and evaluate metamorphic *R. pipiens*, using daytime visual surveys and hand capturing frogs for analysis of skeletal malformations and/or gonadal anomalies. For the

61 wetlands with two years of survey data, there was little difference in presence or breeding *R. pipiens*, with 75% and 82% having the same occupancy and breeding status, respectively, in both years. We identified the year with the most appropriate sampling conditions (based on NAAMP protocol) to include in analyses.

Malformation Surveys. In the intensive sampling area of the Prairie Couteau, we conducted 62 malformation surveys at 35 unique wetlands. We had no prior knowledge of the presence or absence of malformed specimens at these wetlands. Wetlands were surveyed once per year for multiple years, but in each year some wetlands were added or dropped due to hydrologic condition (e.g. drying) or other contingencies. All surveys and examinations were conducted using the protocol described in Schoff et al. (2003). Metamorphic anurans (Gosner stage 42 - 46, Gosner 1960) were collected by dip netting in a time- and effort-constrained protocol, which specified that if fewer than ten metamorphs were observed after 2 person-hours, the survey was abandoned. Collection continued until 100 - 150 metamorphs were obtained, or four person-hours had elapsed. Metamorphs were kept in containers until trained observers examined the live specimens for malformations, snout-vent length, and general condition. Specimens were returned to their wetland of origin, except for voucher specimens and some malformed individuals, which were euthanized by immersion in 1g/L 3-aminobenzoic acid ethyl ester (MS-222), fixed either in Bouin's fixative or 70% EtOH, and examined in the lab to observe skeletal elements. Malformations were classified based on published recommendations (Johnson et al. 2000; Meteyer et al. 2000). Primary abnormalities were assigned if they were: the only observable abnormality; clearly the most severely abnormal aspect in a specimen

containing multiple abnormalities; or the most proximal abnormality of an specimen containing more than one abnormality in a limb or body segment (e.g. if both the femur and tibiofibula of the same leg were malformed, the femur would be assigned as primary). Primary malformations were assigned a 'severe' classification if the abnormality was judged likely to negatively affect the frog's fitness (by impacting survival or ability to avoid predators and hunt for food). For statistical analyses, malformations were summarized to the wetland-level for surveys with a minimum of 50 metamorphic *R. pipiens* evaluated (n = 13 wetlands). Wetland-level endpoints were 1) presence/absence of all malformations, 2) prevalence of all malformations, and 3) prevalence of 'severe' malformations.

Gonadal anomalies. In 2005 metamorphic *R. pipiens* from 11 wetlands in Iowa, Minnesota, North Dakota, and South Dakota (Figure 1) were collected for gonadal analysis, as described in Schoff et al. (*In review*). Briefly, during a fourth field visit, *R. pipiens* at Gosner stage 44-46 (Gosner 1960) were captured, euthanized, and preserved in Bouin's fixative (Sigma, St. Louis, MO). Morphological sex of each specimen was determined by gross gonadal morphology and confirmed by histological analysis. Gonadal anomalies (primarily testicular oocytes, or TOs) were documented and quantified based on evaluation of step-sections of the entirety of each male gonad (5 μm thick sections, 50 μm steps, and standard Harris's hematoxylin and eosin staining). Gonadal anomalies were summarized for the nine wetlands with at least five males. Severity of TOs was scored in a representative median section of each specimen (the validation set for scoring is shown in Appendix C Figure S1). Wetland-level endpoints

were 1) prevalence (# with TOs/# males), 2) maximum severity rank where zero (0) contained zero TOs and five (5) was severely impacted (Appendix C Figure S1, Olker 2014c), and 3) presence/absence of TO severity of 3 or greater.

Characterization of Environmental Gradients

Environmental gradients were characterized based on a suite of 50 environmental variables collected at the wetland, local, and landscape scale (Table 2) that were selected for their potential to directly or indirectly affect the distribution, abundance and health of amphibians. Local- and within-wetland conditions were characterized with general water chemistry (water temperature, pH, specific conductivity), water depth, wetland morphology (wetland size, wetland type, dominant cover), wetland habitat, surrounding habitat, and presence of aquatic predators (fish, crayfish, predaceous diving beetles). Water samples were collected 3 times from each wetland (coinciding with amphibian surveys 1-3) to quantify atrazine concentrations during the period of amphibian development, as described in Schoff et al.(*In review*). Samples were filtered through a 0.2 micron filter and stored at 4° C until analysis with an enzyme-linked immunoabsorbent assay with a nominal detection limit of 0.011 µg/L (Abraxis, Warminster, PA). The average atrazine concentration from surveys 2 and 3 was used for analyses, as this collection period coincided with larval development of *R. pipiens*.

Land use/land cover was characterized from the 2001 National Land Cover Data (NLCD) for multiple buffer widths from 90 m to 3 km from the wetland edge, which encompasses the expected range of movement for *R. pipiens* (1-2 km, Merrell 1977). The 16 NLCD land use/land cover classes were aggregated to the following five categories:

wetland (emergent herbaceous wetlands), urban (developed open space, developed low intensity, developed medium intensity, developed high intensity), natural (deciduous forest, evergreen forest, mixed forest, shrub/scrub, grassland/herbaceous, pasture/hay), agriculture (cultivated crops), and open water. Human population density and stream density (ESRI data, 2000 block census data and all stream linework, respectively) were summarized for 10 km buffers, and road density (TIGER road dataset,and highways/freeways) was summarized for 2, 5, and 10 km buffers surrounding each study wetland. Additional location characteristics were also summarized and included in analyses, including distance to nearest road, latitude, elevation of wetland, standard deviation of elevation within 10 km buffer (measure of 'hilliness'), and proportion of the county planted in corn (to approximate potential exposure to pesticides, e.g. atrazine, and fertilizers).

An index of environmental gradients was derived with a principal components analysis (PCA) to combine these 50 distinct variables to determine the main environmental gradient(s) or environmental condition (Factor analysis, varimax raw rotation of axes, SAS 9.2). Interpretable principal axes were identified based on the 'broken-stick' method (McCune & Grace 2002) and interpreted based on correlations of independent predictor variables with axes.

Statistical Analysis

The following endpoints were used as response variables:

1. Presence of *R. pipiens* (all methods and calling surveys only)
2. Presence of breeding (eggs, larval or metamorphic *R. pipiens* observed)
3. Prevalence and severity of skeletal and eye malformations
4. Prevalence and severity of gonadal anomalies (TOs)

We used an information-theoretic approach (Burnham & Anderson 2002; Mazerolle 2006; Grueber et al. 2011) to evaluate the relationship of amphibian endpoints with environmental gradients and individual variables from each scale. This approach allows for comparison of multiple plausible models, averaging of model coefficients, and identification of the relative influence of individual predictor variables, in a framework that assumes that there is more than one 'best' model. Using the guidelines in Burnham & Anderson (2002), we quantified the relationships of the nine amphibian endpoints with environmental factors with two sets of models, using: 1) index of environmental gradients (primary axes from PCA) to address Goal 1, and 2) individual environmental variables run separately at each spatial scale to address Goal 2. For both sets of models, we evaluated the amount of model uncertainty for amphibian endpoints based on the % of models within 10 AICc (corrected Akaike's information criteria) of the top model (AICcMIN model). We then compared the relative importance of each predictor variable, hereafter called relative variable importance (RVI), which is based on the Akaike's weight of models in which a variable is present. For the models with the index of environmental gradients, we also identified the best-supported model or averaged model

to explain the presence, breeding, or developmental endpoints. See Appendix D for detailed steps of this analysis. Model types were as follows: logistic regression including a spatial term (glmmML, binomial) for presence, calling, and breeding; logistic regression (glm, binomial) for the other binary data (presence of malformations or severe gonadal anomalies), and linear regression (lm) for prevalence data (malformations, severe malformations, and TOs) and maximum TO severity score. Analyses were completed in R 3.0.0.

Results

R. pipiens was present in 58% (87/149) of the study wetland, while presence by ecoregions ranged from 32% to 83% (Table 3). Calling surveys detected *R. pipiens* in 32% (48/149) of individual wetlands and in 19-43% of wetlands in each of the five ecoregions. Of the wetlands with *R. pipiens* detected via visual or calling surveys, 71% (62/87) had evidence of breeding (eggs, larvae, or metamorphic frogs). Notably, calling was not detected in over half (55%, 34/62) of the wetlands with evidence of breeding.

Malformation prevalence and severity

Metamorphic frogs were found in 26 of the 35 unique wetlands in which malformations surveys were conducted in mid to late summer. Of 2679 specimens examined during these malformation surveys, 105 (3.92%) had identifiable abnormalities (Table 4). The vast majority of our specimens were from 22 surveys (at 13 unique wetlands over 1-3 years) during which 50 or more metamorphic *R. pipiens* were collected. Malformed specimens were found in 11 of these 13 wetlands, with average malformation prevalence of 3.24% and average prevalence of severe malformations of

2.54% (Table 4). Fifteen surveys yielded 100 or more metamorphic *R. pipiens;* at least one malformed frog was encountered during each of these surveys (data not shown). 'Severe' malformations were encountered in 74 specimens from 11 unique wetlands.

Gonadal anomalies

Specimens with TOs were found in all 11 of the wetlands surveyed, with number and severity of TOs varying across male metamorphic *R. pipiens* as described in Schoff et al. (*In review*) and Olker (2014a). For the current analysis, we included only the nine wetlands yielding at least five males; for these wetlands, TO prevalence ranged from 15.7% to 94.4% (average of 47.5%) and the maximum TO severity score at a wetland ranged from zero to the maximum score of five (Table 5).

Environmental gradients

The PCA of the wetland, local, and landscape variables identified three interpretable axes characterizing environmental gradients (Table 6). The first three PCA (eigenvectors) explained 46% of the variance, and accounted for three major gradients observed across the study area: developed land (urban, agriculture, and roads), geographic setting (which includes human population density, stream density, elevation, and wetland surface area), and wetland density (Figure 2). PC1 was highly positively correlated with the proportion of natural land cover from 90-3200 m and the number of seasonal wetlands within 2 km, and negatively correlated with the proportion of agriculture from 90-3200 m, urban at 800 m, and road density within 2 km (and thus can be said to reflect the gradient from developed to natural lands). PC2 represents geographic variation across the study area of the Prairie Pothole Region, with a positive

correlation with latitude and longitude, elevation, and wetland surface area, and negative correlation with human population density, road density, and stream density within 10 km, and proportion of county planted with corn. This axis characterizes differences across the ecoregions surveyed, with Northern Short Grasslands on the positive end, Central Tall Grasslands on the negative end, and Northern Mixed Grasslands, Prairie Couteau, and Central Tall Grasslands overlapping and encompassing zero. PC3 was highly positively correlated with proportion of wetlands and open water within 90-3200 m.

Relationship of amphibian metrics to environmental gradients

The relationship of the amphibian metrics to the index of environmental gradients (PCs) was not as strong as hypothesized (Table 7). There was considerable model uncertainty in response to the three principal components axes that represent environmental gradients, indicated by the large number of models within 10 AIC of the model with the lowest AIC value (Appendix D Tables S5-S13). This suggests that a multi-model approach was most appropriate, which entailed a comparison of multiple models and model-averaging when there was not one 'best' model (Burnham & Anderson 2002). Models assessing the relationship of amphibian metrics to the index of environmental gradients are included in Appendix D S5-S13, including AICc, Akaike model weight, and parameter estimates for each model, and selection probability (RVI) and averaged parameter estimates across models within 10 AICc of the AICcMIN model.

PC1 (the natural to developed land gradient) and PC3 (wetland density gradient) were each important variables for only one amphibian metric. PC1 had high RVI (greater

than 0.4) for only skeletal malformation prevalence and PC3 had high RVI for only evidence of breeding. PC2 (geographic setting) was an important variable for five of the nine amphibian metrics, with a negative relationship with presence, calling, and evidence of breeding, and a positive relationship with presence of skeletal malformations and presence of TOs with severity of score of 3 or greater. The interaction of PC1 and PC3 had RVI of less than 0.08 for all amphibian metrics (Table 7).

For *R. pipiens* presence, calling, and breeding, all models were within 10 AIC and Akaike model weights indicated that no model was more likely than any other model. There was little to no evidence that the null models were better than any other model within 2 AICc, based on the ratio of model weights (Appendix D Tables S5-7). *R. pipiens* presence and calling had a negative relationship (based on averaged coefficient across models within 10 AICc) with geographic setting (PC2), which was the most important PC for these amphibian metrics (RVI of 0.552 and 0.406, respectively). For *R. pipiens* breeding, PC2 and PC3 (wetland density) had similar RVI (0.466 and 0.406, respectively). Breeding had a negative relationship with geographic setting and a positive relationship with metric of wetland density (Table 7).

For the malformation endpoints, there were 2-3 models within 2 AICc of the AICcMIN model, with low evidence that any model was more likely than any other model, based on Akaike model weights (Appendix D Tables S8-10). Malformation presence had a positive relationship with PC2, which had the highest RVI (0.45) for this amphibian metric. In contrast, malformation prevalence had a negative relationship with developed to natural land gradient (PC1), which was the PC with the highest RVI (0.45)

for this metric. None of the PCs had RVI of greater than 0.4 for prevalence of 'severe' skeletal malformations (Table 7).

For 2 of the 3 gonadal endpoints (TO prevalence and maximum severity score) there was moderate to strong evidence that the null model was better than other models (Appendix D Tables S11-13). For these three metrics, RVI was less than 0.3 for the PCs that represent the index of environmental gradient. For presence of TOs with a severity score of 3 or greater, wetland density gradient, PC2, was included in one of the two models within 2 AICc of the AICcMIN model, with RVI of 0.468 across all models within 10 AICc and positive relationship with this metric (Table 7).

Relationship of amphibian metrics to environmental variables at each spatial scale

Analysis with individual environmental variables at each spatial scale showed that several of these predictor variables were more highly related to amphibian metrics than the index of environmental gradients (PCs). Variables from all three spatial scales (wetland, local and landscape) had high RVI, with differences across metrics. Within wetland variables had high RVI for *R. pipiens* presence, calling, evidence of breeding, and the three skeletal malformation metrics, with average water depth, wetland area, wetland regime, and water chemistry (temperature, pH, and specific conductivity) important for more than one amphibian metric (Table 8).

At the local spatial scale, the important variables were land use/land cover within 200 m, with proportion wetlands and proportion urban having the highest values across the environmental gradients and individual predictor variables (Table 9). Local variables

had high RVI for *R. pipiens* calling, evidence of breeding, and the three skeletal malformation metrics. Proportion of wetlands had a strong positive relationship with evidence of breeding, and proportion urban had a strong positive relationship with skeletal malformations. Although somewhat weaker based on RVI, proportion agriculture at this scale was an important variable for *R. pipiens* calling (with which it had a positive relationship).

Landscape variables had high RVI for *R. pipiens* presence, the three skeletal malformation metrics, and maximum TO severity score (Table 10). At this spatial scale, proportion of urban had the highest RVI, with a positive relationship with the three skeletal malformation metrics. Proportion wetlands was also important, with a positive relationship with presence of skeletal malformations. The number of semi-permanent wetlands within 1 km had a negative relationship with *R. pipiens* presence. TO metrics overall were poorly related to individual environmental variables, with the only exception being the average distance to wetlands (seasonal and semipermanent) within 2 km.

Discussion

We found that *R. pipiens* distribution and health in the Prairie Pothole Region were influenced by the index of environmental gradients and several individual predictors at each of the three spatial scales. The three types of indicator metrics, however, displayed different relationships to the index of environmental gradients, as well as to the individual variables and spatial scales.

Presence, calling, breeding

R. pipiens presence, calling, and breeding differed geographically, with higher detection at wetlands in the ecoregions at the center of the study area (Table 3). PC2 reflects the geographic gradients including land use and human population density, which are related to ecoregion, with greater agricultural intensity and higher population density in the east (Central Tall and Northern Tall ecoregions) compared to the west (Northern Mixed and Northern Short ecoregions). *R. pipiens* presence, calling, and breeding were negatively related to this measure of geographic setting, which supports the concept that broad scale landscape factors, such as land form or geologic history and regional land cover/land use patterns, are drivers of species distributions and patterns of amphibian presence/absence. The positive relationship of *R. pipiens* breeding with PC3 (wetland density) suggests that distributions of annual recruitment (based on successful reproduction) may differ from presence/absence of a species.

We found that wetland and local characteristics were equally or more important than the PCs representing the index of environmental gradient in models of *R. pipiens* presence, calling, and breeding. This response to wetland and local variables could be due to the fact that this species has a large geographic distribution (NPWRC 1997) and the ability to move across the landscape (albeit not very far relative to the size of the Prairie Pothole Region). Therefore, patterns in presence, calling, and breeding may reflect 'choices' by *R. pipiens* to utilize wetlands for breeding or non-breeding activities. In this study, we found that *R. pipiens* were more likely to be present in smaller, seasonal wetlands; however, likelihood of breeding increased with water temperature and average

water depth, and decreased with specific conductivity. These breeding-specific relationships match published *R. pipiens* breeding preferences (Merrell 1977; Smith & Keinath 2007) and suggest that *R. pipiens* select wetlands for breeding differentially from wetlands for non-breeding use. *R. pipiens* interact with the within-wetland and local habitat and stressors (e.g. water levels, predators), and have the opportunity to leave in search of more suitable or preferred conditions or wetlands. Landscape scale factors also influence these patterns because wetland density, land use/land cover, and barriers (e.g. roads) may alter or limit the ability of *R. pipiens* to access suitable/preferred habitat.

These results show that *R. pipiens* in the Prairie Pothole Region are influenced by multiple factors at multiple scales, as has been documented for North American amphibian species in the Midwest U. S. (Johnson et al. 2002a), Great Lakes (Price et al. 2004), and Colorado (Johnson et al. 2011), as well as in Australia (Lemckert & Mahony 2010), Belgium (Declerck et al. 2006), and Romania (Hartel et al. 2009). Unlike many of these previous studies, we did not find a strong relationship of *R. pipiens* presence, calling, or breeding with the land use at the local or landscape scale, with the exception of a higher likelihood of breeding *R. pipiens* in areas with higher proportion of wetlands across the spatial scales (PC3) and, specifically, within 200 m. Notably, this positive relationship appears to be driven by the much higher proportion of wetland land cover at wetlands with breeding in several areas (Figure 3). Multiple studies have elucidated species-specific relationships with wetland and local habitat, and more generalized relationships with landscape scale land use/land cover, including a negative relationship with urban land use and roads (Findlay & Houlahan 1997; Johnson et al. 2002a, 2011;

Price et al. 2004; Porej et al. 2004; Hartel et al. 2009) and a positive relationship with forest cover (Findlay & Houlahan 1997; Porej et al. 2004; Otto et al. 2007). In our study, the influence of land cover was not strongly detected in the individual predictor variables at the local and landscape scale, likely because the historic natural state for Prairie Pothole Region was grassland with little forest cover, which was a strong predictor in other studies. Additionally, our study wetlands were selected primarily in rural areas using a stratified random sampling design to characterize amphibian responses across the ecoregions of the Prairie Pothole Region. Because of the low human population density across much of the Prairie Pothole Region, this sampling did not occur across a strong gradient from urban to agriculture land use, as was the case in other U.S. Midwest and Eastern studies (Johnson et al. 2002a; Price et al. 2004; Porej et al. 2004; Otto et al. 2007). Rather, our study encompassed a gradient of natural habitat (grassland, forest, wetlands) to human developed (urban, agriculture, roads). We also did not find a strong response to road density, which could be due to the low urban development in our study area in combination with the relatively uniform road density due to the presence of section lines throughout this rural study area.

Physiological state metrics

Measures of health or physiological state of organisms in a wetland may be better measures than presence for estimating population-level impacts. For example, several physiological state indicators (body condition and stress hormone levels) were found to be responding to habitat quality and availability at a finer spatial scale than presence in common toad (*Bufo bufo*) populations (Janin et al. 2011). We used metrics that

incorporate the larval response to the aquatic environment beyond survival to metamorphosis as a measure of 'health' of the metamorphic frogs, which is important for annual recruitment and juvenile survival, and possibly reproductive capacity.

Skeletal malformations. The overall malformation prevalence (3.9%) in Prairie Pothole Region wetlands was higher than the recent analysis of amphibian malformations across U.S. National Wildlife Refuges (2% skeletal or eye abnormalities, Reeves et al. 2013), but within a range (0-10.2%) comparable to previous reports (Taylor et al. 2005; Piha et al. 2006; Reeves et al. 2008). Although our malformation study wetlands were selected as part of our overall randomized sampling design, these surveys were conducted in a small area of the Prairie Pothole Region with a limited range across the geographical gradient and small range of urban development. Despite the localized nature of this sampling, study wetlands still had a broad range across the other two environmental gradients (natural to agriculture/urban; wetland density).

Prevalence of skeletal malformations was the only metric for which the natural to agriculture/urban gradient was an important variable; however, independent predictor variables appear to be more related to this set of metrics with average wetland depth and proportion urban development within 200 m and 800 m as important variables for the three skeletal malformation metrics (Figure 4). Urbanization has been reported to have an influence on overall anuran abundance and diversity (Pillsbury & Miller 2008) and presence of gonadal anomalies (Skelly et al. 2010). Malformations, however, have more often been associated with other factors, such as proximity to agriculture (Taylor et al. 2005) or roads (Reeves et al. 2008), organic and inorganic contaminants (Reeves et al.

2010), presence of predators (Reeves et al. 2008) or parasites (Johnson et al. 2002b), and a combination of factors (Rohr et al. 2008). These natural to developed gradient (PC1), and associated local and landscape variables likely contribute to or alter within-wetland characteristics (e.g. nutrients, presence and concentrations of suites of agrochemicals) that are directly or indirectly related to malformations, but were not measured in this study. For example, this positive relationship of malformations with PC1 could be due to a direct relationship with a teratogenic pesticide or an indirect effect of fertilizers and herbicides increasing parasitic trematode infections via increased nutrients, with contaminants entering the aquatic ecosystem via wet or dry deposition or run-off from the surrounding agricultural or urban landscapes (Mann et al. 2009).

Gonadal anomalies (TOs). Gonadal anomalies were detected in frogs from all 11 wetlands that were evaluated, which covered a range of local and landscape characteristics. The geographic gradient, PC2, was an important PC variable for the presence of severe anomalies; however, prevalence and maximum severity score were unrelated to the environmental gradients and prevalence was unrelated to the measured independent predictors (Figure 5, Tables 7-10).

This lack of response to measured habitat variables at the local and landscape scale matches the results of a previous analysis of *R. pipiens* (Schoff et al. *In review*) and of the closely related Plains Leopard Frog (*Rana blairi*) in the agricultural region of Nebraska (Papoulias et al. 2013). In these studies, TOs were present in frogs from all wetlands sampled and TO presence or severity was unrelated to atrazine concentration or proportion agriculture in the landscape. This result also matches other studies that did not

find reproductive effects of atrazine in exposure experiments (Coady et al. 2004; Jooste et al. 2005; Orton et al. 2006; Oka et al. 2008; Olker 2014a). However, gonadal anomalies have been associated with land use (agriculture and urban development) in field-collected amphibians (McDaniel et al. 2008; Skelly et al. 2010), suggesting that TOs may be related to variables not measured in this study (e.g. synthetic or natural estrogens in the environment).

Comparison of metrics

There were considerable differences across amphibian metrics in response to the index of environmental gradients and individual variables at each scale (wetland, local, and landscape). As a result of this research, we recommend amphibian breeding presence or success as a better indicator than species presence or calling. Breeding was related to geographic location (more likely in certain ecoregions) as well as overall wetland density and several individual variables within the wetland and at the local scale. This metric incorporates presence of females and adequate or desirable breeding habitat, which is not included in presence or calling alone.

The different results obtained from surveys of breeding evidence and presence/absence emphasizes the need for amphibian population assessments to consist of more than anuran calling surveys. While calling surveys can indicate presence and population size of males, they do not estimate the quality of breeding habitat or annual recruitment. Evaluating responses to local and landscape habitat based on calling surveys or other presence/absence measures does not provide adequate information to manage amphibian populations (or the habitat they depend on). For example, in two of the

ecoregions (Central Tall and Northern Mixed Grasslands), *R. pipiens* were breeding in only 40-58% of the wetlands in which they were present (Table 3), demonstrating that adult *R. pipiens* are present (calling or non-breeding activity) in unsuitable breeding habitat. Clearly, presence can create a false positive if used as the only indicator in occupancy or habitat suitability assessments. Visual searches for evidence of breeding, or even more intensive sampling methods, have recently been included in studies because these metrics provide a better indication of how local and landscape habitat could impact amphibian populations. For example, egg mass counts have been used to estimate population size and evaluate influence of local and landscape habitat (Hartel et al. 2009), and evidence of breeding was used in conjunction with presence to assess local and landscape influences on decline of *R. pipiens* in Colorado (Johnson et al. 2011).

The metrics of physiological condition (malformations and gonadal anomalies) could be informative indicators; however, these metrics are only possible if the species is present and breeding is successful, as many of these metrics typically include only metamorphic individuals from a known breeding location. Our results suggest that skeletal malformations may be a good additional indicator, as there were strong positive relationships with the gradient of natural to developed land (agriculture, urban, and roads). However, assessing this metric is dependent on successful breeding and is labor intensive. TO prevalence and severity are not recommended metrics, based on the lack of relationship with environmental variables measured, in addition to the uncertainty of impacts on reproductive capacity and the high cost of collection and histopathology.

Summary/Conclusion

We recommend evidence of amphibian breeding (presence or success) as a better indicator of local population status (and recruitment) than species presence or calling. Skeletal malformations may be a good indicator of human influence, and along with breeding presence/absence may be effective measures of environmental conditions. However, more research is needed on direct and indirect causes of amphibian malformations.

Based on reported amphibian responses to environmental change, we assume that evidence of amphibian breeding indicates the quality of the aquatic and surrounding terrestrial habitat. Specifically, calling males (or presence of adults) indicates that: 1) overwintering habitat is available in the landscape for breeding adults, 2) terrestrial food and shelter are available, 3) the wetland is accessible to adults (distance and land cover acceptable for migration to breeding pond), and 4) the wetland contains habitat (e.g. vegetation, depth) to attract breeding adults. The addition of evidence of breeding expands the indicator to include that wetland habitat is amenable for larval development (e.g. appropriate hydrology, limited predators and contaminants).

In this study we used any evidence of breeding (eggs, larvae of any age, or metamorphic frogs); however, breeding success (presence of metamorphic frogs or some measure of number of metamorphic frogs) would be a further measure of current wetland condition (Guzy et al. 2012). Additionally, data about successful annual recruitment (number or density of metamorphic amphibians dispersing from a wetland) would ultimately be necessary to understand and manage amphibian populations (Semlitsch 2000). Another component of recruitment with indicator potential is the health of

individuals through measures of physiological state. While very informative, these metrics are only possible if the species is present and breeding is successful, as many of these metrics typically include only metamorphic individuals from a known breeding location. Additionally, measures of physiological state respond differently to environmental changes, such as the differences between malformations and gonadal anomalies found in the Prairie Pothole Region, and more research is needed to understand if each of these endpoints are responding to or indicating habitat quality or environmental condition.

While amphibian metrics included here provide a snapshot of the amphibian population and distribution, sampling across this large area allowed identification of relationships with within-wetland, local, and landscape scale factors. In this analysis, we could only compare amphibian metrics to the measured environmental and habitat variables. However, the set included a fairly comprehensive list (with the exception of nutrient and parasite data) of variables reported in previous studies as directly or indirectly influencing amphibians. Presence, breeding, and health of metamorphic *R. pipiens* are essential components of measuring population sustainability; however, long-term population monitoring, including breeding success, is the only way to determine population trends and identify areas at risk of local extirpation that may lead to greater population discontinuity and decline. Clearly, to understand amphibian populations and preserve aquatic ecosystems, there is a need for greater support to monitor populations and conduct surveys/sampling across large geographic scales using statistically determined sampling points.

Table 1. Description of response variables of *Rana pipiens* with sample size and type of statistical model.

Type	Variable	Description	Model
Habitat use n = 149 wetlands	Presence	*Rana pipiens* heard or seen in nighttime calling or daytime visual encounter surveys	logistic regression with spatial term[a]
	Calling	*Rana pipiens* calling during 10 min nighttime calling surveys following NAAMP protocol	logistic regression with spatial term[a]
	Breeding	presence of breeding observed during daytime visual encounter surveys: eggs, larval or metamorphic *R. pipiens* observed	logistic regression with spatial term[a]
Malformations n = 13 wetlands[b]	Presence	skeletal or eye malformation observed in examination of at least 50 metamorphic *R. pipiens* from a wetland	logistic regression[d]
	Prevalence (all)[c]	# of specimens with of skeletal or eye malformations/ total # examined, from examination of at least 50 metamorphic *R. pipiens* from a wetland	linear regression[e]
	Prevalence of severe malformations[c]	prevalence of 'severe' malformations (likely to impact survival or ability to avoid predators and hunt for food) from examination of at least 50 metamorphic *R. pipiens* from a wetland	linear regression[e]
Gonadal anomalies n = 9 wetlands[f]	Prevalence[g]	# of males with testicular oocytes/total # males examined, from gonadal histological analysis from at least 5 male metamorphic *R. pipiens* collected at a wetland.	linear regression[e]
	Presence of severe (score ≥3)	presence of severity score of 3 or greater observed in male metamorphic *R. pipiens* collected from a wetland, validation set used for scoring shown in Appendix C Figure S1	logistic regression[d]
	Maximum severity score	maximum severity score in male metamorphic *R. pipiens* collected from a wetland, validation set used for scoring shown in Appendix C Figure S1	linear regression[e]

[a] Generalized linear mixed model in R (glmmML, binomial) with spatial term (cell ID) included as a random effect to account for spatial dependencies of wetlands within a general area (12 by 12 mile cell); [b] 35 wetlands sampled for malformation assessments, 13 wetlands included in analysis (50 or more metamorphic *R. pipiens* evaluated in at least one year); [c] square-root transformation; [d] Generalized linear model in R (glm, binomial); [e] Linear model in R (lm); [f] 11 wetlands sampled for gonadal assessments, 9 wetlands included in analysis (at least five males evaluated); [g] TOs were found at all wetlands in at least one specimen, therefore TO presence/absence could not be included as a response variable.

Table 2. Environmental variables (within wetland, local, and landscape scale) included in Principle Components Analysis and models

Scale	Abbrev	Predictor variable description	in models (VIF < 3)
within	HYDRO	hydroregime (seasonal or semi-permanent)	X
	AqPredYN	aquatic predators: odonata, crayfish, predaceous diving beetles	X[c]
	ModYN	wetland modified: Yes/No	X[d]
	AREA	surface area of wetland (m2)[a]	X[d]
	Avg_Depth	average water depth measured at pond center	X
	SD_Depth	SD of water depth measured at pond center[a]	X[d]
	Basin_Wet	percent of basin wet[b]	X[d]
	Emerg	percent of wetland that is emergent vegetation[b]	X[d]
	pH	pH	X
	Sp_Cond	specific conductivity (µS)[a]	X
	DayWaterT	water temperature (degrees C) during day surveys	X[d]
	CallWaterT	water temperature (degrees C) during calling surveys	X
	AvgATZ23	average of atrazine from survey 2 and 3 (in sample yr used) [a]	X
local	LC	landcover category in 90 m buffer (grass or crop)	X[d]
	Rd_dist	distance to nearest road[a]	X[c]
	pAg90	proportion of 90 m buffer that is Agriculture[b]	
	pAg200	proportion of 200 m buffer that is Agriculture[b]	X
	pNatural90	proportion of 90 m buffer that is Natural[b]	
	pNatural200	proportion of 200 m buffer that is Natural[b]	
	pUrban90	proportion of 90 m buffer that is Urban[b]	
	pUrban200	proportion of 200 m buffer that is Urban[b]	X
	pWet90	proportion of 90 m buffer that is Wetland[b]	
	pWet200	proportion of 200 m buffer that is Wetland[b]	X
	pWetOW90	proportion of 90 m buffer that is Open Water or Wetland[b]	
	pWetOW200	proportion of 200 m buffer that is Open Water or Wetland[b]	
	SS100n	# of seasonal wetlands within 100 m buffer[a]	X
	SP100n	# of semipermanent wetlands within 100 m buffer[a]	X

Table 2, continued. Environmental variables at the within wetland, local, and landscape scales.

Landscape	Elev	elevation of wetland (m)	X[d]
	Lat_N	latitude (decimal degrees)	X[d]
	Long_W	longitude (decimal degrees)	
	propcorn	proportion of county planted in corn (2005) [b]	
	Strm_km_km2	km of stream per square km (within 10 km buffer) [a]	
	PPSK	# people per square km (within 10 km buffer) [a]	X[d]
	ELEV_STD	standard deviation of elevation in meters (within 10 km buffer) [a]	X[d]
	RdDensity_all2k	road density (km/km2), all road types within 2 km buffer [a]	X[d]
	RdDensity_all10k	road density (km/km2), all road types within 10 km buffer[a]	
	RdDensity_HF2k	road density (km/km2), highways & freeways within 2 km	X[d]
	RdDensity_HF10k	road density (km/km2) of highways & freeways within 10 km	X
	pAg800	proportion of 800 m buffer that is Agriculture[b]	X
	pAg3200	proportion of 3200 m buffer that is Agriculture[b]	
	pNatural800	proportion of 800 m buffer that is Natural[b]	
	pNatural3200	proportion of 3200 m buffer that is Natural[b]	
	pUrban800	proportion of 800 m buffer that is Urban[b]	X
	pUrban3200	proportion of 3200 m buffer that is Urban[b]	X[d]
	pWet800	proportion of 800 m buffer that is Wetland[b]	X
	pWet3200	proportion of 3200 m buffer that is Wetland[b]	
	pWetOW800	proportion of 800 m buffer that is Open Water or Wetland[b]	
	pWetOW3200	proportion of 3200 m buffer that is Open Water or Wetland[b]	
	SS1000n	# of seasonal wetlands within 1000 m[a]	X[e]
	SS2000n	# of seasonal wetlands within 2000 m[a]	
	SP1000n	# of semipermanent wetlands within 1000 m[a]	X[e]
	SP2000n	# of semipermanent wetlands within 2000 m[a]	
	SPSS1000d	average distance (m) to seasonal and semipermanent wetlands	X[d]
	SPSS2000d	average distance (m) to seasonal and semipermanent wetlands	X

[a] square root transformation; [b] arcsin square root transformation; [c] not included in gonadal models (based on VIF >3); [d] not included in malformation or gonadal models (based on VIF >3); [e] not included in malformation models (based on VIF >3)

Table 3. Summary of wetland types and *Rana pipiens* presence, calling, and breeding across the Prairie Pothole Region ecoregions (U.S. portions east of Montana) included in study area.

	Ecoregion					
	Northern Short	**Northern Mixed**	**Prairie Couteau**	**Northern Tall**	**Central Tall**	**All**
wetland regime	31[a]	39 (39)	42	14	23	149[a]
# of semipermanent	15	13	22	10	13	73
# of seasonal	16	26	20	4	10	76
surrounding landcover (within 90 m)						
# 'crop'	17	19	16	4	7	63
# grassland or pasture	14	20	26	10	16	86
Rana pipiens:						
Present						
# wetlands	10	24	35	8	10	87
% wetlands	32%	62%	83%	57%	43%	58%
Calling						
# wetlands	6	13	18	5	6	48
% wetlands	19%	33%	43%	36%	26%	32%
Breeding						
# wetlands	8	14	30	6	4	62
% wetlands	26%	36%	71%	43%	17%	42%

[a] 3 wetlands dried during the breeding season and were excluded.

Table 4. Summary of malformation prevalence and severity in metamorphic *Rana pipiens* found in seasonal and semi-permanent wetlands with at least 50 specimens evaluated in at least one survey.

Wetland ID	# years with >50 specimens	Malformation prevalence all	severe	limb (fore/hind)	cranial-facial
CSSPC-205	1	0.015	0.015	0.008	0.007
CSSPg-4	1	0.000	0.000	0.000	0.000
CSSSg-5	1	0.030	0.030	0.030	0.000
SPg-202	2	0.032	0.032	0.014	0.015
SPg-206	1	0.030	0.022	0.030	0.000
SPg-T11	1	0.021	0.021	0.014	0.007
SPg-T20	1	0.060	0.045	0.052	0.007
SPg-T8	2	0.014	0.011	0.014	0.000
SSC-T25	3	0.082	0.042	0.072	0.008
SSg-1707	2	0.102	0.089	0.076	0.027
SSg-1751	2	0.000	0.000	0.000	0.000
SSg-204	2	0.016	0.016	0.013	0.003
SSg-T35	2	0.019	0.007	0.012	0.007

Table 5. Summary of testicular oocyte prevalence and severity in metamorphic *Rana pipiens* found in seasonal and semi-permanent wetlands.

Wetland ID#	#. metamorphic *R. pipiens* captured	Testicular oocytes			
	Males (all)	# of males evaluated for TOs	prev.	severity score ≥3 present?	max severity score
2	18 (31)	17	0.944	Y	5
5	5 (10)	3	0.600	N	2
6	61 (130)	10	0.164	Y	4
12	15 (26)	8	0.533	N	1
13	7 (13)	2	0.286	N	2
14	51 (98)	8	0.157	N	2
15	2 (2)	1	0.500	Y	3
16	7 (9)	3	0.429	N	2
17	1 (2)	1	1.00	N	0
18	55 (88)	49	0.891	Y	4
19	40 (58)	11	0.275	Y	3

Table 6. Loadings of environmental variables on first three axes from the Principle Components Analysis (varimax raw rotated factor pattern) with primary axis shaded grey for each variable.

Predictor variable	PC1	PC2	PC3
pNatural800	0.8641	0.14739	-0.11383
pNatural200	0.85644	0.04545	-0.08329
pNatural90	0.8245	-0.00477	-0.10181
pNatural3200	0.76117	0.3678	-0.17314
SS2000n	0.60826	0.53291	-0.03587
SS1000n	0.59351	0.44355	-0.04074
rd_dist	0.54528	-0.14202	-0.02029
ELEV_STD	0.49454	-0.06518	-0.27646
SP1000n	0.4245	0.31674	0.12367
SS100n	0.32896	0.17824	0.00884
emerg	0.24539	-0.17588	-0.12389
CallWaterT	0.20536	0.00209	-0.05549
DayWaterT	0.17819	-0.05128	0.00146
RdDensity_HF2k	-0.14458	0.04714	-0.07913
Avg_Depth	-0.20896	-0.04736	-0.07209
basin_wet	-0.3112	0.15618	0.2625
pUrban90	-0.55541	0.1708	-0.05229
pUrban3200	-0.56235	-0.35872	0.01565
RdDensity_all2k	-0.57697	-0.15505	0.12912
pUrban200	-0.5947	0.14688	-0.02915
pUrban800	-0.62568	-0.06185	0.05374
pAg90	-0.71864	-0.07654	-0.24142
pAg3200	-0.75554	-0.41729	-0.01721
pAg200	-0.7874	-0.07342	-0.21593
pAg800	-0.83882	-0.20185	-0.13288

Table 6, continued.

Predictor variable	PC1	PC2	PC3
Long_W	-0.04618	0.85823	0.07672
Lat_N	-0.14671	0.7994	0.3763
Elev	0.25427	0.70779	-0.12822
area	-0.26748	0.69696	0.21371
SP2000n	0.44136	0.4573	0.07708
spcond	0.04142	0.43817	0.34526
SPSS2000d	0.09708	0.42894	0.05107
SPSS1000d	0.00422	0.39578	-0.15087
sd_depth	-0.04401	-0.22905	0.00469
RdDensity_HF10k	-0.01605	-0.35045	-0.00388
AvgATZ23	0.01573	-0.38602	-0.07394
strm_km_km2	0.32723	-0.63036	-0.29653
PPSK	-0.20652	-0.77083	-0.05072
RdDensity_all10k	-0.2926	-0.79912	0.07005
propcorn	0.08993	-0.82051	-0.33772
pWetOW200	-0.03975	-0.03165	0.91777
pWet200	0.01647	-0.1071	0.91629
pWet90	-0.11863	-0.03099	0.87289
pWetOW90	-0.14594	0.06826	0.85721
pWetOW800	-0.01037	0.17278	0.81871
pWet800	0.08588	0.09084	0.78769
pWet3200	0.16027	0.32689	0.61485
pWetOW3200	0.13695	0.3779	0.59723
SP100n	0.27376	-0.19322	0.32182
pH	-0.15585	0.15041	0.194
eigenvalue	9.501	7.389	6.320
% variance explained	21.6%	15.6%	9.3%
cumulative % explained	21.6%	37.1%	46.4%

Table 7. Relationship of index of environmental gradients (PCs) with amphibian metrics: relative variable importance (>0.4 in bold) based on inclusion in and weight of models within 10 AICc of AICcMIN model (* indicates inclusion in models within 2 AICc of AICcMIN model); direction of average parameter coefficients from models within 10 AICc of AICcMIN model (+, -, or +/-) included in parentheses.

	Index of Environmental Gradient: Predictor variables			
Amphibian metric (response variable)	***PC1***	***PC2***	***PC3***	***PC1*PC3***
Presence/absence	0.277	**0.552***	0.292	0.024
	(+/-)	**(-)**	(+)	(+)
Calling	0.315*	**0.406***	0.349*	0.056
	(+/-)	**(-)**	(+)	(+)
Evidence of breeding	0.380*	**0.466***	**0.460***	0.048
	(+)	**(-)**	**(+)**	(+)
Presence of skeletal malformations	0.310*	**0.450***	0.170	0.000
	(-)	**(+)**	(+/-)	(-)
Prevalence of skeletal malformations	**0.450***	0.130	0.180	0.000
	(-)	(+)	(-)	(+)
Prevalence of 'severe' skeletal malformations	0.330*	0.134	0.139	NA
	(-)	(+)	(+/-)	
Prevalence of testicular oocytes	0.072	0.070	0.096	NA
	(+)	(+)	(-)	
Presence of testicular oocytes with severity score of 3 or greater	0.179	**0.468***	0.222	0.072
	(+/-)	**(+)**	(+/-)	(+)
Maximum testicular oocyte severity score	0.065	0.246	0.105	NA
	(+/-)	(+)	(-)	

Table 8. Relationship of wetland variables with amphibian metrics: relative variable importance (>0.4 in bold) based on inclusion in and weight of models within 10 AICc of AICcMIN model (* indicates inclusion in models within 2 AICc of AICcMIN model); direction of average parameter coefficients from models within 10 AICc of AICcMIN model (+, -, or +/-) included in parentheses; shaded rows indicate variables not included in information-theoretic models due to variance inflation factor (VIF) >3.

	Within wetland: Predictor variables												
Amphibian metrics	***Aq Pred***	***Basin_Wet***	***Emerg***	***Avg_Depth***	***Call WaterT***	***Day WaterT***	***HYDRO***	***Mod***	***AREA***	***Avg ATZ23***	***pH***	***SD_Depth***	***Sp cond***
Presence/absence	0.345* (+)	0.362* (+)	0.263 (+/-)	**0.557*** **(+)**	0.352* (+)	0.246* (+/-)	**0.454*** **(+)**	0.340* (+)	**0.441*** **(-)**	0.283* (+)	0.334* (-)	0.250* (+/-)	0.267 (+/-)
Calling	0.302* (+)	**0.587*** **(+)**	0.379* (-)	0.338* (+)	0.299* (-)	0.273* (+/-)	0.228 (+)	0.255* (+)	**0.500*** **(-)**	0.308* (+)	0.287* (+/-)	0.277* (+)	0.231* (+/-)
Evidence of breeding	0.253* (+)	0.314* (+)	0.263* (+/-)	**0.549*** **(+)**	**0.530*** **(+)**	0.244 (+/-)	0.279* (+)	0.238 (+)	0.230 (+/-)	0.340* (+)	0.280* (+)	0.222 (+/-)	**0.738*** **(-)**
Presence of skeletal malformations	0.124 (+)			**0.518*** **(+)**	0.272* (+/-)		0.153 (+/-)			0.251* (+/-)	0.308* (-)		0.639* (-)
Prevalence of skeletal malformations	0.442 (+)			**0.792*** **(+)**	0.049* (+/-)		**0.491*** **(+)**			0.047 (+/-)	**0.572*** **(-)**		0.236 (-)
Prevalence of 'severe' skeletal malformations	0.381* (+)			**0.597*** **(+)**	0.066 (+/-)		0.341* (+)			0.065 (+/-)	**0.410*** **(-)**		0.264* (-)
Prevalence of testicular oocytes				0.083 (-)	0.054 (+/-)		0.100 (+)			0.116 (+)	0.093 (+)		0.056 (+/-)
Presence of testicular oocytes with severity score of 3 or greater				0.266* (+)	0.055 (+/-)		0.053 (+)			0.051 (+/-)	0.052 (+)		0.079 (+)
Maximum testicular oocyte severity score				0.140 (+)	0.088 (-)		0.055 (+)			0.061 (+)	0.055 (+)		0.055 (+)

Table 9. Relationship of local variables with amphibian metrics: relative variable importance (>0.4 in bold) based on inclusion in and weight of models within 10 AICc of AICcMIN model (* indicates inclusion in models within 2 AICc of AICcMIN model); direction of average parameter coefficients from models within 10 AICc of AICcMIN model (+, -, or +/-) included in parentheses; shaded rows indicate variables not included in information-theoretic models due to variance inflation factor (VIF) >3.

	Local (within 200 m): Predictor variables						
Amphibian metrics	***pAg200***	***pUrban200***	***pWet200***	***LC***	***rd_dist***	***SP100n***	***SS100n***
Presence/ absence	0.279 (+)	0.261 (-)	0.329* (+)	0.283* (+)	0.262 (-)	0.271 (-)	0.277* (+)
Calling	**0.517*** **(+)**	0.303* (-)	0.303* (+)	0.294* (+)	0.375* (+)	0.302* (+)	0.341* (-)
Evidence of breeding	0.262 (+/-)	0.359* (-)	**0.830*** **(+)**	0.262 (+)	0.307* (-)	0.272* (+/-)	0.252 (+/-)
Presence of skeletal malformations	0.401* (+/-)	**0.729*** **(+/-)**	0.201* (+/-)		0.230* (+/-)	0.290* (+/-)	0.346* (-)
Prevalence of skeletal malformations	0.121 (-)	**0.980*** **(+)**	0.079 (+/-)		0.142 (+)	0.082 (+/-)	0.185 (-)
Prevalence of 'severe' skeletal malformations	0.161 (-)	**0.964*** **(+)**	0.072 (+)		0.107 (+/-)	0.073 (+/-)	0.398* (-)
Prevalence of testicular oocytes	0.060 (+)	0.061 (-)	0.090 (-)			0.067 (+)	0.062 (+)
Presence of testicular oocytes with severity score of 3 or greater	0.069 (-)	0.060 (-)	0.060 (-)			0.095 (-)	0.068 (+)
Maximum testicular oocyte severity score	0.059 (+)	0.059 (+)	0.093 (-)			0.080 (-)	0.059 (-)

Table 10. Relationship of landscape variables with amphibian metrics: relative variable importance (>0.4 in bold) based on inclusion in and weight of models within 10 AICc of AICcMIN model (* indicates inclusion in models within 2 AICc of AICcMIN model); direction of average parameter coefficients from models within 10 AICc of AICcMIN model (+, -, or +/-) included in parentheses; shaded rows indicate variables not included in information-theoretic models due to variance inflation factor (VIF) >3.

	Landscape (800 m – 10 km): Predictor variables													
Amphibian metrics	***pAg 800***	***pUrban 3200***	***pUrban 800***	***pWet 800***	***Elev***	***ELEV_STD***	***PPSK***	***Rd Density_all2k***	***Rd Density_HF10k***	***Rd Density_HF2k***	***SP 1000n***	***SPSS 1000d***	***SPSS 2000d***	***SS 1000n***
Presence/ absence	0.251* (-)	0.229 (+/-)	0.315* (+)	0.246* (+)	0.350* (-)	0.365* (+)	0.227 (+/-)	0.237* (+/-)	0.255* (+)	0.223 (+/-)	**0.542*** **(-)**	0.329* (-)	0.219 (+/-)	0.246* (+/-)
Calling	0.340* (+)	0.212 (+/-)	0.229* (+/-)	0.210 (+/-)	0.224* (+/-)	0.342* (+)	0.217 (+/-)	0.215 (+/-)	0.273* (+)	0.211 (+/-)	0.214 (+/-)	0.353* (-)	0.232* (+/-)	0.256* (+/-)
Evidence of breeding	0.333 (-)	0.328 (+/-)	0.241 (+/-)	0.260 (+)	0.358 (-)	0.355 (+)	0.284 (+/-)	0.355 (-)	0.235 (+/-)	0.233 (+/-)	0.321 (-)	0.260 (-)	0.223 (+/-)	0.269 (+/-)
Presence of skeletal malformations	0.240 (+)		**0.688*** **(+)**	**0.576*** **(+)**					0.414 (-)				0.180 (+/-)	
Prevalence of skeletal malformations	0.101 (+/-)		**0.871*** **(+)**	0.134 (+)					0.085 (+/-)				0.301* (+)	
Prevalence of 'severe' skeletal malformations	0.100 (+/-)		**0.802** **(+)**	0.217 (+)					0.095 (+/-)				0.289 (+)	
Prevalence of testicular oocytes	0.090 (+)		0.100 (-)	0.090 (-)					0.089 (-)		0.217* (+)		0.096 (-)	0.085 (-)
Presence of testicular oocytes with severity score of 3 or greater	0.084 (+/-)		0.093 (-)	0.099 (-)					0.092 (+/-)		0.082 (+/-)		0.271* (+)	0.090 (+)
Maximum testicular oocyte severity score	0.065 (+/-)		0.064 (+/-)	0.323* (-)					0.077 (+/-)		0.071 (-)		**0.572*** **(+)**	0.062 (+/-)

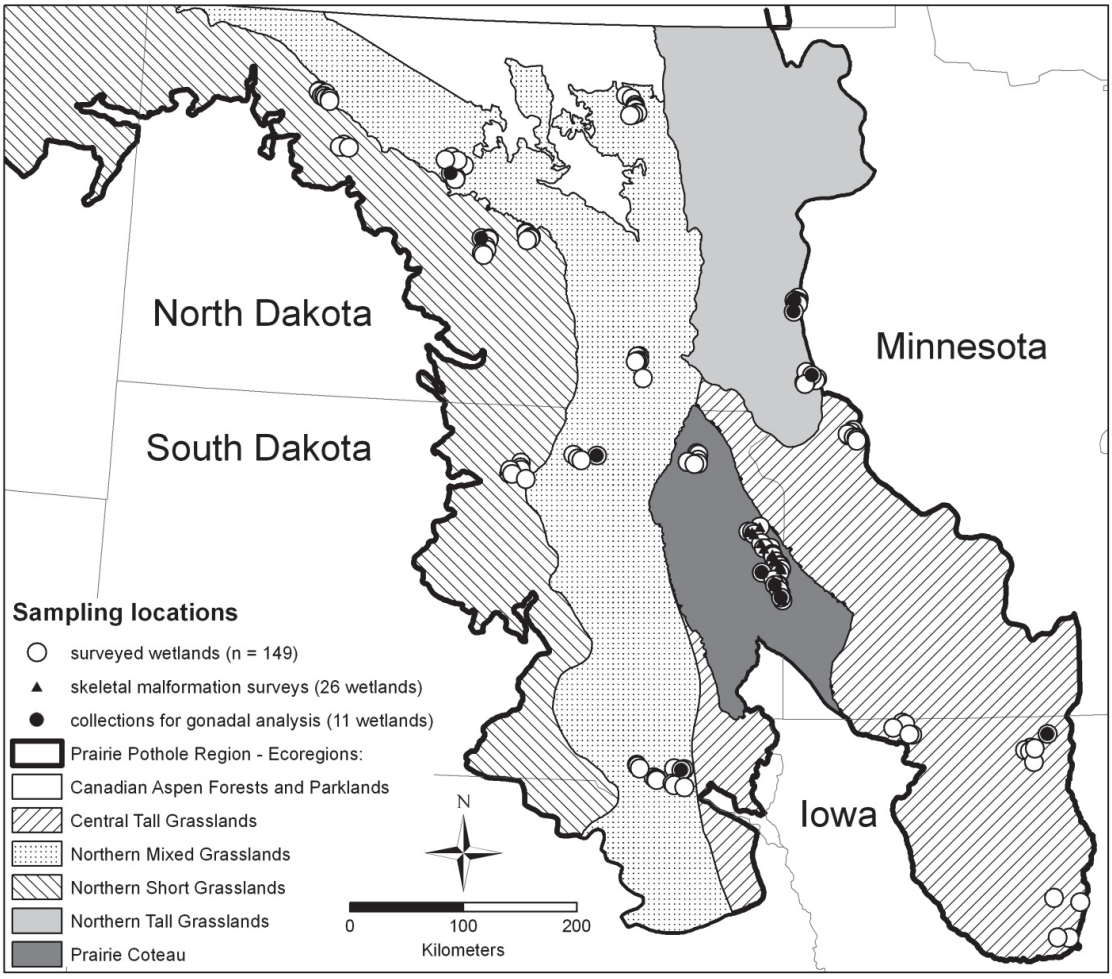

Figure 1. Map of study wetlands (all circles) within the Prairie Pothole Region, with wetlands with additional endpoints identified with closed triangles for skeletal malformations and closed circles for gonadal analysis.

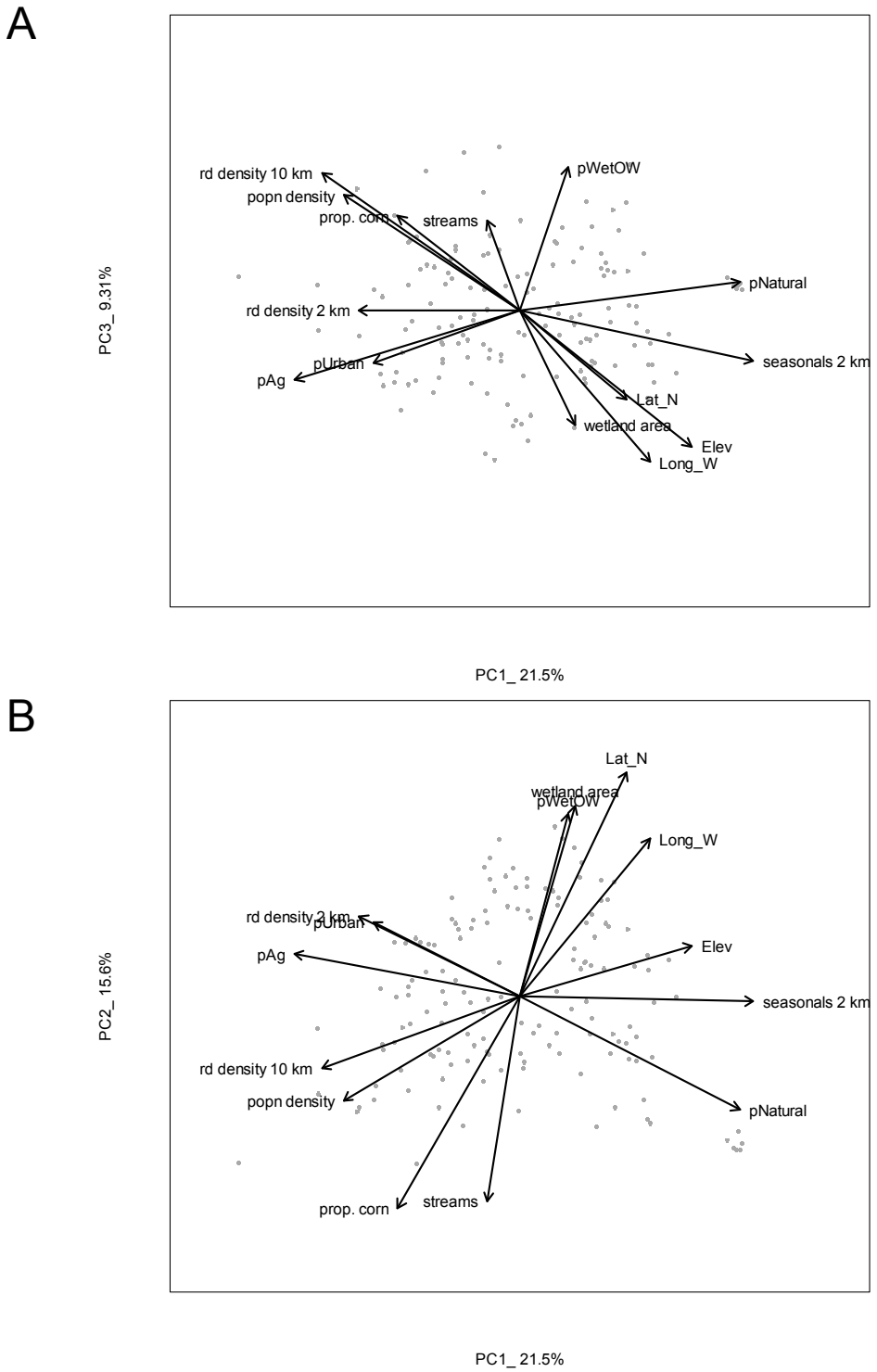

Figure 2. The three major axes from Principle Components Analysis used to develop index of environmental gradients, with % variance explained by each axis and arrows indicating the direction and magnitude of correlation with the axes (average across groups of closely related variables, e.g. proportion natural at multiple buffers).

Details of Figure 2: PC1 represents range of developed land, with highly positive correlations with proportion natural land cover from 90-3200 m and number of seasonal wetlands within 2 km and negative correlations with proportion of agriculture from 90-3200 m, urban at 800 m, and road density within 2 km; PC2 represents geographic variation (across ecoregions) across the study area of the Prairie Pothole Region, with positive correlation with latitude and longitude, elevation, and wetland surface area, and negative correlated with human population density, road density, and stream density within 10 km, and proportion of county planted in corn; PC3 represents range in wetland density, with high positive correlations with proportion of wetlands and open water within 90-3200 m.

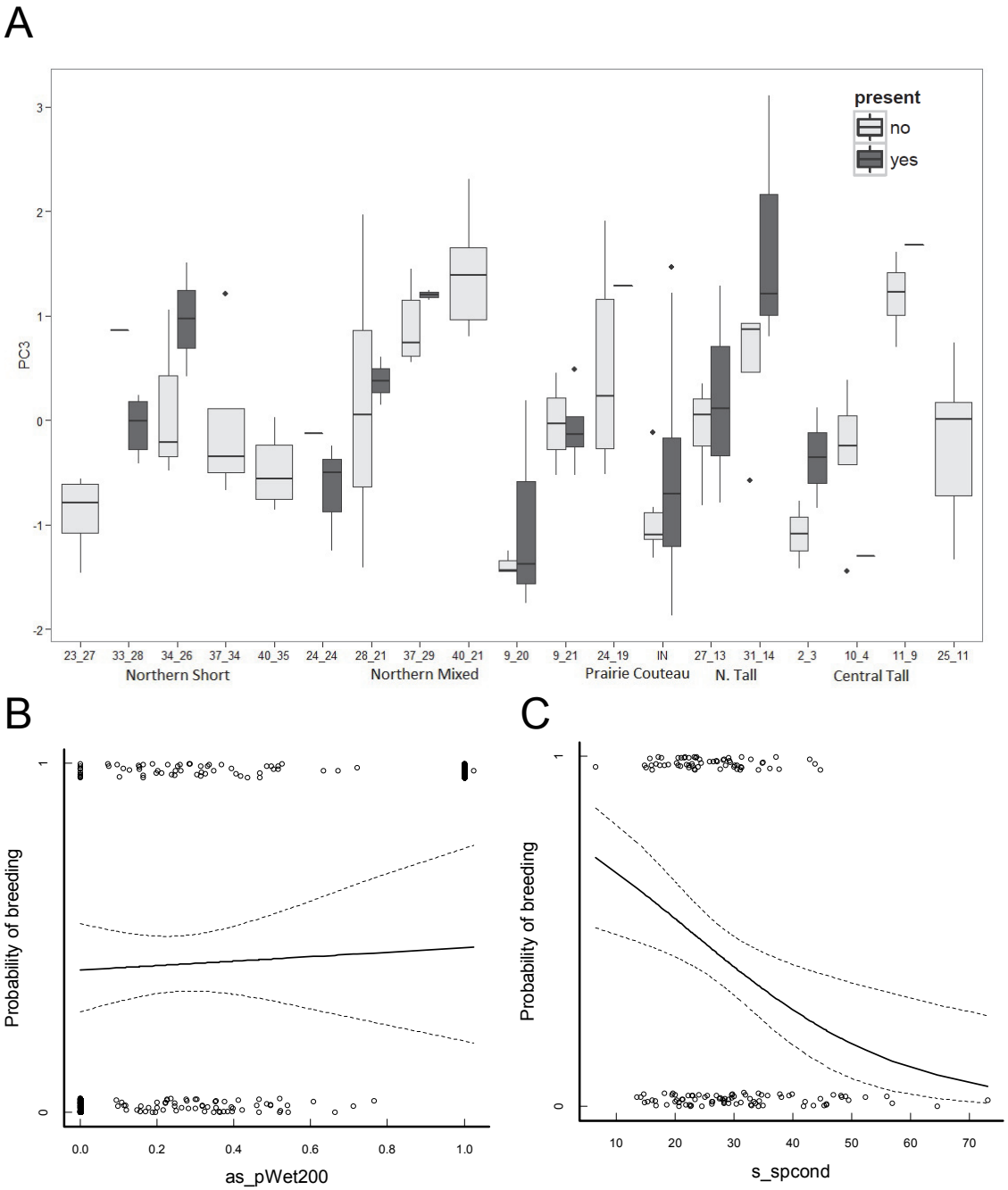

Figure 3. Presence of *Rana pipiens* breeding evidence compared to variables with high relative importance at each level: A) PC3 from index of environmental gradients (median with interquartile range box, non-outlier range whiskers, dots for outliers), B) proportion wetlands in 200 m buffer at local scale (with logistic regression line and 95% C.I.), and C) specific conductivity at wetland scale (with logistic regression line and 95% C.I.).

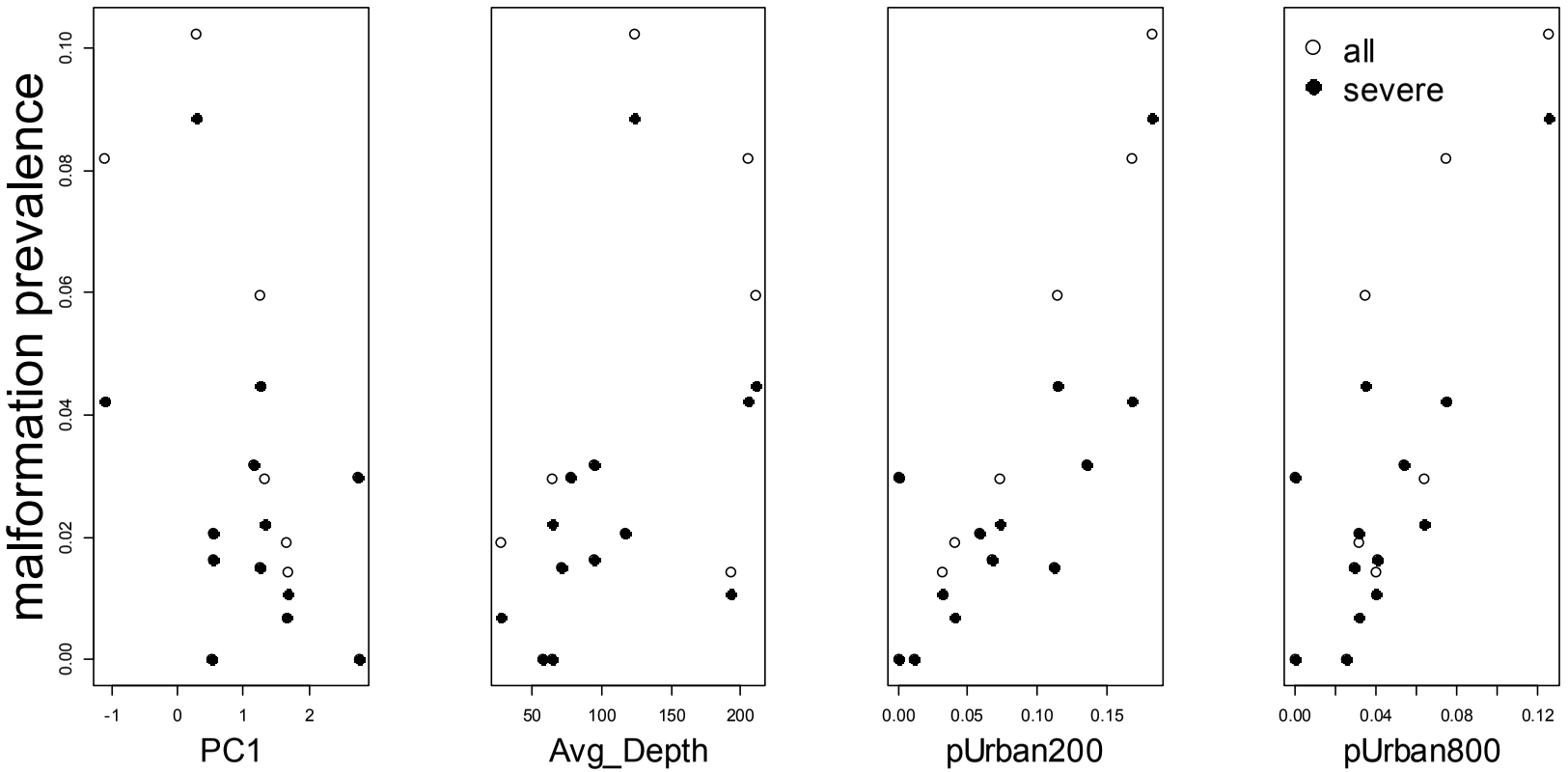

Figure 4. Prevalence of skeletal malformations (all and severe) in metamorphic *Rana pipiens* compared to variables with high relative importance at each level: PC1 from index of environmental gradients, average wetland depth at wetland scale, proportion urban in 200 m buffer at local scale, and proportion urban in 800 m buffer at landscape scale.

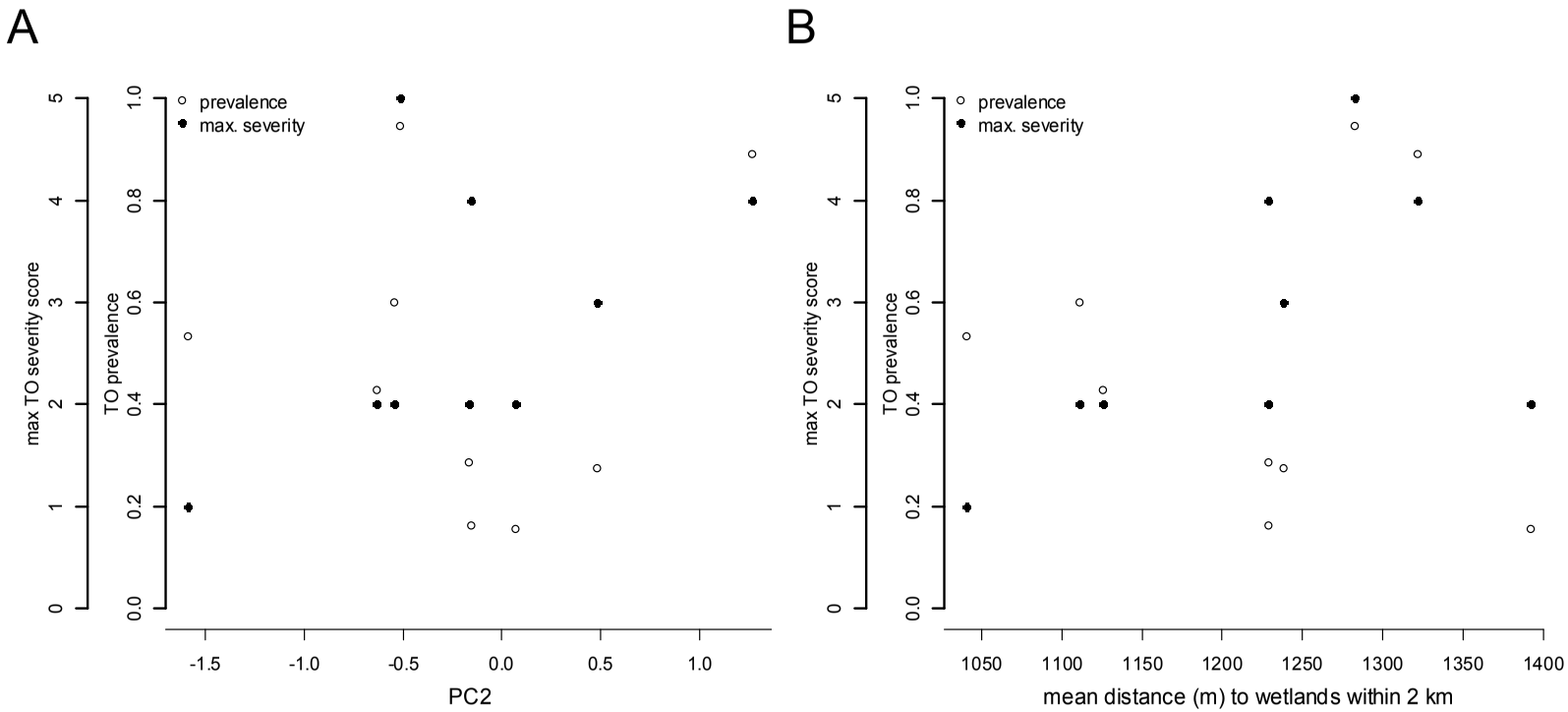

Figure 5. Prevalence of testicular oocytes in male metamorphic *Rana pipiens*, presence of testicular oocytes with a severity score of three or greater, and maximum testicular oocyte severity score compared to variables with high relative importance at each level: A) PC2 from index of environmental gradients and B) average distance to wetlands (seasonal and semipermanent) within 2 km at landscape scale.

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Appendix A – Chapter 1 Supplemental Materials

Supplemental Tables and Figures for Chapter 1: Effects of atrazine and accelerated pond-drying rate on amphibian larval development and growth

Table S1. Endpoints to assess atrazine impacts and accelerated drawdown rate on survival, growth, and development in two North American amphibian species.

Endpoint	Predicted Responses		Potential impacts on individuals	Potential impacts on populations
	Atrazine	Accel. Pond-drying		
Survival to metamorphosis	↓ Survival	↓ Survival	Death; cannot reproduce	Reduced recruitment; genetic diversity lost
Size (length and weight at metamorphosis)	Smaller	Smaller	Increased predation risk and juvenile mortality; older reproductive age; reduced fecundity	Reduced recruitment and population size; overall lower reproductive rates in population
Development (time to forelimb emergence and metamorphosis)	Slower or faster development	Faster development	Increased or decreased exposure to contaminants; increased risk of desiccation	Reduced recruitment; reduced population size

Table S2. Experimental design of *Rana pipiens* and *Rana sylvatica* mesocosm exposure experiments with number of replicate mesocosms for each species.

		# replicate mesocosms per treatment			
		R. pipiens[a]		*R. sylvatica*[b]	
	Drawdown rate:	**Normal**	**Accel.**	**Normal**	**Accel.**
Atrazine (µg/L)	Control (0)	**5**	**0**	**6**	6
	Acetone (0)	**5**	**5**	**0**	**0**
	0.1	**5**	**5**	**6**	**5**
	20	**5**	**5**	**6**	**6**
	200	**5**	**5**	**6**	**6**

[a] initial density of 5 tadpoles per mesocosm
[b] initial density of 6 tadpoles per mesocosm

Table S3. Pearson correlation coefficients of survival, growth and development endpoints of *Rana pipiens* in mesocosm exposure experiment.

Rana pipiens by specimen	*BCI = Weight/Snout-to-vent length*	*snout-to-vent length, mm*[a]	*weight, g*[a]	*days to reach Gosner stage 42*[a]	*days to reach Gosner stage 45*[a]
BCI = Weight/Snout-to-vent length	1.000	0.696	0.974	-0.117	-0.059
snout-to-vent length, mm[a]	0.696	1.000	0.831	0.018	0.046
weight, g[a]	0.974	0.831	1.000	-0.191	-0.143
days to reach Gosner stage 42[a]	-0.117	0.018	-0.191	1.000	0.458
days to reach Gosner stage 45[a]	-0.059	0.046	-0.143	0.458	1.000
by mesocosm	*proportion survived to end of experiment*[a]	*proportion to reach Gosner stage 42*[a]	*proportion died during metamorphosis, Gosner stage 41-45*[a]	*proportion to reach Gosner stage 45*[a]	*variance of days to reach Gosner stage 45*[a]
proportion survived to end of experiment[a]	1.000	0.275	-0.597	0.415	-0.045
proportion to reach Gosner stage 42[a]	0.275	1.000	-0.012	0.936	0.346
proportion died during metamorphosis, Gosner stage 41-45[a]	-0.597	-0.012	1.000	-0.095	-0.036
proportion to reach Gosner stage 45[a]	0.415	0.936	-0.095	1.000	0.360
variance of days to reach Gosner stage 45[a]	-0.045	0.346	-0.036	0.360	1.000

[a]Natural log transformation

Table S4. Pearson correlation coefficients of survival, growth and development endpoints of *Rana sylvatica* in mesocosm exposure experiment.

Rana sylvatica by specimen	*BCI = Weight/Snout-to-vent length*	*snout-to-vent length, mm*[a]	*weight, g*[a]	*days to reach Gosner stage 42*[a]	*days to reach Gosner stage 45*[a]
BCI = Weight/Snout-to-vent length	1.00	0.56	0.93	-0.10	-0.11
snout-to-vent length, mm[a]	0.56	1.00	0.81	-0.19	-0.22
weight, g[a]	0.93	0.81	1.00	-0.17	-0.18
days to reach Gosner stage 42[a]	-0.10	-0.19	-0.17	1.00	0.95
days to reach Gosner stage 45[a]	-0.11	-0.22	-0.18	0.95	1.00

by mesocosm	*proportion survived to end of experiment*[a]	*proportion to reach Gosner stage 42*[a]	*proportion died during metamorphosis, Gosner stage 41-45*[a]	*proportion to reach Gosner stage 45*[a]	*variance of days to reach Gosner stage 45*[a]
proportion survived to end of experiment[a]	1.00	0.74	-0.69	0.73	-0.14
proportion to reach Gosner stage 42[a]	0.74	1.00	-0.09	0.14	0.11
proportion died during metamorphosis, Gosner stage 41-45[a]	-0.69	-0.09	1.00	-0.94	0.37
proportion to reach Gosner stage 45[a]	0.73	0.14	-0.94	1.00	-0.35
variance of days to reach Gosner stage 45[a]	-0.14	0.11	0.37	-0.35	1.00

[a]Natural log transformation

Table S5. Treatment and interaction of fixed effects on specimen and mesocosm outcomes of growth, development, and survival in *Rana pipiens.* Results for significant fixed factors reported in Chapter 1, Table 2.

		Fixed effects p-values (mixed model or ANOVA)		
Rana pipiens	**Endpoint**	**Atrazine**	**Drawdown rate**	**Atrazine X Drawdown rate**
by specimen	*BCI = Weight/Snout-to-vent length*	0.6555	0.2345	0.2031
	snout-to-vent length, mm[a]	0.7931	0.1218	0.2613
	weight, g[a]	0.9926	0.4155	0.6324
	days to reach Gosner stage 45[a]	0.6791	0.7975	0.826
	Gosner stage attained by end of experiment (or collection at metamorphosis)	0.0018[b]	0.3957	0.0338[b]
by mesocosm	*proportion survived to end of experiment*[a]	0.3616	0.6624	0.1133
	proportion to reach Gosner stage 42[a]	0.0044[b]	0.5374	0.0485[b]
	proportion died during metamorphosis, Gosner stage 41-45[a]	0.8857	0.5481	0.2183
	proportion to reach Gosner stage 45[a]	0.0388[b]	0.962	0.2397
	variance of days to reach Gosner stage 45[a]	0.2688	0.083	0.3014

[a]Natural log transformation

[b] Significant at $\alpha = 0.05$. The most conservative test would include the Bonferroni adjustment for multiple tests (for the above 10 models the cut-off value for significance would be $\alpha/10 = 0.05/10 = 0.005$); however, this adjustment was not applied because the response variables were not independent (survival, growth and development are correlated) and the Bonferroni adjustment greatly increases the probability of false negatives (β), and, thus, greatly reduces power.

Table S6. Treatment and interaction of fixed effects on specimen and mesocosm outcomes of growth, development, and survival in *Rana sylvatica*. Results for significant fixed factors reported in Chapter 1, Table 2.

		Fixed effects p-values (mixed model or ANOVA)		
Rana sylvatica	**Endpoint**	**Atrazine**	**Drawdown rate**	**Atrazine X Drawdown rate**
by specimen	*BCI = Weight/Snout-to-vent length*	0.0029[b]	0.8511	0.2097
	snout-to-vent length, mm[a]	0.106	0.9975	0.1193
	weight, g[a]	0.0111[b]	0.9595	0.1444
	days to reach Gosner stage 45[a]	0.0017[b]	0.2462	0.9957
	Gosner stage attained by end of experiment (or collection at metamorphosis)	0.2091	0.362	0.1499
by mesocosm	*proportion survived to end of experiment*[a]	0.0026[b]	0.4419	0.4286
	proportion to reach Gosner stage 42[a]	0.2138	0.2513	0.0686
	proportion died during metamorphosis, Gosner stage 41-45[a]	0.0052[b]	0.8164	0.5275
	proportion to reach Gosner stage 45[a]	0.0523	0.9388	0.4804
	variance of days to reach Gosner stage 45[a]	0.0701	0.7641	0.3995

[a] Natural log transformation, NA = not applicable

[b] Significant at $\alpha = 0.05$. The most conservative test would include the Bonferroni adjustment for multiple tests (for the above 10 models the cut-off value for significance would be $\alpha/10 = 0.05/10 = 0.005$); however, this adjustment was not applied because the response variables were not independent (survival, growth and development are correlated) and the Bonferroni adjustment greatly increases the probability of false negatives (β), and, thus, greatly reduces power.

Table S7. Mean daily water temperature for normal and accelerated drawdown treatments in non-atrazine exposed outdoor mesocosms measured every 30 min with HOBO dataloggers (Onset).

		Rana pipiens		*Rana sylvatica*	
	drawdown:	**Normal**	**Accelerated**	**Normal**	**Accelerated**
Entire experiment[a]	n	10	10	12	12
	Min	15.6	15.4	15.3	15.3
	Max	20.7	20.6	19.4	19.5
	Mean	18.2	18.0	17.5	17.3
	SD	2.5	2.5	1.9	1.8
Before drawdown	Min	20.3	20.2	15.3	15.3
	Max	20.7	20.6	16.0	15.8
	Mean	20.5	20.4	15.7	15.6
	SD	0.2	0.1	0.2	0.2
After drawdown[b]	Min	15.6	15.4	18.9	18.3
	Max	16.1	16.0	19.4	19.5
	Mean	15.8	15.7	19.2	19.1
	SD	0.2	0.3	0.2	0.4
After drawdown: Summer[c]	Min	21.7	21.6		
	Max	22.2	22.3		
	Mean	22.0	21.9	NA	
	SD	0.2	0.3		
After drawdown: Fall[c]	Min	9.0	8.6		
	Max	9.6	9.2		
	Mean	9.2	8.8	NA	
	SD	0.2	0.3		

[a] *R. pipiens*: May 26 – Nov 3, 2006; *R. sylvatica*: May 16 – July 31, 2009
[b] *R. pipiens*: July 12 – Nov 3, 2006; *R. sylvatica*: July 2 – July 31, 2009
[c] *R. pipiens*: After drawdown divided into Summer: July 12 – August 31, 2006 and Fall: September 1 – Nov 3, 2006

Table S8. Chlorophyll-A (µg/L) and relative *in vivo* chlorophyll-A on five sampling dates in the *Rana sylvatica* experimental mesocosms.

		Chlorophyll-A (µg/L)					Mean *in vivo* chlorophyll-A (± SD) [relative measure]: by atrazine concentration			
					Outlier removed					
Date	**n**	**min**	**max**	**mean (± SD)**	**max**	**mean (± SD)**	**0 µg/L**	**0.1 µg/L**	**20 µg/L**	**200 µg/L**
June 11	10	0.4	8.1	3.9 (± 2.8)	8.1	3.9 (± 2.8)	0.523 (± 0.137)	0.487 (± 0.089)	0.799 (± 0.199)	0.632 (± 0.114)
June 18	7	1.4	32.4	8.1 (± 10.1)	8.1	4.5 (± 2.2)	0.784 (± 0.694)	0.551 (± 0.152)	0.543 (± 0.219)	0.401 (± 0.264)
June 25	8	0	73.2	11.6 (± 23.4)	9.9	3.9 (± 4.0)	0.962 (± 1.457)	0.614 (± 0.188)	0.442 (± 0.191)	0.721 (± 0.272)
July 7	[a]						0.888 (± 0.861)	0.708 (± 0.343)	0.492 (± 0.211)	0.509 (± 0.338)
July 29	10	0	42.7	6.0 (± 12.3)	5.3	2.3 (± 1.7)	2.702 (± 4.575)	0.427 (± 0.278)	0.688 (± 1.173)	0.420 (± 0.594)

[a] no samples collected for chlorophyll-A analysis

Table S9. Periphyton growth (mg/cm^2) on 23 cm long acrylic rods (0.635 cm diameter) deployed in *Rana sylvatica* experimental mesocosms from June 11-July 23, 2009.

Atrazine conc. (µg/L)	All mesocosms					Normal drawdown					Accelerated drawdown				
	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD
0	12	0.0050	0.0702	0.0201	0.0181	6	0.0050	0.0150	0.0109	0.0049	6	0.0050	0.0702	0.0292	0.0223
0.1	12	0.000	0.1153	0.0205	0.0323	6	0.0000	0.0451	0.0125	0.0167	6	0.0050	0.1153	0.0284	0.0432
20	11	0.0050	0.0852	0.0428	0.0274	5[a]	0.0150	0.0752	0.0451	0.0256	6	0.0050	0.0852	0.0409	0.0311
200	12	0.0100	0.0451	0.0196	0.0114	6	0.0100	0.0351	0.0192	0.0107	6	0.0100	0.0451	0.0201	0.0131

[a] not measured in one mesocosm due to drying of acrylic rod

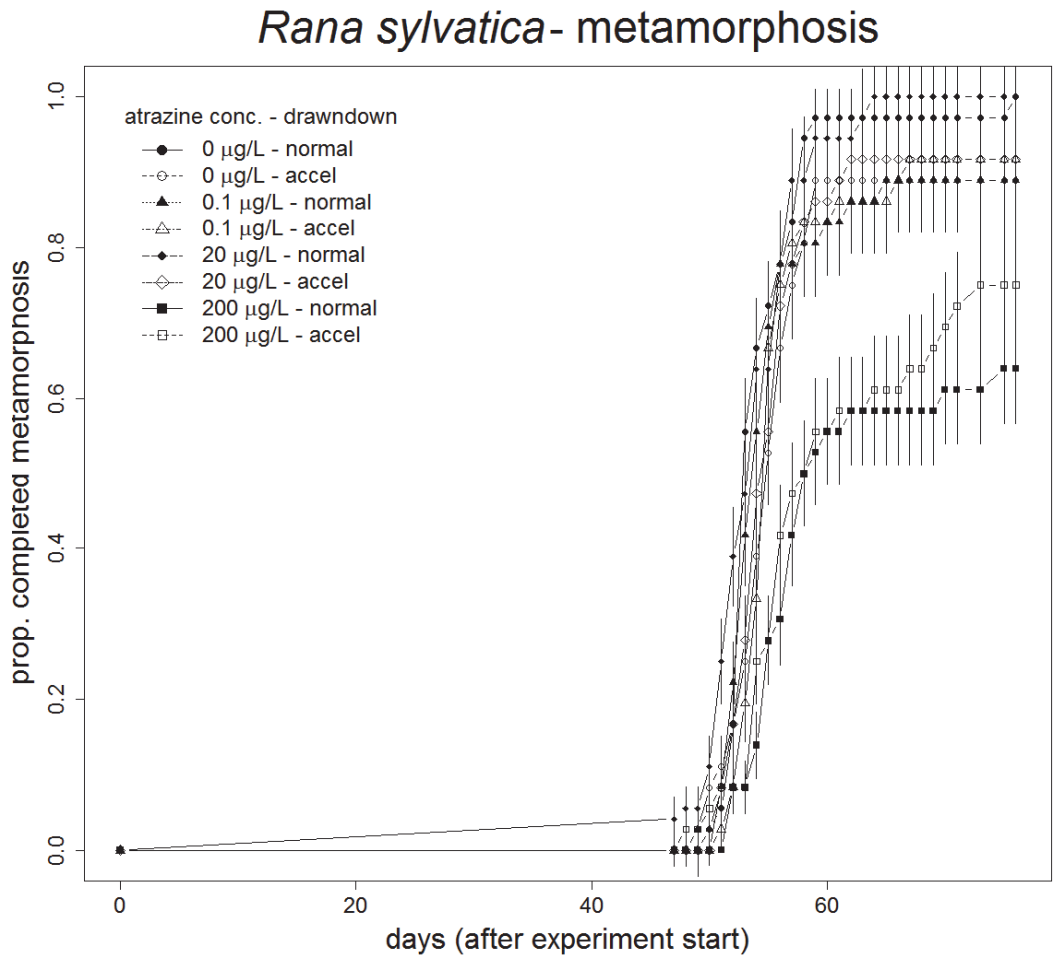

Figure S1. Metamorphosis curves by treatment with standard error bars for *Rana sylvatica*. Circle for 0 µg/L (or ppb), triangle for 0.1 µg/L, diamond for 20 µg/L, square for 200 µg/L, with filled symbols for normal and open symbols for accelerated drawdown rate.

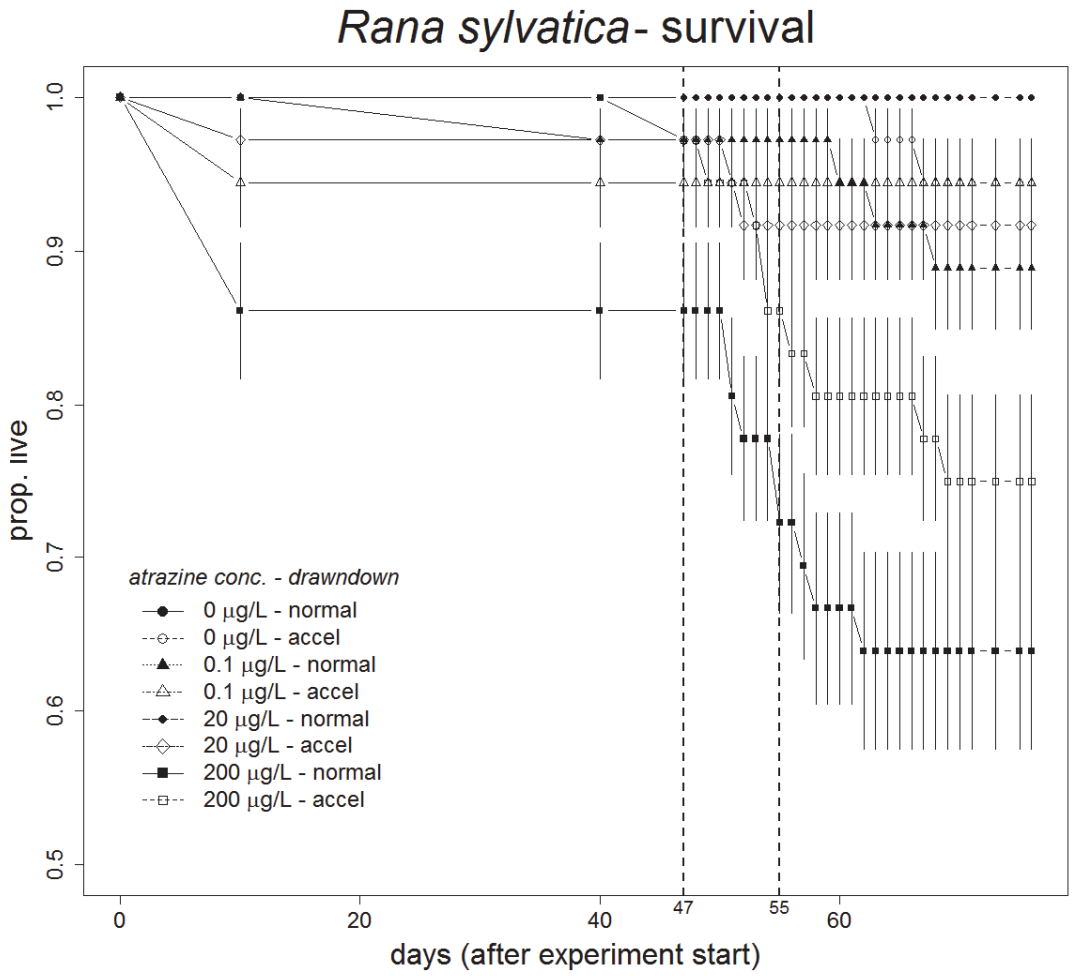

Figure S2. Survival curves by treatment with standard error bars for *Rana sylvatica*. Circle for 0 µg/L (or ppb), triangle for 0.1 µg/L, diamond for 20 µg/L, square for 200 µg/L, with filled symbols for normal and open symbols for accelerated drawdown rate. Dashed lines for the earliest (47) and average (55) number of days to metamorphosis.

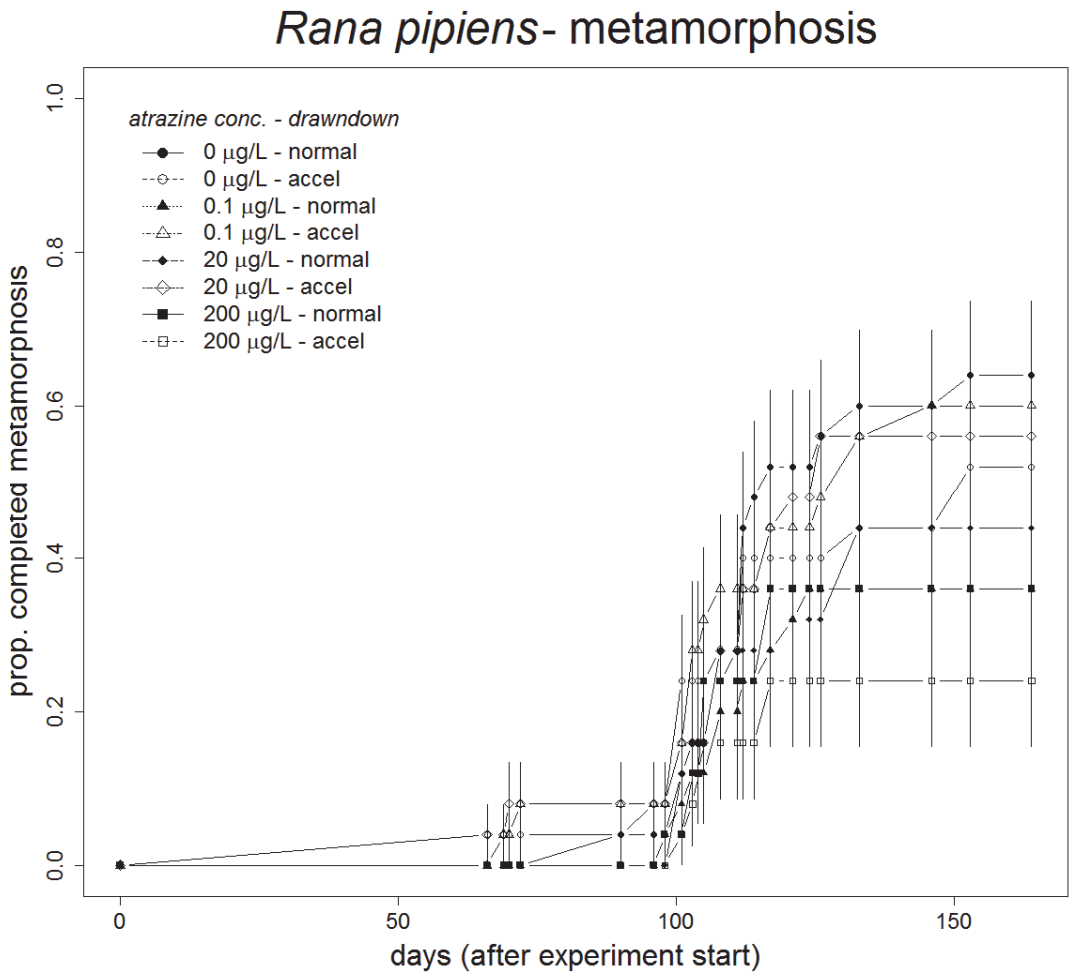

Figure S3. Metamorphosis curves by treatment with standard error bars for *Rana pipiens*. Circle for 0 µg/L (or ppb), triangle for 0.1 µg/L, diamond for 20 µg/L, square for 200 µg/L, with filled symbols for normal and open symbols for accelerated drawdown rate.

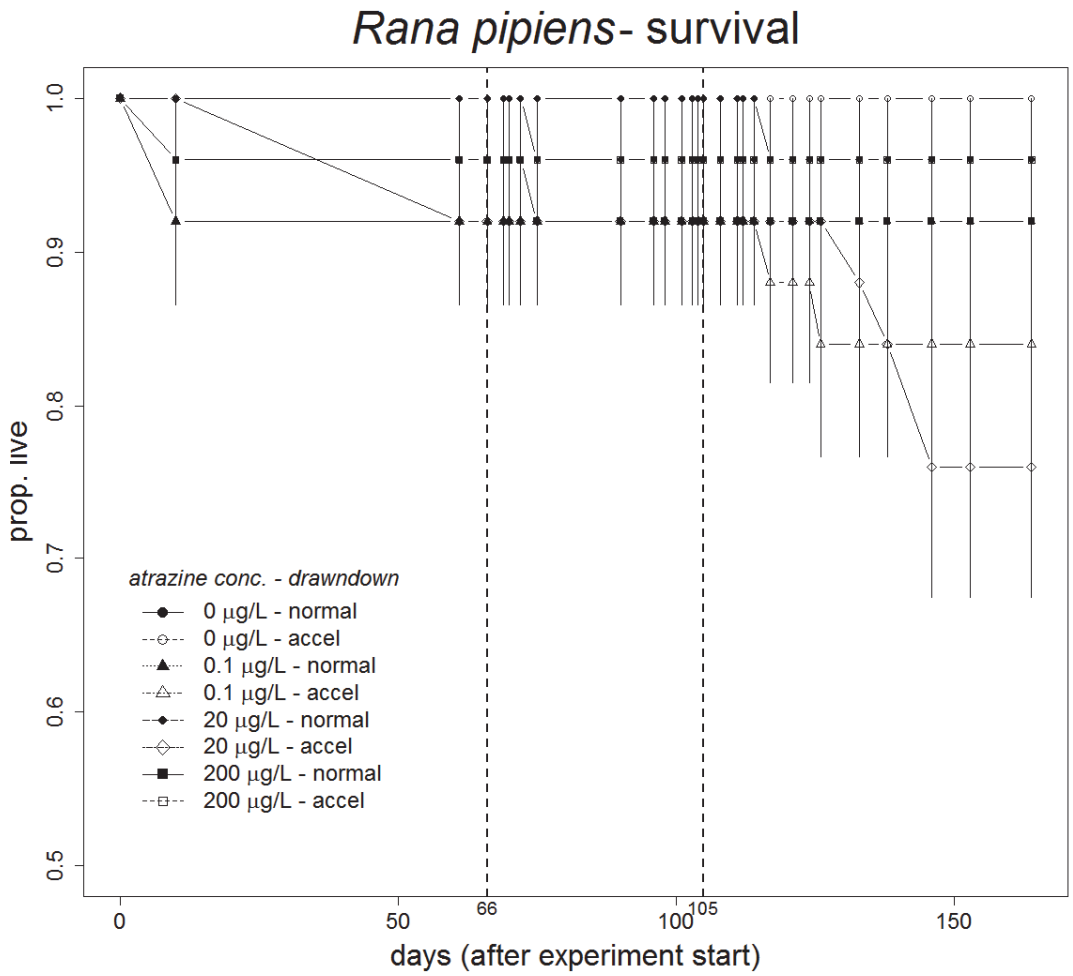

Figure S4. Survival curves by treatment with standard error bars for *Rana pipiens*. Circle for 0 µg/L (or ppb), triangle for 0.1 µg/L, diamond for 20 µg/L, square for 200 µg/L, with filled symbols for normal and open symbols for accelerated drawdown rate. Dashed lines for the earliest (66) and average (105) number of days to metamorphosis.

Appendix B – Chapter 2 Supplemental Materials

Supplemental Tables and Figures for Chapter 2: An assessment of testicular oocytes in field-captured *Rana pipiens*

Table S1. Methods for testicular ooctye (TO) assessments at the specimen-level.

Specimen-level			
All sections	TO presence and counts for all sections from 50 µm longitudinal step-sections across entirety of gonad	**# TOs (total)**	Total # of TOs detected in the gonad
		Mean TOs	Average # of TOs per section
		#TOs/ area	Total # of TOs normalized for gonad size (based on aerial extent of largest median section).
Middle 80%	TO presence and counts for the middle 80% of sections from 50 µm longitudinal step-sections	**# TOs**	# of TOs detected in the middle portion of the gonad
		Mean TOs	Average # of TOs per section of middle portion of gonad
Sub-sample: 25%	TO presence and counts for sub-sample of 25% of sections (every 4th section) from the middle 80% of the gonad (average of all possible sub-samples)	**# TOs**	# of TOs detected in sub-sample consisting of 25% of sections from the middle 80% of the gonad (mean of 4 sub-samples).
		Mean TOs	Average # of TOs per section in sub-sample consisting of 25% of sections from the middle 80% of the gonad (mean of 4 sub-samples).
Sub-sample: 10%	TO presence and counts for sub-sample of 10% of sections (every 10th section) from the middle 80% of the gonad (average of all possible sub-samples)	**# TOs**	# of TOs detected in sub-sample consisting of 10% of sections from the middle 80% of the gonad (mean of 10 sub-samples).
		Mean TOs	Average # of TOs per section in sub-sample consisting of 10% of sections from the middle 80% of the gonad (mean of 10 sub-samples).
Representative section	Representative section from the mid-portion of the gonad was selected for determination of severity and establishing relative gonadal size. This section was identified as the largest section within two sections (~100 µm) of the median section.	**# TOs**	# of TOs detected in representative section (largest section within 100 µm of the median section).
		#TOs/ area	# of TOs in representative section normalized for gonad size (based on aerial extent of largest median section),
		Max Sev	Maximum TO severity score for the representative sections for the left and right gonads.

Table S2. Methods for testicular ooctye (TO) assessments at the site-level.

Site-level			
All specimens	All males, all stages (Gosner stage 44-46) to mimic realistic field collection. n = 1 to 61 at 12 natural breeding wetlands (total n=261 with 4 specimens excluded due to damage or different collection date).	**TO presence**	Presence of TOs at a site, based on all males, all stages of *Rana pipiens*; evaluated for the 5 specimen-level assessments (see above).
		TO prevalence	Site TO prevalence, based on all males, all stages of *Rana pipiens*; evaluated for the 5 specimen-level assessments (see above).
		TO severity	Average TO severity at a site, based on all males, all stages of *Rana pipiens*; evaluated for the 5 specimen-level assessments (see above).
Sub-sample: 10 males per site	Sub-sample for 10 males, with 10 resampling events to randomly collect 10 males for the 6 sites with at least 10 males in original sampling event.	**TO presence**	Presence of TOs at a site, average of 10 sub-samples of 10 males; evaluated for the 5 specimen-level assessments (see above).
		TO prevalence	Site TO prevalence, average of 10 sub-samples of 10 males; evaluated for the 5 specimen-level assessments (see above).
		TO severity	Maximum TO severity at a site, range across 10 sub-samples of 10 males.

Table S3. Distribution of testicular oocytes within gonads of each specimen, mean ± SE, two-sided t-tests (mean TOs/section) and paired t-tests (total # TOs). $\alpha = 0.05$

Comparison		Mean # TOs per section*	Total # TOs*
Left v. Right gonad	**Left**	0.499 ± 0.033	7.99 ± 1.61
	Right	0.488 ± 0.035	7.94 ± 1.47
	p=	0.222	0.98
Middle 80% of gonad v. outer 20%	**Middle 80%**	0.902 ± 0.049	14.43 ± 2.59
	Outer 20%	0.573 ± 0.102	1.36 ± 0.34
	p=	<0.0001	<0.0001

*****similar results for analysis of each specimen individually

Table S4. Frequency of testicular oocyte occurrence in regions of representative sections (% of total for each row and column), not significantly different from even distribution (χ^2 test, α=0.05).

	Medullar	***Cortical***	**Total**
Anterior	35	43	**78 (49%)**
Posterior	33	47	**80 (50%)**
Total	**68 (43%)**	**90 (57%)**	

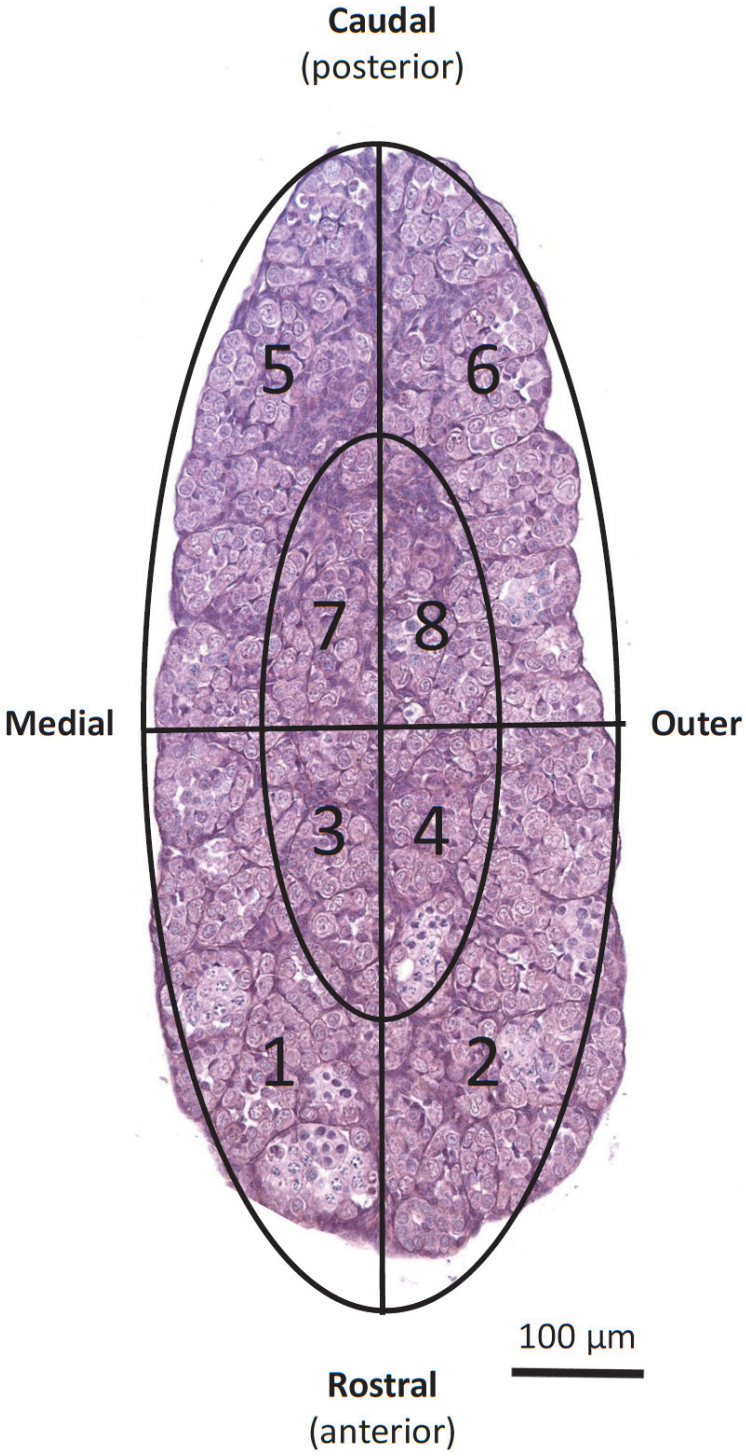

Figure S1. Diagram for documenting location of TOs within representative section (~median) from middle of *Rana pipiens* gonad (1-4 = anterior; 5-8=posterior; 1,2,5,6=cortical; 3,4,7,8=medullar; 1,3,5,7= medial; 2,4,6,8=outer).

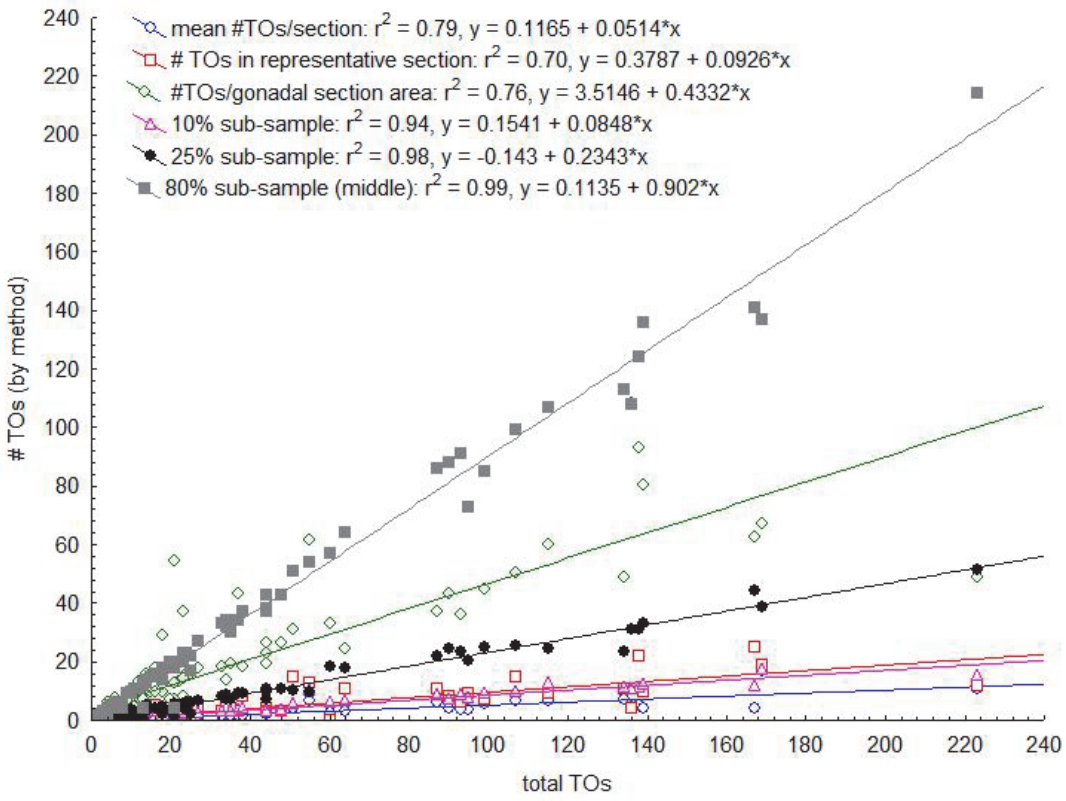

Figure S2. Linear relationship between methods of testicular oocyte (TO) assessment with total number of TOs from all sections ($p<0.001$, $n=114$). Total number of TOs based on evaluation of all sections (5 µm thick longitudinal step sections every 50 µm through the entirety of the gonad). Other methods to evaluate TOs compared were: mean number of TOs per section, number of TOs in representative section (identified as the largest section within 100 µm of the median section), number of TOs normalized for relative gonad size (based on aerial area of representative section), and number of TOs in sub-samples of sections, with 10% (every 10th section), 25% (every 4th section) and the middle 80% of the gonad.

Appendix C – Chapter 3 Supplemental Materials

Supplemental Figure for Chapter 3: Effects of atrazine on testicular oocyte presence and severity in two North American amphibian species

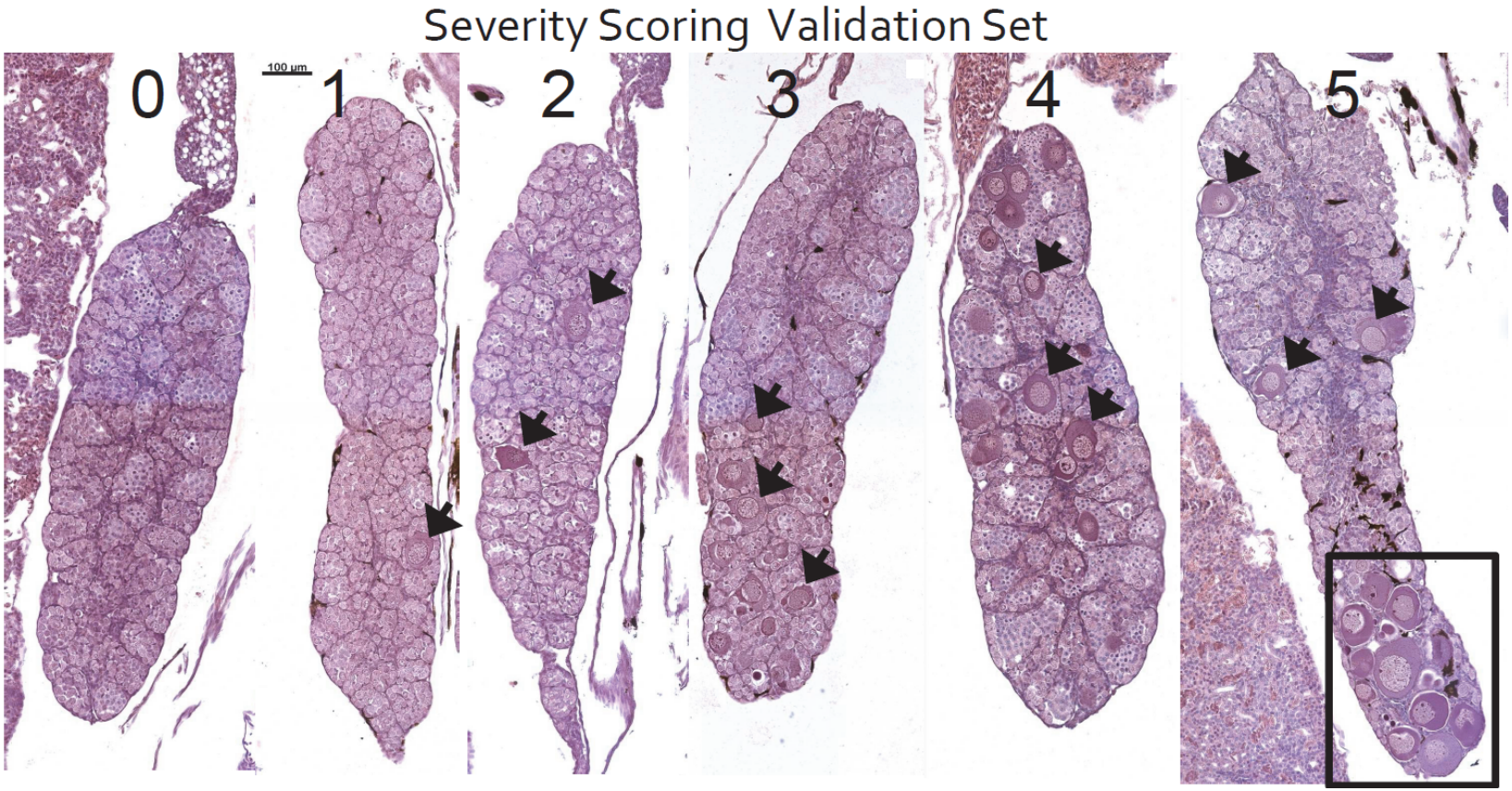

Figure S1. Severity scoring from 0 (no testicular oocytes) to 5 (severely impacted). Validation set examples from experimental *Rana pipiens*. Arrows indicate examples of testicular oocytes, box around section of testis dominated by oocytes.

Appendix D – Chapter 4 Supplemental Materials

Supplemental Methods, Results, Tables, and Figures for Chapter 4: Landscape factors influencing *Rana pipiens* presence, breeding, skeletal malformations, and gonadal development

Environmental Variables

Table S1. Summary of response variables and environmental predictor variables across ecoregions sampled in the U.S. Prairie Pothole Region, with environmental gradients from Principle Components Analysis and individual predictor variables from within-wetland, local, and landscape scales. *reduced sample size for some variables due to wetlands dry during sampling period.

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
	# of wetlands	**31**	**39**	**42**	**14**	**23**	**149**
	*n – reduced**	28	39	42	14	23	146
response							
***Rana pipiens* present**	***# wetlands***	**10**	**24**	**35**	**8**	**10**	**87**
	% wetlands	**32%**	**62%**	**83%**	**57%**	**43%**	**58%**
***Rana pipiens* calling**	***# wetlands***	**6**	**13**	**18**	**5**	**6**	**48**
	% wetlands	**19%**	**33%**	**43%**	**36%**	**26%**	**32%**
***Rana pipiens* breeding**	***# wetlands***	**8**	**14**	**30**	**6**	**4**	**62**
	% wetlands	**26%**	**36%**	**71%**	**43%**	**17%**	**42%**
Env. Gradient							
PC1 (development gradient)	***average***	**-0.124**	**-0.168**	**0.805**	**-0.755**	**-0.575**	**0.000**
	min	-1.569	-1.563	-1.163	-1.555	-1.419	-1.569
	max	1.192	1.036	2.882	0.437	0.433	2.882
	SE	0.150	0.104	0.183	0.143	0.110	0.083
	95% CI LL	-0.431	-0.380	0.436	-1.064	-0.804	-0.164
	95% CI UP	0.183	0.043	1.175	-0.446	-0.346	0.164
PC2 (geographic variation gradient)	***average***	**1.357**	**0.059**	**-0.057**	**-0.406**	**-1.401**	**0.000**
	min	0.446	-1.351	-1.055	-1.061	-3.026	-3.026
	max	2.039	0.986	1.163	0.104	-0.294	2.039
	SE	0.076	0.109	0.071	0.096	0.162	0.083
	95% CI LL	1.201	-0.163	-0.200	-0.614	-1.737	-0.164
	95% CI UP	1.512	0.280	0.087	-0.198	-1.065	0.164
PC3 (wetland density gradient)	***average***	**-0.171**	**0.335**	**-0.349**	**0.535**	**-0.049**	**0.000**
	min	-1.464	-1.756	-1.867	-0.819	-1.442	-1.867
	max	1.521	2.318	1.915	3.118	1.682	3.118
	SE	0.130	0.177	0.148	0.274	0.199	0.083
	95% CI LL	-0.438	-0.023	-0.647	-0.057	-0.462	-0.164
	95% CI UP	0.097	0.694	-0.051	1.127	0.364	0.164

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
wetland							
wetland regime	***# of semipermanent***	**15**	**13**	**22**	**10**	**13**	**73**
	# of seasonal	**16**	**26**	**20**	**4**	**10**	**76**
modification	*# wetlands*	7	16	12	0	3	38
	% wetlands	**22.6%**	**41.0%**	**28.6%**	**0.0%**	**13.0%**	**25.5%**
presence of aquatic predators (fish, crayfish, predaceous diving beetles)	*# wetlands*	16	24	20	11	15	86
	% wetlands	**51.6%**	**61.5%**	**47.6%**	**78.6%**	**65.2%**	**57.7%**
surface area of wetland (m^2)	***average***	**1735.9**	**1072.0**	**718.6**	**1641.1**	**574.5**	**1087.2**
	min	1151.8	528.7	633.6	1057.7	408.2	408.2
	max	2302.7	1874.8	1080.1	2224.6	718.7	2302.7
	SE	77.8	63.4	23.2	161.8	26.5	46.0
	95% CI LL	1577.0	943.7	671.6	1291.6	519.6	996.2
	95% CI UP	1894.9	1200.3	765.5	1990.7	629.3	1178.2
average water depth at pond center (cm)	***average***	**68.6**	**84.1**	**81.1**	**110.1**	**83.9**	**82.4**
	min	0.0	15.3	15.8	23.8	11.7	0.0
	max	150.0	150.0	241.0	150.0	150.0	241.0
	SE	8.8	6.5	8.4	12.1	9.2	3.9
	95% CI LL	50.6	71.0	64.1	83.9	64.8	74.6
	95% CI UP	86.7	97.2	98.1	136.2	103.0	90.2
SD of water depth at pond center (cm)	***average***	**13.7**	**23.9**	**10.6**	**18.2**	**23.9**	**17.5**
	min	0.0	0.0	0.7	0.0	0.0	0.0
	max	52.5	77.4	33.0	57.7	56.0	77.4
	SE	2.5	3.5	1.0	5.6	3.3	1.4
	95% CI LL	8.6	16.7	8.6	6.1	17.1	14.7
	95% CI UP	18.7	31.1	12.6	30.3	30.7	20.2
percent of basin wet	***average***	**67.5**	**74.7**	**71.0**	**78.3**	**75.0**	**72.5**
	min	0.0	38.3	33.3	68.3	31.7	0.0
	max	96.7	88.3	91.7	88.3	90.0	96.7
	SE	5.5	1.9	2.1	1.6	3.5	1.5
	95% CI LL	56.2	70.9	66.7	74.8	67.8	69.6
	95% CI UP	78.7	78.6	75.3	81.8	82.2	75.5

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
percent of wetland that is emergent vegetation	***average***	**43.9**	**42.4**	**58.3**	**58.0**	**44.1**	**48.9**
	min	3.3	1.7	10.0	20.0	0.0	0.0
	max	100.0	90.0	96.3	91.7	95.0	100.0
	SE	5.8	4.2	4.1	5.7	5.7	2.3
	95% CI LL	32.1	34.0	50.0	45.8	32.3	44.4
	95% CI UP	55.8	50.9	66.7	70.2	55.8	53.5
pH *	***average***	**7.7**	**7.8**	**7.3**	**7.4**	**7.6**	**7.6**
	min	6.7	6.9	5.9	6.9	7.0	5.9
	max	9.4	9.0	9.2	8.7	9.5	9.5
	SE	0.1	0.1	0.1	0.1	0.1	0.1
	95% CI LL	7.5	7.6	7.1	7.1	7.3	7.5
	95% CI UP	7.9	8.0	7.5	7.6	7.9	7.7
specific conductivity (uS/cm^2)*	***average***	**1430.6**	**1274.0**	**676.2**	**653.7**	**446.4**	**942.2**
	min	512.0	229.6	256.4	215.8	42.1	42.1
	max	5343.3	4167.0	2396.7	1912.7	1088.0	5343.3
	SE	184.7	139.8	57.2	113.2	59.3	63.5
	95% CI LL	1051.5	991.1	560.8	409.1	323.4	816.8
	95% CI UP	1809.6	1557.0	791.6	898.3	569.4	1067.7
water temperature (°C) from day surveys*	***average***	**17.0**	**19.4**	**18.9**	**18.5**	**16.7**	**18.3**
	min	10.6	13.7	13.2	12.3	10.0	10.0
	max	24.3	26.2	25.0	22.3	22.8	26.2
	SE	0.8	0.6	0.4	0.7	0.7	0.3
	95% CI LL	15.3	18.3	18.1	16.9	15.2	17.7
	95% CI UP	18.7	20.6	19.7	20.1	18.2	18.9
water temperature (°C) from nighttime calling surveys*	***average***	**15.3**	**16.0**	**15.7**	**15.6**	**13.7**	**15.4**
	min	10.0	11.5	10.7	12.0	8.3	8.3
	max	22.0	22.3	19.3	20.5	22.3	22.3
	SE	0.6	0.4	0.3	0.6	0.7	0.2
	95% CI LL	14.1	15.1	15.1	14.3	12.3	14.9
	95% CI UP	16.5	16.9	16.4	16.9	15.2	15.8

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
Average atrazine concentration from surveys 2 and 3 (µg/L)*	***average***	**0.047**	**0.609**	**0.158**	**0.333**	**0.239**	**0.287**
	min	0.006	0.006	0.019	0.057	0.041	0.006
	max	0.155	16.963	1.572	2.859	0.480	16.963
	SE	0.007	0.432	0.036	0.196	0.025	0.117
	95% CI LL	0.032	-0.265	0.086	-0.091	0.188	0.054
	95% CI UP	0.062	1.482	0.230	0.756	0.290	0.519
Local							
Surrounding landcover (within 90 m)	***# 'crop'***	**17**	**19**	**16**	**4**	**7**	**63**
	# grassland or pasture	**14**	**20**	**26**	**10**	**16**	**86**
Distance to nearest road (m)*	***average***	**32.6**	**22.9**	**77.3**	**19.3**	**38.4**	**42.4**
	min	1.0	0.0	0.0	1.0	3.0	0.0
	max	200.0	80.0	500.0	100.0	125.0	500.0
	SE	8.5	3.3	17.0	7.6	7.4	5.6
	95% CI LL	15.1	16.2	43.0	2.8	23.0	31.2
	95% CI UP	50.0	29.5	111.5	35.7	53.8	53.6
Proportion of agriculture within 90 m	***average***	**0.223**	**0.295**	**0.206**	**0.552**	**0.446**	**0.302**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.885	0.852	0.990	0.905	0.882	0.990
	SE	0.051	0.051	0.045	0.070	0.065	0.026
	95% CI LL	0.120	0.192	0.115	0.401	0.312	0.251
	95% CI UP	0.326	0.398	0.298	0.702	0.581	0.354
Proportion of agriculture within 200 m	***average***	**0.285**	**0.350**	**0.244**	**0.623**	**0.482**	**0.353**
	min	0.000	0.000	0.000	0.127	0.000	0.000
	max	0.863	0.904	0.936	0.916	0.913	0.936
	SE	0.055	0.051	0.047	0.064	0.062	0.026
	95% CI LL	0.172	0.247	0.150	0.484	0.354	0.301
	95% CI UP	0.397	0.454	0.339	0.762	0.610	0.405

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
Proportion of natural within 90 m	***average***	**0.478**	**0.394**	**0.596**	**0.138**	**0.293**	**0.429**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.981	0.886	1.000	0.920	1.000	1.000
	SE	0.057	0.052	0.052	0.066	0.058	0.028
	95% CI LL	0.362	0.290	0.491	-0.004	0.172	0.374
	95% CI UP	0.594	0.499	0.701	0.280	0.414	0.484
Proportion of natural within 200 m	***average***	**0.511**	**0.402**	**0.593**	**0.121**	**0.273**	**0.432**
	min	0.020	0.000	0.000	0.000	0.000	0.000
	max	0.952	0.919	1.000	0.786	0.868	1.000
	SE	0.057	0.048	0.050	0.056	0.051	0.027
	95% CI LL	0.395	0.304	0.493	-0.001	0.167	0.379
	95% CI UP	0.628	0.500	0.693	0.243	0.379	0.485
Proportion of urban within 90 m	***average***	**0.141**	**0.120**	**0.095**	**0.139**	**0.111**	**0.118**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.456	0.274	0.313	0.281	0.417	0.456
	SE	0.023	0.012	0.014	0.022	0.020	0.008
	95% CI LL	0.093	0.096	0.067	0.090	0.070	0.102
	95% CI UP	0.189	0.145	0.123	0.187	0.152	0.133
Proportion of urban within 200 m	***average***	**0.102**	**0.092**	**0.076**	**0.087**	**0.111**	**0.092**
	min	0.026	0.000	0.000	0.026	0.000	0.000
	max	0.332	0.192	0.211	0.157	0.421	0.421
	SE	0.013	0.007	0.010	0.010	0.020	0.005
	95% CI LL	0.075	0.077	0.056	0.065	0.071	0.082
	95% CI UP	0.129	0.106	0.097	0.109	0.152	0.103
Proportion of wetland within 90 m	***average***	**0.095**	**0.166**	**0.089**	**0.171**	**0.115**	**0.122**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.470	0.600	0.481	0.657	0.458	0.657
	SE	0.019	0.024	0.020	0.045	0.026	0.011
	95% CI LL	0.057	0.117	0.048	0.075	0.060	0.100
	95% CI UP	0.133	0.215	0.130	0.268	0.170	0.144

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
Proportion of wetland within 200 m	***average***	**0.054**	**0.126**	**0.082**	**0.162**	**0.081**	**0.095**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.251	0.480	0.435	0.730	0.350	0.730
	SE	0.010	0.018	0.019	0.049	0.018	0.009
	95% CI LL	0.033	0.090	0.043	0.055	0.044	0.076
	95% CI UP	0.075	0.162	0.121	0.268	0.117	0.114
Proportion of wetland and open water within 90 m	***average***	**0.158**	**0.190**	**0.102**	**0.172**	**0.150**	**0.151**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.470	0.600	0.615	0.657	0.505	0.657
	SE	0.021	0.026	0.022	0.045	0.033	0.012
	95% CI LL	0.116	0.137	0.057	0.075	0.081	0.126
	95% CI UP	0.200	0.243	0.147	0.268	0.219	0.175
Proportion of wetland and open water within 200 m	***average***	**0.102**	**0.154**	**0.087**	**0.169**	**0.133**	**0.122**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.369	0.480	0.435	0.730	0.407	0.730
	SE	0.015	0.021	0.019	0.049	0.025	0.010
	95% CI LL	0.071	0.112	0.048	0.064	0.081	0.102
	95% CI UP	0.133	0.196	0.126	0.274	0.185	0.143
# of seasonal wetlands within 100 m	***average***	**1.5**	**1.6**	**2.1**	**1.1**	**0.7**	**1.5**
	min	0.0	0.0	0.0	0.0	0.0	0.0
	max	5.0	8.0	7.0	3.0	2.0	8.0
	SE	0.3	0.3	0.3	0.3	0.2	0.1
	95% CI LL	1.0	1.1	1.5	0.5	0.3	1.3
	95% CI UP	2.1	2.1	2.6	1.7	1.0	1.8
# of semi-permanent wetlands within 100 m	***average***	**0.3**	**0.4**	**0.7**	**0.9**	**0.5**	**0.5**
	min	0.0	0.0	0.0	0.0	0.0	0.0
	max	1.0	5.0	4.0	3.0	3.0	5.0
	SE	0.1	0.2	0.2	0.3	0.2	0.1
	95% CI LL	0.1	0.1	0.4	0.3	0.1	0.4
	95% CI UP	0.4	0.7	1.0	1.5	0.8	0.7

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
Landscape							
Elevation	***average***	**605.7**	**451.1**	**536.9**	**333.7**	**381.0**	**485.6**
	min	520.0	393.0	484.0	325.0	241.0	241.0
	max	693.0	506.0	589.0	353.0	457.0	693.0
	SE	9.4	5.3	3.6	2.3	10.6	7.9
	95% CI LL	586.6	440.3	529.7	328.7	359.1	470.0
	95% CI UP	624.8	461.9	544.1	338.7	402.9	501.2
Latitude	***average***	**47.200**	**46.017**	**44.863**	**46.499**	**43.918**	**45.659**
	min	45.362	42.954	44.441	46.152	41.750	41.750
	max	48.373	48.437	45.595	46.841	45.788	48.437
	SE	0.193	0.352	0.052	0.085	0.305	0.144
	95% CI LL	46.805	45.304	44.759	46.315	43.285	45.375
	95% CI UP	47.594	46.729	44.968	46.682	44.550	45.943
Longitude	***average***	**100.476**	**98.504**	**96.722**	**96.244**	**94.784**	**97.625**
	min	99.363	97.527	96.466	96.082	93.261	93.261
	max	101.967	100.435	97.545	96.355	96.811	101.967
	SE	0.197	0.147	0.054	0.024	0.220	0.169
	95% CI LL	100.073	98.206	96.613	96.193	94.328	97.292
	95% CI UP	100.879	98.802	96.831	96.296	95.241	97.959
Proportion of county planted with corn	***average***	**0.046**	**0.114**	**0.178**	**0.075**	**0.328**	**0.147**
	min	0.000	0.008	0.096	0.057	0.121	0.000
	max	0.151	0.251	0.249	0.093	0.490	0.490
	SE	0.011	0.016	0.006	0.005	0.027	0.010
	95% CI LL	0.024	0.082	0.166	0.065	0.273	0.128
	95% CI UP	0.067	0.146	0.189	0.086	0.384	0.167
Stream density (km/km^2) within 10 km buffer	***average***	**0.109**	**0.383**	**0.723**	**0.445**	**0.557**	**0.455**
	min	0.000	0.045	0.015	0.300	0.422	0.000
	max	0.290	0.843	1.217	0.627	0.973	1.217
	SE	0.016	0.038	0.049	0.023	0.026	0.025
	95% CI LL	0.076	0.306	0.624	0.394	0.503	0.404
	95% CI UP	0.141	0.460	0.822	0.495	0.612	0.505

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
People per km^2 within 10 km	***average***	**0.809**	**2.600**	**2.754**	**10.514**	**20.610**	**5.794**
	min	0.428	0.578	1.013	2.479	4.664	0.428
	max	1.440	14.701	5.210	35.159	105.859	105.859
	SE	0.055	0.418	0.187	2.726	5.281	1.015
	95% CI LL	0.696	1.755	2.376	4.626	9.658	3.789
	95% CI UP	0.922	3.445	3.131	16.402	31.562	7.799
Std. dev. of elevation within 10 km	***average***	**17.770**	**12.242**	**26.991**	**21.202**	**13.361**	**18.564**
	min	9.706	4.442	7.813	13.493	5.893	4.442
	max	26.694	34.664	67.581	28.252	20.251	67.581
	SE	0.823	1.225	1.753	1.497	1.064	0.808
	95% CI LL	16.089	9.762	23.451	17.967	11.154	16.967
	95% CI UP	19.450	14.722	30.530	24.437	15.568	20.161
Road density within 2 km	***average***	**1.083**	**1.220**	**1.120**	**1.250**	**1.324**	**1.182**
	min	0.583	0.459	0.195	0.946	0.687	0.195
	max	1.966	1.810	1.870	1.475	1.996	1.996
	SE	0.066	0.043	0.067	0.043	0.056	0.028
	95% CI LL	0.948	1.132	0.984	1.158	1.207	1.126
	95% CI UP	1.219	1.307	1.256	1.342	1.441	1.238
Road density (km/km^2) within 10 km	***average***	**0.945**	**1.253**	**1.131**	**1.382**	**1.496**	**1.204**
	min	0.802	1.015	0.808	1.208	1.220	0.802
	max	1.057	1.493	1.412	1.674	2.067	2.067
	SE	0.013	0.019	0.023	0.037	0.039	0.018
	95% CI LL	0.919	1.214	1.085	1.302	1.414	1.168
	95% CI UP	0.970	1.291	1.176	1.462	1.577	1.240
Highway and freeway density (km/km^2) within 2 km	***average***	**0.093**	**0.046**	**0.094**	**0.000**	**0.077**	**0.070**
	min	0.000	0.000	0.000	0.000	0.000	0.000
	max	0.671	0.310	0.352	0.000	0.376	0.671
	SE	0.032	0.016	0.021	0.000	0.027	0.011
	95% CI LL	0.028	0.013	0.052		0.021	0.048
	95% CI UP	0.157	0.079	0.136		0.133	0.091

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
Highway and freeway density (km/km^2) within 10 km	***average***	**0.060**	**0.086**	**0.085**	**0.053**	**0.117**	**0.082**
	min	0.000	0.007	0.000	0.000	0.000	0.000
	max	0.132	0.149	0.178	0.115	0.355	0.355
	SE	0.007	0.006	0.006	0.011	0.021	0.004
	95% CI LL	0.046	0.074	0.074	0.030	0.074	0.073
	95% CI UP	0.073	0.098	0.096	0.077	0.160	0.091
Proportion of agriculture within 800 m	***average***	**0.350**	**0.420**	**0.279**	**0.662**	**0.573**	**0.412**
	min	0.000	0.000	0.000	0.232	0.283	0.000
	max	0.843	0.908	0.868	0.834	0.921	0.921
	SE	0.047	0.038	0.043	0.043	0.038	0.022
	95% CI LL	0.254	0.343	0.192	0.569	0.494	0.368
	95% CI UP	0.445	0.498	0.366	0.756	0.653	0.456
Proportion of agriculture within 3200 m	***average***	**0.361**	**0.445**	**0.273**	**0.710**	**0.686**	**0.441**
	min	0.069	0.089	0.024	0.613	0.318	0.024
	max	0.681	0.701	0.627	0.830	0.915	0.915
	SE	0.033	0.029	0.028	0.017	0.034	0.019
	95% CI LL	0.292	0.387	0.217	0.673	0.616	0.403
	95% CI UP	0.429	0.503	0.329	0.748	0.756	0.479
Proportion of natural within 800 m	***average***	**0.485**	**0.383**	**0.583**	**0.119**	**0.241**	**0.414**
	min	0.036	0.013	0.037	0.006	0.001	0.001
	max	0.854	0.835	0.946	0.620	0.547	0.946
	SE	0.046	0.034	0.043	0.043	0.028	0.022
	95% CI LL	0.391	0.314	0.495	0.025	0.183	0.370
	95% CI UP	0.579	0.452	0.671	0.212	0.300	0.458
Proportion of natural within 3200 m	***average***	**0.469**	**0.380**	**0.544**	**0.122**	**0.156**	**0.386**
	n	31.000	39.000	42.000	14.000	23.000	149.000
	min	0.148	0.065	0.198	0.044	0.014	0.014
	max	0.795	0.848	0.819	0.189	0.372	0.848
	SE	0.033	0.030	0.029	0.011	0.018	0.018
	95% CI LL	0.402	0.321	0.485	0.097	0.119	0.350
	95% CI UP	0.536	0.440	0.603	0.147	0.193	0.422

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
Proportion of wetland within 800 m	***average***	**0.050**	**0.114**	**0.076**	**0.134**	**0.054**	**0.083**
	n	31.000	39.000	42.000	14.000	23.000	149.000
	min	0.000	0.000	0.000	0.012	0.002	0.000
	max	0.183	0.370	0.244	0.398	0.226	0.398
	SE	0.007	0.017	0.009	0.025	0.012	0.007
	95% CI LL	0.035	0.079	0.058	0.079	0.030	0.070
	95% CI UP	0.065	0.148	0.095	0.188	0.079	0.096
Proportion of wetland within 3200 m	***average***	**0.053**	**0.097**	**0.075**	**0.083**	**0.038**	**0.071**
	n	31.000	39.000	42.000	14.000	23.000	149.000
	min	0.006	0.002	0.003	0.016	0.001	0.001
	max	0.152	0.294	0.144	0.151	0.122	0.294
	SE	0.006	0.015	0.005	0.011	0.007	0.005
	95% CI LL	0.040	0.068	0.064	0.059	0.023	0.062
	95% CI UP	0.066	0.127	0.086	0.107	0.054	0.081
Proportion of wetland and open water within 800 m	***average***	**0.113**	**0.150**	**0.099**	**0.178**	**0.119**	**0.126**
	n	31.000	39.000	42.000	14.000	23.000	149.000
	min	0.010	0.001	0.000	0.088	0.002	0.000
	max	0.390	0.376	0.420	0.412	0.473	0.473
	SE	0.014	0.019	0.013	0.024	0.022	0.008
	95% CI LL	0.084	0.112	0.072	0.127	0.072	0.110
	95% CI UP	0.143	0.188	0.126	0.229	0.165	0.142
Proportion of wetland and open water within 3200 m	***average***	**0.129**	**0.134**	**0.148**	**0.124**	**0.101**	**0.131**
	n	31.000	39.000	42.000	14.000	23.000	149.000
	min	0.035	0.006	0.003	0.061	0.001	0.001
	max	0.233	0.314	0.303	0.203	0.466	0.466
	SE	0.010	0.016	0.011	0.011	0.024	0.007
	95% CI LL	0.107	0.101	0.125	0.100	0.052	0.117
	95% CI UP	0.150	0.166	0.170	0.149	0.150	0.144

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
Proportion of urban within 800 m	***average***	**0.052**	**0.046**	**0.039**	**0.041**	**0.065**	**0.048**
	min	0.021	0.024	0.000	0.021	0.026	0.000
	max	0.117	0.091	0.125	0.070	0.127	0.127
	SE	0.005	0.003	0.004	0.004	0.006	0.002
	95% CI LL	0.043	0.040	0.030	0.033	0.052	0.044
	95% CI UP	0.062	0.051	0.047	0.049	0.077	0.052
Proportion of urban within 3200 m	***average***	**0.042**	**0.041**	**0.035**	**0.043**	**0.056**	**0.042**
	min	0.024	0.025	0.018	0.037	0.037	0.018
	max	0.066	0.067	0.054	0.048	0.073	0.073
	SE	0.003	0.002	0.002	0.001	0.002	0.001
	95% CI LL	0.036	0.037	0.032	0.041	0.051	0.040
	95% CI UP	0.047	0.044	0.038	0.045	0.060	0.044
# of seasonal wetlands within 1000 m	***average***	**39.4**	**27.7**	**45.3**	**19.8**	**13.1**	**32.1**
	min	4.0	5.0	4.0	4.0	3.0	3.0
	Max	75.0	70.0	118.0	37.0	25.0	118.0
	SE	3.0	2.4	4.6	3.1	1.5	1.9
	95% CI LL	33.2	22.8	36.0	13.0	10.1	28.4
	95% CI UP	45.6	32.6	54.6	26.6	16.2	35.8
# of seasonal wetlands within 2000 m	***Average***	**139.9**	**92.7**	**151.8**	**54.4**	**39.4**	**107.4**
	Min	38.0	24.0	17.0	14.0	3.0	3.0
	Max	239.0	189.0	377.0	99.0	80.0	377.0
	SE	7.8	6.5	17.2	8.1	4.7	6.5
	95% CI LL	123.9	79.5	117.1	36.9	29.6	94.6
	95% CI UP	155.8	105.9	186.5	71.9	49.2	120.1
# of semipermanent wetlands within 1000 m	***Average***	**10.1**	**5.5**	**11.6**	**9.4**	**5.1**	**8.5**
	Min	0.0	0.0	1.0	0.0	0.0	0.0
	Max	41.0	16.0	27.0	33.0	19.0	41.0
	SE	1.5	0.6	1.1	2.2	1.1	0.6
	95% CI LL	7.0	4.3	9.4	4.6	2.8	7.3
	95% CI UP	13.3	6.6	13.8	14.2	7.4	9.6

		Northern Short	Northern Mixed	Prairie Couteau	Northern Tall	Central Tall	All
# of semipermanent wetlands within 2000 m	***average***	**33.3**	**16.3**	**34.5**	**28.3**	**14.1**	**25.8**
	Min	6.0	1.0	7.0	8.0	0.0	0.0
	max	115.0	32.0	64.0	70.0	57.0	115.0
	SE	4.3	1.3	2.7	5.3	3.0	1.6
	95% CI LL	24.6	13.7	29.0	16.9	7.9	22.7
	95% CI UP	42.1	19.0	40.1	39.7	20.4	28.9
Average distance (m) to seasonal and semipermanent wetlands within 1000 m	***average***	**617.1**	**563.7**	**592.9**	**577.6**	**551.6**	**582.5**
	Min	541.0	305.8	456.4	398.6	311.3	305.8
	max	709.2	690.5	696.8	818.6	738.9	818.6
	SE	8.3	12.6	8.2	30.0	22.8	6.5
	95% CI LL	600.1	538.2	576.4	512.7	504.3	569.7
	95% CI UP	634.2	589.2	609.4	642.4	599.0	595.3
Average distance (m) to seasonal and semipermanent wetlands within 2000 m	***average***	**1286.6**	**1253.3**	**1245.1**	**1180.3**	**1133.4**	**1232.6**
	Min	1062.8	940.1	1044.2	809.0	633.0	633.0
	Max	1593.8	1425.4	1486.3	1373.1	1447.6	1593.8
	SE	19.1	16.7	12.2	36.1	47.1	11.2
	95% CI LL	1247.5	1219.6	1220.5	1102.3	1035.7	1210.5
	95% CI UP	1325.6	1287.1	1269.7	1258.3	1231.1	1254.6

Skeletal malformations

Classification and distribution.

For analytical purposes, malformations were classified as occurring in the axial skeleton, which included eye, jaw, and skull, or in the appendicular skeleton, including forelimb or hindlimb (Table S3). Furthermore, although many malformed individuals had multiple abnormalities, most of these were limited to a single body part or region, and it was usually possible to specify one to be the most significant, which we refer to as the primary malformation (Tables S3, S4). Notably, only 2 out of the 27 metamorphic *Rana pipiens* with multiple malformations had abnormalities in two distinct body regions, and in only one of these could each malformation be considered primary and severe. Each abnormality was assessed to determine whether the abnormality might interfere with fitness (survival or reproduction). Based on this necessarily subjective judgment, we determined 76 (72%) of the individuals had severe malformations.

Hindlimb malformations predominated (Tables S3, S4), with the majority consisting of missing or shortened limbs and limb segments (ectromely, ectrodactyly, brachymely, brachydactyly, and ectrophalangy; Table S3). Three individuals lacked entire limbs (amely). Forelimb abnormalities constituted about 4% of all malformed specimens, with a distribution of types similar to that seen in hindlimbs. Notably, multiple limbs or limb segments represented a very small percentage of all abnormalities, with no instances of polymely and only two of polydactyly or polysyndactyly identified throughout all of the surveys (Table S4).

The most common non-limb malformations were missing or reduced eyes and a small number of jaw and spinal abnormalities (Tables S3, S4). Only one specimen was found with cutaneous fusion, in which the right forelimb failed to emerge through the skin of the opercular chamber, resulting in an apparently intact limb detectable underneath the pharyngeal skin. In one individual the spine was twisted enough to cause a visible curve to the pelvis or trunk, and was classified as scoliosis.

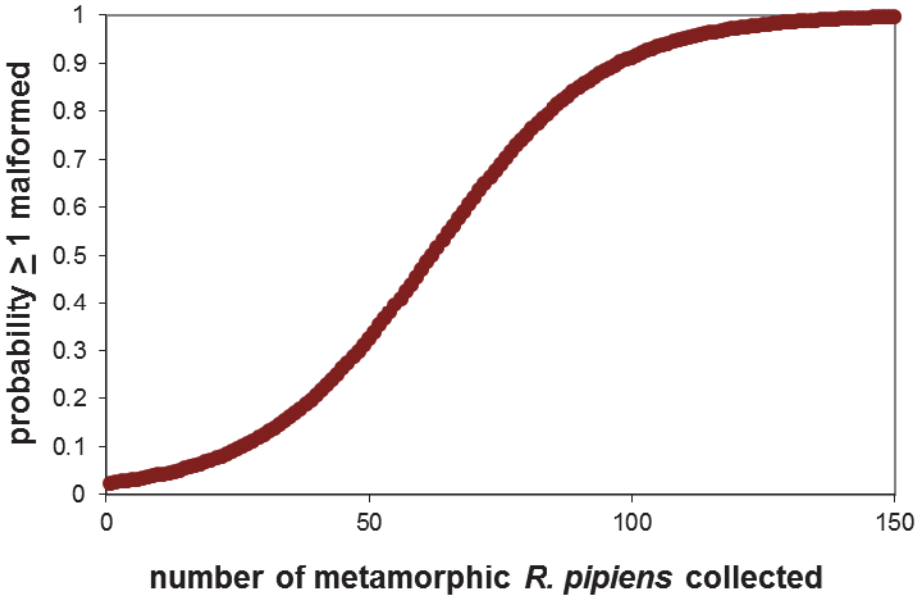

Figure S1. Probability of collecting malformed metamorphs based on sample size in an individual collection, based on observed probabilities in sampled wetlands in the Prairie Pothole Region. Analysis of all individual surveys indicated that the probability of obtaining a malformed specimen in collections of 100 or more metamorphic *Rana pipiens* was about 90%.

Table S2. Malformed metamorphic *Rana pipiens* found in seasonal and semi-permanent wetlands in eastern South Dakota (Prairie Couteau ecoregion of the Prairie Pothole Region).

	Total surveys	**Unique wetlands**	**Wetlands w/ metas**	**Surveys w/ metas**	**Surveys w/ malfs**	**Wetland malf prev**	**Total Metas**	**Total Malfs**	**Malf prev. (%)**
All surveys	62	35	26	43	20	44.4%	2679	105	3.92%
Surveys with ≥ 50 metamorphic *R. pipiens* examined			13	22	19	86.4%	2593	104	4.01%

Table S3. Summary of primary malformations.

Region of primary malformation	Num of each type	Percent of each type
Axial skeleton		
jaw	4	3.81%
eye	15	14.29%
spine	1	0.95%
Appendicular skeleton		
forelimb	5	4.76%
hindlimb	80	76.19%
Total	**105**	

Table S4. Classifications of primary malformations

Region/classification		Num of each type	Percent (%) of each type
Hindlimb	Amely	3	2.86
	asymmetry	1	0.95
	Atrophy	3	2.86
	brachydactyly	1	0.95
	brachymely	22.5[a]	21.43
	ectrodactyly	15	14.29
	ectromely	6	5.71
	ectrophalangy	23	21.90
	phocomelia	3	2.86
	syndactyly	1	0.95
	polysyndactyl	1	0.95
	Total hindlimb	**80**	**76.19**
Forelimb	brachymely	1	0.95
	ectrodactyly	2	1.90
	ectromely	1	0.95
	Total forelimb	**4**	**3.81**
Craniofacial	anophthalmia	4	3.81
	microphthalmia	10.5[a]	10.00
	brachygnathia	4	3.81
	Total craniofacial	**19**	**18.10**
Other	cutaneous fusion[b]	1	0.95
	scoliosis	1	0.95
	Total other	**2**	**1.90**

[a] One specimens had malformations in two regions (hindlimb and eye in each case); each of these was designated as half of a primary malformation for purposes of calculating proportions.
[b] Intact forearm contained within a sealed opercular chamber.

Gonadal anomalies (testicular oocytes)

TO severity was assessed based on a scoring system developed from the observed range of TO abundance, aggregation, and extent across testes in the *Rana pipiens* experiment (Olker 2014c). TO severity was scored from zero (no TOs) to five (severely impacted with portions of the gonad section dominated by ooctyes). A validation set of photos of each severity was used to score a representative median section of the left and right gonad for each male specimen.

Environmental gradients with Principle Components Analysis

PC1 represents range of developed land, with highly positive correlations with proportion natural land cover from 90-3200 m and number of seasonal wetlands within 2 km and negative correlations with proportion of agriculture from 90-3200 m, urban at 800 m, and road density within 2 km; PC2 represents geographic variation (across ecoregions) across the study area of the Prairie Pothole Region, with positive correlation with latitude and longitude, elevation, and wetland surface area, and negative correlated with human population density, road density, and stream density within 10 km, and proportion of county planted in corn; PC3 represents range in wetland density, with high positive correlations with proportion of wetlands and open water within 90-3200 m.

Statistical Analysis Details and Results

We used an information-theoretic approach (Burnham & Anderson 2002; Mazerolle 2006; Grueber et al. 2011) to evaluate the relationship of amphibian endpoints with environmental gradients and individual variables from each scale. Specifically, we used the following modeling protocols.

First, variables were tested for normality and collinearity. If necessary, variables were transformed to improve normality, with arcsin square root transformation for land cover proportions and square root transformation for other non-normal variables. Collinearity of predictor variables was evaluated with Pearson correlation coefficients and variance inflation factors (VIF) following recommendations in Zuur et al. (2009). Variables were grouped by spatial scale (Principle Component axes, within wetland, local, and landscape) and variables with high VIF values were removed until all were below 3.

Second, we tested for spatial autocorrelation by fitting the null generalized linear model and testing the residuals for autocorrelation with Moran's I, for the continuous response variables, and evaluating spline correlograms for binary response variables (Turner et al. 2001; Zuur et al. 2009). *R. pipiens* presence, calling, and breeding exhibited spatial autocorrelation to approximately 70 km, suggesting that a spatial term was necessary in the model. The generalized linear mixed model (GLMM) with the inclusion of a spatial term (cell ID = general location in the Prairie Pothole Region) resolved spatial autocorrelation for these response variables. The rest of the response variables did not appear to exhibit any spatial autocorrelation, with spline correlograms showing a line around zero for the binary endpoints (presence of malformations and presence of TO severity of 3 or greater) and p-values > 0.5 for Moran's I for malformation and TO prevalence and severity endpoints.

Third, we fitted the full set of models for each amphibian endpoint, compared them with the corrected Akaike's information criteria (AICc, which is appropriate for small samples sizes), and identified the set of top models. The 'best' fitting model has the minimum AICc value (typically called the AICcMIN model) and models with different numbers of parameters can be directly compared based on differences in AICc (delta AICc). Models with small delta AICc (less than 2) have substantial support as plausible/comparable to the AICcMIN model, whereas those with delta AICc greater than 10 having no support (Burnham & Anderson 2002).

Finally, we compared relative importance of predictor variables (based on Akaike's weights of models in which variables are present) to identify the most influential factors on presence, breeding, or developmental endpoints for *R. pipiens* in the Prairie Pothole Region. For the environmental gradient PCs, we created model-averaged coefficients for predictor variables with high importance. For each spatial scale, influential individual predictor variables were identified based on relative variable importance and interpreted based on direction of relationship for the models within 10 AIC of the AICcMIN model.

Table S5. Information-theoretic models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* presence/absence. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Presence models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
PC2	**0.175**	**NA**	**-0.508**	**NA**	**NA**	**3**	**-82.052**	**170.273**	**0**	**0.294**
null model	**0.169**	**NA**	**NA**	**NA**	**NA**	**2**	**-83.3458**	**170.775**	**0.502**	**0.229**
PC2 + PC3	**0.168**	**NA**	**-0.498**	**0.072**	**NA**	**4**	**-82.0172**	**172.318**	**2.045**	**0.106**
PC1 + PC2	**0.183**	**0.035**	**-0.508**	**NA**	**NA**	**4**	**-82.0447**	**172.373**	**2.100**	**0.103**
PC3	**0.157**	**NA**	**NA**	**0.147**	**NA**	**3**	**-83.2032**	**172.575**	**2.302**	**0.0932**
PC1	**0.174**	**0.015**	**NA**	**NA**	**NA**	**3**	**-83.3446**	**172.858**	**2.585**	**0.0809**
PC1 + PC2 + PC3	**0.173**	**0.019**	**-0.499**	**0.068**	**NA**	**5**	**-82.0151**	**174.458**	**4.185**	**0.03633**
PC1 + PC2	**0.151**	**-0.022**	**NA**	**0.151**	**NA**	**4**	**-83.2005**	**174.684**	**4.411**	**0.0325**
PC1 + PC2 + PC3+PC1*PC3	**0.175**	**0.044**	**-0.493**	**0.078**	**0.068**	**6**	**-81.992**	**176.588**	**6.315**	**0.0125**
PC1 + PC3 + PC1*PC3	**0.154**	**0.021**	**NA**	**0.165**	**0.115**	**5**	**-83.1324**	**176.693**	**6.420**	**0.0119**
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.277	0.552	0.292	0.024					
parameter estimates (B) averaged across models	0.171	0.020	-0.505	0.108	0.091					
unconditional SE	0.389	0.295	0.316	0.280	0.315					
unconditional 95% CI	-0.591	-0.557	-1.125	-0.441	-0.528					
	0.932	0.598	0.114	0.657	0.709					

Table S6. Information-theoretic models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* calling. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Calling models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
null model	**-0.941**	**NA**	**NA**	**NA**	**NA**	**2**	**-89.7561**	**183.596**	**0**	**0.274**
PC2	**-0.938**	**NA**	**-0.306**	**NA**	**NA**	**3**	**-89.0272**	**184.223**	**0.627**	**0.200**
PC3	**-0.964**	**NA**	**NA**	**0.172**	**NA**	**3**	**-89.4725**	**185.114**	**1.518**	**0.128**
PC1	**-0.958**	**-0.081**	**NA**	**NA**	**NA**	**3**	**-89.6926**	**185.554**	**1.958**	**0.103**
PC2 + PC3	**-0.952**	**NA**	**-0.289**	**0.130**	**NA**	**4**	**-88.8602**	**186.004**	**2.408**	**0.082**
PC1 + PC2	**-0.954**	**-0.074**	**-0.305**	**NA**	**NA**	**4**	**-88.9734**	**186.230**	**2.634**	**0.073**
PC1 + PC3	**-0.994**	**-0.121**	**NA**	**0.197**	**NA**	**4**	**-89.3388**	**186.961**	**3.365**	**0.051**
PC1 + PC3+PC1*PC3	**-0.987**	**0.014**	**NA**	**0.180**	**0.303**	**5**	**-88.5741**	**187.577**	**3.981**	**0.037**
PC1 + PC2 + PC3	**-0.979**	**-0.106**	**-0.284**	**0.152**	**NA**	**5**	**-88.7574**	**187.943**	**4.347**	**0.031**
PC1 + PC2 + PC3+PC1*PC3	**-0.974**	**0.009**	**-0.240**	**0.147**	**0.267**	**6**	**-88.1759**	**188.956**	**5.360**	**0.019**
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		**0.315**	**0.406**	**0.349**	0.056					
parameter estimates (B) averaged across models	-0.953	-0.072	-0.298	0.164	0.291					
unconditional SE	0.325	0.240	0.255	0.233	0.250					
unconditional 95% CI	-1.590	-0.541	-0.797	-0.293	-0.199					
	-0.316	0.398	0.202	0.620	0.781					

Table S7. Information-theoretic models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* breeding. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Breeding models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
null model	**-0.904**	**NA**	**NA**	**NA**	**NA**	**2**	**-82.2062**	**168.496**	**0**	**0.167**
PC3	**-0.991**	**NA**	**NA**	**0.411**	**NA**	**3**	**-81.1662**	**168.501**	**0.005**	**0.167**
PC2	**-0.941**	**NA**	**-0.474**	**NA**	**NA**	**3**	**-81.1745**	**168.518**	**0.022**	**0.165**
PC2 + PC3	**-1.005**	**NA**	**-0.430**	**0.348**	**NA**	**4**	**-80.4345**	**169.153**	**0.656**	**0.120**
PC1 + PC2 + PC3	**-0.839**	**0.311**	**-0.462**	**NA**	**NA**	**4**	**-80.5699**	**169.423**	**0.927**	**0.105**
PC1	**-0.813**	**0.292**	**NA**	**NA**	**NA**	**3**	**-81.6568**	**169.483**	**0.986**	**0.102**
PC1 + PC3	**-0.919**	**0.184**	**NA**	**0.346**	**NA**	**4**	**-80.9753**	**170.234**	**1.738**	**0.070**
PC1 + PC2 + PC3	**-0.917**	**0.227**	**-0.438**	**0.271**	**NA**	**5**	**-80.1476**	**170.724**	**2.227**	**0.055**
PC1 + PC3+PC1*PC3	**-0.903**	**0.267**	**NA**	**0.351**	**0.171**	**5**	**-80.8204**	**172.069**	**3.573**	**0.0280**
PC1 + PC2 + PC3+PC1*PC3	**-0.905**	**0.291**	**-0.421**	**0.280**	**0.133**	**6**	**-80.0566**	**172.718**	**4.221**	**0.020**
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.380	0.466	0.460	0.048					
parameter estimates (B) averaged across models	-0.922	0.266	-0.453	0.358	0.155					
unconditional SE	0.438	0.295	0.342	0.300	0.312					
unconditional 95% CI	-1.780	-0.312	-1.123	-0.230	-0.457					
	-0.065	0.845	0.216	0.947	0.766					

Table S8. Information-theoretic models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* malformation presence. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Malformation presence models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
null model	**1.705**	**NA**	**NA**	**NA**	**NA**	**1**	**-5.5812**	**13.526**	**0**	**0.332**
PC2	**2.111**	**NA**	**4.922**	**NA**	**NA**	**2**	**-4.50772**	**14.215**	**0.689**	**0.235**
PC1 + PC2	**5.040**	**-1.748**	**9.019**	**NA**	**NA**	**3**	**-3.22325**	**15.113**	**1.587**	**0.150**
PC1	**2.639**	**-0.699**	**NA**	**NA**	**NA**	**2**	**-5.234**	**15.668**	**2.142**	**0.114**
PC3	**1.705**	**NA**	**NA**	**0.000**	**NA**	**2**	**-5.5812**	**16.362**	**2.836**	**0.080**
PC2 + PC3	**1.308**	**NA**	**5.608**	**-0.974**	**NA**	**3**	**-4.3953**	**17.457**	**3.931**	**0.046**
PC1 + PC3	**3.405**	**-0.808**	**NA**	**0.678**	**NA**	**3**	**-5.17737**	**19.021**	**5.495**	**0.021**
PC1 + PC2 + PC3	**5.206**	**-1.768**	**8.947**	**0.162**	**NA**	**4**	**-3.22232**	**19.445**	**5.919**	**0.017**
PC1 + PC3+PC1*PC3	**4.513**	**-1.725**	**NA**	**1.543**	**-0.920**	**4**	**-4.99909**	**22.998**	**9.472**	**0.003**
PC1 + PC2 + PC3+PC1*PC3	6.342	-2.900	9.263	1.252	-1.438	5	-3.07797	24.727	11.201	0.001
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.310	0.450	0.170	0.000					
parameter estimates (B) averaged across models	2.494	-1.293	6.517	-0.140	-0.920					
unconditional SE	2.183	1.305	5.178	2.229	1.469					
unconditional 95% CI	-2.188	-4.146	-4.805	-5.077	-4.242					
	7.177	1.560	17.840	4.797	2.403					

Table S9. Information-theoretic models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* malformation prevalence. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Malformation prevalence models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
null model	**0.156**	**NA**	**NA**	**NA**	**NA**	**2**	**12.87235**	**-20.544**	**0**	**0.353**
PC1	**0.205**	**-0.044**	**NA**	**NA**	**NA**	**3**	**14.59566**	**-20.525**	**0.020**	**0.349**
PC3	**0.104**	**NA**	**NA**	**-0.057**	**NA**	**3**	**13.50369**	**-18.341**	**2.204**	**0.117**
PC2	**0.156**	**NA**	**0.001**	**NA**	**NA**	**3**	**12.87238**	**-17.078**	**3.467**	**0.062**
PC1 + PC2	**0.212**	**-0.050**	**0.073**	**NA**	**NA**	**4**	**14.88894**	**-16.778**	**3.767**	**0.054**
PC1 + PC3	**0.178**	**-0.039**	**NA**	**-0.023**	**NA**	**4**	**14.70779**	**-16.416**	**4.129**	**0.045**
PC2 + PC3	**0.101**	**NA**	**0.026**	**-0.060**	**NA**	**4**	**13.53839**	**-14.077**	**6.468**	**0.014**
PC1 + PC2 + PC3	**0.181**	**-0.045**	**0.077**	**-0.027**	**NA**	**5**	**15.04057**	**-11.510**	**9.035**	**0.004**
PC1 + PC3+PC1*PC3	**0.174**	**-0.034**	**NA**	**-0.027**	**0.005**	**5**	**14.71399**	**-10.857**	**9.688**	**0.003**
PC1 + PC2 + PC3+PC1*PC3	0.175	-0.038	0.078	-0.031	0.007	6	15.05332	-4.107	16.438	<0.001
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.450	0.130	0.180	0.000					
parameter estimates (B) averaged across models	0.170	-0.044	0.034	-0.048	0.005					
unconditional SE	0.052	0.025	0.115	0.057	0.051					
unconditional 95% CI	0.061	-0.100	-0.218	-0.173	-0.110					
	0.280	0.012	0.287	0.077	0.120					

Table S10. Models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* severe malformation prevalence. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Severe malformation prevalence models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
null model	**0.138**	**NA**	**NA**	**NA**	**NA**	**2**	**14.55076**	**-23.902**	**0**	**0.475**
PC1	**0.174**	**-0.032**	**NA**	**NA**	**NA**	**3**	**15.6853**	**-22.704**	**1.198**	**0.261**
PC3	**0.116**	**NA**	**NA**	**-0.024**	**NA**	**3**	**14.69293**	**-20.719**	**3.182**	**0.097**
PC2	**0.138**	**NA**	**0.008**	**NA**	**NA**	**3**	**14.55429**	**-20.442**	**3.459**	**0.084**
PC1 + PC2	**0.180**	**-0.037**	**0.061**	**NA**	**NA**	**4**	**15.92794**	**-18.856**	**5.046**	**0.038**
PC1 + PC3	**0.178**	**-0.033**	**NA**	**0.004**	**NA**	**4**	**15.68859**	**-18.377**	**5.524**	**0.030**
PC2 + PC3	**0.114**	**NA**	**0.019**	**-0.026**	**NA**	**4**	**14.71404**	**-16.428**	**7.473**	**0.0113**
PC1 + PC2 + PC3	0.181	-0.038	0.061	0.001	NA	5	15.92826	-13.285	10.616	0.002
PC1 + PC3+PC1*PC3	0.187	-0.042	NA	0.009	-0.008	5	15.71154	-12.852	11.050	0.002
PC1 + PC2 + PC3+PC1*PC3	0.188	-0.045	0.060	0.006	-0.007	6	15.9445	-5.889	18.012	<0.001
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.330	0.134	0.139	NA					
parameter estimates (B) averaged across models	0.148	-0.033	0.024	-0.018	NA					
unconditional SE	0.038	0.023	0.101	0.051	NA					
unconditional 95% CI	0.066	-0.083	-0.199	-0.131	NA					
	0.230	0.018	0.246	0.094	NA					

Table S11. Models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* testicular oocyte prevalence. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Testicular oocyte prevalence models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
null model	**0.475**	**NA**	**NA**	**NA**	**NA**	**2**	**-1.20233**	**8.40465**	**0**	**0.756**
PC3	**0.432**	**NA**	**NA**	**-0.071**	**NA**	**3**	**-0.877**	**12.554**	**4.149**	**0.095**
PC1	**0.490**	**0.035**	**NA**	**NA**	**NA**	**3**	**-1.16689**	**13.134**	**4.729**	**0.071**
PC2	**0.480**	**NA**	**0.023**	**NA**	**NA**	**3**	**-1.18441**	**13.169**	**4.764**	**0.070**
PC1 + PC3	0.453	0.078	NA	-0.089	NA	4	-0.70471	19.409	11.005	0.003
PC2 + PC3	0.434	NA	0.070	-0.090	NA	4	-0.72711	19.454	11.050	0.003
PC1 + PC2	0.490	0.030	0.009	NA	NA	4	-1.16477	20.330	11.925	0.002
PC1 + PC3+PC1*PC3	0.456	0.050	NA	-0.108	-0.052	5	-0.60393	31.208	22.803	<0.001
PC1 + PC2 + PC3	0.449	0.057	0.046	-0.097	NA	5	-0.64961	31.299	22.895	<0.001
PC1 + PC2 + PC3+PC1*PC3	0.451	0.020	0.057	-0.120	-0.059	6	-0.52033	55.041	46.636	<0.001
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.072	0.070	0.096	NA					
parameter estimates (B) averaged across models	0.473	0.035	0.023	-0.071	NA					
unconditional SE	0.103	0.148	0.140	0.098	NA					
unconditional 95% CI	0.233	-0.315	-0.307	-0.304	NA					
	0.712	0.384	0.354	0.161	NA					

Table S12. Models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* presence of testicular oocyte severity score of three (3) or greater. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Presence of testicular oocyte severity score of 3 or more models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
PC2	**0.221**	**NA**	**2.506**	**NA**	**NA**	**2**	**-4.4256**	**14.851**	**0**	**0.345**
null model	**-0.223**	**NA**	**NA**	**NA**	**NA**	**1**	**-6.18265**	**14.937**	**0.086**	**0.330**
PC2 + PC3	**-0.625**	**NA**	**7.053**	**-2.108**	**NA**	**3**	**-3.44385**	**17.688**	**2.836**	**0.083**
PC1 + PC3+PC1*PC3	**68.081**	**437.538**	**NA**	**142.947**	**394.127**	**4**	**-1.25E-09**	**18.000**	**3.149**	**0.071**
PC1	**-0.102**	**0.298**	**NA**	**NA**	**NA**	**2**	**-6.13393**	**18.268**	**3.417**	**0.062**
PC3	**-0.297**	**NA**	**NA**	**-0.120**	**NA**	**2**	**-6.16612**	**18.332**	**3.481**	**0.060**
PC1 + PC2	**-0.056**	**-0.879**	**2.995**	**NA**	**NA**	**3**	**-4.19867**	**19.197**	**4.346**	**0.039**
PC1 + PC3	**-0.193**	**0.405**	**NA**	**-0.215**	**NA**	**3**	**-6.08741**	**22.975**	**8.124**	**0.006**
PC1 + PC2 + PC3	-0.636	0.057	7.191	-2.170	NA	4	-3.44337	24.887	10.036	0.002
PC1 + PC2 + PC3+PC1*PC3	-21.635	18.680	40.681	-11.326	43.126	5	-2.17E-10	30.000	15.149	<0.001
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.179	0.468	0.222	0.072					
parameter estimates (B) averaged across models	4.793	174.400	3.359	45.290	394.100					
unconditional SE	18900.000	221100.00	3.865	69680.000	310500.00					
unconditional 95% CI	-48584.590	-568121.20	-5.694	-179084.00	-797891.80					
	48594.176	568470.07	12.411	179174.59	798680.06					

Table S13. Information-theoretic models for environmental gradients (from Principle Components Analysis) for *Rana pipiens* maximum testicular oocyte severity score. For each model: parameter estimates for each predictor in the model, degrees of freedom (df), corrected Aikake's Information criterion (AICc), log likelihood, delta AICc (compared to AICcMIN model), and Aikake model weight; with the models within 10 AICc of the AICcMIN model in bold. For the set of models within 10 AICc of the AICcMIN model: selection probability (or relative variable importance) based on inclusion in and weight of models, average parameter estimates with unconditional standard errors (SE) and 95% confidence intervals (CI).

Maximum testicular oocytes severity score Models	(Intercept)	PC1	PC2	PC3	PC1*PC3	df	logLik	AICc	delta	weight
null model	**2.778**	**NA**	**NA**	**NA**	**NA**	**2**	**-14.6135**	**35.227**	**0**	**0.620**
PC2	**2.942**	**NA**	**0.826**	**NA**	**NA**	**3**	**-13.3083**	**37.417**	**2.190**	**0.208**
PC3	**2.591**	**NA**	**NA**	**-0.304**	**NA**	**3**	**-14.3138**	**39.428**	**4.201**	**0.076**
PC1	**2.791**	**0.032**	**NA**	**NA**	**NA**	**3**	**-14.612**	**40.024**	**4.797**	**0.056**
PC2 + PC3	**2.628**	**NA**	**1.142**	**-0.615**	**NA**	**4**	**-11.6829**	**41.366**	**6.139**	**0.029**
PC1 + PC2	**2.770**	**-0.541**	**1.081**	**NA**	**NA**	**4**	**-12.852**	**43.704**	**8.477**	**0.009**
PC1 + PC3	2.646	0.201	NA	-0.350	NA	4	-14.2564	46.513	11.286	0.002
PC1 + PC2 + PC3	2.529	-0.380	1.299	-0.571	NA	5	-11.3752	52.750	17.523	<0.001
PC1 + PC3+PC1*PC3	2.617	0.525	NA	-0.136	0.597	5	-13.5621	57.124	21.897	<0.001
PC1 + PC2 + PC3+PC1*PC3	2.515	-0.103	1.219	-0.398	0.444	6	-10.6613	75.323	40.096	<0.001
For model set within 10 AICc of AICc min model (in bold)										
selection probability (relative variable importance)		0.065	0.246	0.105	NA					
parameter estimates (B) averaged across models	2.794	-0.046	0.872	-0.389	NA					
unconditional SE	0.454	0.690	0.551	0.445	NA					
unconditional 95% CI	1.742	-1.664	-0.430	-1.433	NA					
	3.846	1.571	2.175	0.654	NA					