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Title.

Effects of hydroperiod on growth, development, survival, and immune defenses in a temperate amphibian

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Abstract.

1. The many and varied effects of human induced environmental change have the potential to threaten animal biodiversity and species abundance. Importantly, human land use and global climate change are predicted to reduce water availability, which might have negative consequences for freshwater organisms.
2. In this study, we tested for an effect of a shortened hydroperiod on larval growth and development, and post-metamorphic survival and immune function in a temperate frog, *Rana pipiens*.
3. Animals developing under pond drying conditions metamorphosed at a smaller size and had lower survival after metamorphosis. We found sex-specific differences in larval period in our fastest drying treatment, with males metamorphosing more quickly than females. Individuals that developed under drying conditions also showed reduced skin swelling after phytohemagglutinin injection, indicating a compromised immune response. We found support for trade-offs between growth, development, and post-metamorphic immune function across hydroperiod treatments. Whole blood from animals with shorter larval periods had lower bacterial killing ability, and small-bodied juveniles had lower antibody titers.
4. Overall, our results indicate that a shortened hydroperiod can affect the rate of larval amphibian growth and development, and might negatively impact the condition of species that rely on freshwater for development. Our work improves understanding of the complex impacts that environmental stressors might have on the health of animal populations.

Keywords.
Climate change, Ecoimmunology, Environmental change, Immune response, Hydroperiod, Larval period, Pond drying, *Rana pipiens*

Introduction.

One particularly important effect of anthropogenic environmental change is alteration of the availability of freshwater, which can have serious implications for both human and wildlife health. Global climate change is predicted to increase average air temperatures, bring more variable annual precipitation, and increase the frequency of drought (Karl & Trenberth, 2003; Sheffield & Wood, 2008). Increases in agricultural water requirements and human population growth are predicted to further decrease water availability in the urbanized and natural environment (Peterson & Keller, 1990; Sun, McNulty, Moore Myers, & Cohen, 2008; Wilk & Hughes, 2002). These changes are poised to impact aquatic habitats in many ways, such as permanent ponds becoming more ephemeral, and ephemeral ponds experiencing increasingly shorter hydroperiods (Brooks, 2009). Thus, human-

induced environmental change poses an important challenge to the survival and persistence of species that depend on the availability of surface freshwater.

It has been well established that changes in hydroperiod can be an important environmental stressor for amphibians during their development (for meta-analyses, see: Tejedo et al. 2010, Richter-Boix et al. 2011). Many aquatic larval amphibians speed up their development and metamorphose more quickly in response to pond drying and other environmental stressors (Alford & Harris, 1988); however, individuals that do so often incur a cost, such as smaller size at metamorphosis (Cabrera-Guzmán, Crossland, Brown, & Shine, 2013; Gervasi & Foufopoulos, 2008; Kirschman, Mccue, Boyles, & Warne, 2017; O'Regan, Palen, & Anderson, 2013). Other amphibian species do not exhibit this type of developmental plasticity (i.e. they do not speed up development in order to escape an environmental stressor). For these species, the cost of developing in a stressful larval environment might be reduced survival to or after metamorphosis (Richter-Boix et al., 2011). If pond hydroperiods shorten as a result of human-induced environmental changes, the health of populations that rely on these water bodies is likely to suffer.

An individual's immunological condition can impact its ability to resist or tolerate pathogens, which is especially important in cases where infectious disease resistance is known to increase long term survival (Acevedo-Whitehouse & Duffus, 2009). Developmental stressors such as predation (e.g., in dragonflies: Moore et al. 2018), high densities of conspecifics (e.g. in frogs: Echaubard et al. 2010), a shortened hydroperiod (e.g., in frogs: Gervasi and Foufopoulos 2008), and pathogen exposure (Warne et al. 2011; in crickets: Rantala and Roff 2005) can act alone or in concert (e.g., in damselflies: Stoks et al. 2006) to compromise immune function, and potentially affect survival and reproduction. However, studies of the impacts of environmental stressors on the immune function of aquatic-developing organisms are rare and have often measured just one or two aspects of immune function (e.g., swelling response, leucocyte counts). Because animal immune systems are complex, a multifaceted approach to measuring immune function is required to understand the total impact of an environmental stressor (Demas, Zysling, Beechler, Muehlenbein, & French, 2011) and to clarify the relationship between developmental stress and immune function. For amphibians, the threat that pathogens such as *Batrachochytrium* fungi (Scheele et al., 2019) and ranaviruses (Brunner, Storfer, Gray, & Hoverman, 2015) pose to population health and persistence makes research on this topic particularly timely. To date, only one study has explored the effects of pond drying on immune function (Gervasi & Foufopoulos, 2008), and more research is needed to discern the effects of pond drying on amphibians more broadly (Kohli et al., 2019).

Using a replicated mesocosm experiment, we assessed how changes in hydroperiod impact the immune function and other fitness-related traits of a temperate frog species that is dependent on freshwater for its larval development: the northern leopard frog (*Rana* [*Lithobates*] *pipiens*; Yuan et al., 2016). *Rana pipiens* prefers to breed in flooded grasslands and permanent ponds (Noland & Ultsch, 1981) and is broadly distributed in the northern United States and southern Canada, though populations of *R. pipiens* are declining in the northern part of this range (Corn & Fogleman, 1984). While the cause(s) of decline remains largely unknown, infectious disease has been suggested as a potential driver (Voordouw, Adama, Houston, Govindarajulu, & Robinson, 2010).

We tested the effect of hydroperiod on development time, growth, and survival pre- and post-metamorphosis for *R. pipiens* exposed to one of three hydroperiod treatments. To assess the impact of hydroperiod on post-metamorphic immune development, we conducted assays of both the innate and adaptive immune system. We hypothesized that animals developing under shortened hydroperiods would develop faster, metamorphose at a smaller size, demonstrate reduced survival to/after metamorphosis and exhibit reduced immune function after metamorphosis. By examining the indirect effects of early-life stressors on post-metamorphic immune function and survival, this work extends previous findings of detrimental effects of a shortened hydroperiod on larval amphibians.

Methods.

Experimental design.

We collected four egg masses from a nearby pond and placed them in outdoor plastic pools at the Pymatuning Laboratory of Ecology in Northwest Pennsylvania to develop and hatch (see Appendix S1 in Supporting Information). At the same site, we established replicate pond mesocosms by filling 770L cattle tanks (n=21) to an initial depth of 41cm (600L) with well water, seeding them with dried leaf litter, pond water and rabbit feed, and covering them with 50% shade cloth (see Appendix S1).

On day 0 of the experiment we placed 40 tadpoles [Gosner stage 25, 3-4 weeks (w) after egg collection, Gosner, 1960] into each mesocosm and randomly assigned mesocosms to one of three drying treatments: no drying control, moderate drying, or fast drying (mesocosms: n=7 per treatment; tadpoles: n=280 per treatment). Initial tadpole density was one tadpole per 15L of water, which is similar to other mesocosm studies (Boone & James, 2003; Detenbeck, Hermanutz, Allen, & Swift, 1996). Every 5 days (d) we removed water from drying treatment mesocosms to reach a pre-determined depth (Appendix S1), which was repeated until the depth reached 10cm (a 145.5L volume), after which we considered drying to be complete (day 50 for fast drying, and day 90 for moderate

drying). Larval period for this species ranges approximately 50-150d depending on temperature and latitude (McKinnell, Hoppe, & McKinnell, 2005).

When forelimbs began to emerge (Gosner stage 42) we moved individuals into an onsite laboratory (husbandry details provided in Appendix S1). We considered the larval period to be the time between placement of animals in the mesocosm and full tail absorption (Gosner stage 46; n=212 no drying, n=211 moderate and n=221 fast drying). We ended the experiment on day 120, when all but 18 animals had either died or completed metamorphosis.

Tests of immune function.

We tested the immune function of a subset of juveniles that emerged from each of our mesocosm treatments using five different immune assays. To compare aspects of the innate immune system, we tested the bacterial killing ability (BKA) of the whole blood (n=29 no drying, n=19 moderate, and n=22 fast), examined leucocyte quality and phagocytosis using a lavage assay (n=10 no drying, n=8 moderate, and n=8 fast), and examined granular gland morphology histologically (n=18 no drying, n=17 moderate, and n=18 fast). To compare aspects of the adaptive immune system we measured the phytohemagglutinin (PHA) swelling response (n=20 no drying, n=14 moderate, and n=14 fast) and the total abundance of immunoglobulin antibodies (IgM and IgY; n=25 no drying, 20 moderate, and 23 fast). We conducted the PHA swelling response, gland morphology and BKA assays 6-7w after the animals metamorphosed. We performed the lavage assay 16-20w post-metamorphosis, and 3w later we measured total antibody abundance. Additional details for each assay are provided in the Appendix S1, Table S1 and Fig S1. The individual assays are described briefly below.

Innate immune assays.

BKA is a common *in vitro* immunological assay that can be done using blood or serum samples and is suitable for a wide range of taxa (Demas et al., 2011). This test measures the constitutive innate immune function of the blood, as killing invasive bacteria is a fundamental function of the immune system (Matson, Tieleman, & Klasing, 2015). To measure BKA we euthanized animals with 0.1% buffered tricaine methanosulfonate (MS-222, Sigma-Adrich, St Louis, MO, USA) until reflexes ceased and collected blood via cardiac puncture. Collected blood was immediately used in the assay and BKA was assessed using an absorbance plate reader (Savage et al., 2016).

To quantify both the extravasation and phagocytic activity of leukocytes in response to an antigen, we used a peritoneal lavage extraction assay with fluorescent beads adapted from Cary et al.

(2014). We quantified the concentration of leukocytes, and used a fluorescence microscope to determine the number of microbeads engulfed by the leukocytes.

To assess the morphology of granular glands, which produce antimicrobial peptides that are secreted onto the skin, we examined dorsal and ventral skin samples using standard histological methods (Woods & Ellis, 1994). For each individual, we randomly chose five glands to measure morphologically. We measured the total gland area and the area that contained peptides using ImageJ (Schneider, Rasband, & Eliceiri, 2012).

Adaptive immune assays.

PHA elicits an immune response by promoting swelling, and this swelling is used as a surrogate for measuring the T-cell mediated immune response at the site of injection (Tella, Lemus, Carrete, & Blanco, 2008). While PHA can reflect both innate and adaptive immune function, if the animal is primed 1w prior to measurement, the swelling is much larger, and largely the result of an adaptive immune response (Fites et al. 2014). We anesthetized animals using 0.05% buffered MS-222 prior to injection and at each measurement period. One week prior to measurements we primed the animals with 100µL of a 1mg/mL PHA solution. Then, we intramuscularly injected one thigh of each animal with PHA (20µL of 25mg/ml PHA) and the other with amphibian phosphate buffered saline as a control. We measured the width of the thigh three times to the nearest 0.01mm using digital calipers at 0, 12, 24 and 48h after injection.

We collected plasma from animals and measured the relative abundance of IgY and IgM general antibodies using an enzyme-linked immune-sorbent assay (ELISA) modified from Cary et al. (2014).

Sex ratio and determination.

We dissected a subset of post-metamorphic animals post-euthanasia (n=272) to determine sex via gross examination of the gonads. Gonads were only easily distinguishable if the animal was older than approximately 12w post metamorphosis. We were unable to determine the sex of 9 individuals examined.

Statistical analysis.

All statistical analyses (see Table S1) were performed using R Studio (RStudio Team, 2016) and R version 3.3.3 (R Core Team 2017). We used Maximum Likelihood (Pinheiro & Bates, 2000) for fitting of mixed effect models and likelihood ratio tests (function 'anova') for comparisons of nested

models. Goodness of fit for linear mixed models is presented as an approximation of R^2 (function 'r2', package 'sjstats', (Byrnes, 2008; Lüdecke, 2018)). We used a quasi-distribution to account for overdispersion in generalized models if necessary.

To compare larval period across drying treatments, we used a linear mixed-effects model (LMM) with larval period (d) as the dependent variable, drying treatment as the fixed effect and mesocosm as the random effect (package: 'nlme', function: 'lme': Pinheiro *et al.*, 2017). To compare survival to metamorphosis across drying treatments, we concatenated the number of animals successfully completing metamorphosis and the number that remained in the mesocosm at day 120 into a 2-vector response variable, which we compared across drying treatments using a generalized linear model (GLM) with a binomial error structure (package: 'lme4', function: 'glmer'; Bates, Maechler, Bolker, & Walker, 2015).

We compared survival through 42d post-metamorphosis among drying treatments using a Cox proportional hazards model (package: 'survival', function: 'coxph'; Therneau, 2019), with drying treatment, body size (SVL, mm) and their interaction as factors, clustered by mesocosm.

To compare size at metamorphosis (SVL and mass, log-transformed) across the three drying treatments we used LMM with drying treatment and larval period as interactive fixed effects, and mesocosm as a random effect.

We compared larval period between the sexes and among drying treatments using a generalized linear mixed effects model (GLMM) with a quasi-Poisson error distribution (package: 'MASS, function: 'glmmPQL'; Ripley et al., 2019). Larval period was the dependent variable, and the independent variable was a single interaction term between sex and drying treatment. We used a Tukey post-hoc test to make pairwise comparisons among all factor levels (package: 'multcomp', function: 'glht'; Hothorn et al., 2019). We did not test for an effect of sex on body size or survival because sex could only be determined in a subset of animals.

To compare BKA across drying treatments we used a LMM, with drying treatment and larval period as fixed effects, and mesocosm as a random effect. We compared leukocyte extravasation and phagocytic activity across drying treatments using GLMMs with a quasi-binomial error distribution. The proportion of cells that contained fluorescent beads was the dependent variable, treatment and larval period were the independent variables, and mesocosm was the random effect. We also compared the number of live leukocytes per injection volume (log-transformed) across drying treatments and larval period using a LMM, with mesocosm as a random effect.

To compare granular gland size and fullness across drying treatments we used GLMMs (binomial error structure) and LMMs, where the dependent variables were gland fullness (GLMM) or

the total area of the gland (log transformed, LMM). Frog ID nested within mesocosm was included as a random effect, and the fixed effects were drying treatment, SVL and gland location (dorsal or ventral).

The greatest difference in swelling between PHA and saline injected legs occurred at 24h post-injection (Fig. S3). We compared the proportional increase in leg swelling at 24h post-PHA injection across drying treatments using a LMM with mesocosm as a random effect. PHA-induced swelling was compared between all treatments using post hoc pairwise comparisons. Leg swelling of the saline-injected leg was compared in a similar manner. We also examined the variation in relative IgM and IgY antibodies across treatments using a separate LMM. Adjusted absorbances were the dependent variable, drying treatment and SVL were the fixed effects, and plate number was a random effect.

Results.

Of the animals that metamorphosed within the experimental time frame, larval period ranged from 62 to 127d, with a mean and standard deviation of 85.75±14.51d. (Fig. 1a). Overall, there was no difference in larval period among drying treatments (LMM: moderate drying, $\beta = -0.023$, $p=0.45$; no drying, $\beta=0.013$, $p=0.70$, Table S2).

A total of 645 animals (76.78%) reached the tail-absorption stage by day 127; 18 animals remained as tadpoles (2.14%) and the rest (21.08%) did not survive through to metamorphosis. There were no differences in the proportion of animals that successfully metamorphosed per mesocosm among drying treatments (GLMM: moderate drying, $\beta=0.10$, $p=0.67$; no drying, $\beta=0.03$, $p=0.90$, Table S3). However, survival to 6w post-metamorphosis was higher in the no drying treatment (95.19%, 95% CI: 91.37-97.37%; COXPH: $\beta= -0.8$, $p=0.017$) than in the moderate (89.04%, 95% CI: 85.50 - 93.30%) or fast drying treatments (89.04%, 95% CI: 85.60 - 93.55%, Fig. 1b). SVL (mm) was a significant predictor of survival probability, with larger frogs more likely to survive than smaller frogs (COXPH: $\beta=-0.25$, $p=0.016$, Table S4).

Animals in the fast and moderate drying regimes were similar in size, but were on average (±SD) 17.08% smaller in mass (0.73±0.17g) and 4.94% smaller in SVL (21.71±1.69mm) at metamorphosis than animals in the no drying treatment (mass: 0.86±0.21g; SVL: 22.78±1.75mm; LMM: SVL, $\beta=-0.043$, $p=0.032$, mass, $\beta=0.064$, $p=0.016$, Fig. 1c; Table S5). There was a significant interaction between larval period and drying treatment (LMM: $\beta=-0.001$, $p=0.0004$), where the size (SVL) of the animal increased with larval period in the no drying treatment but remained similar over time in the two drying treatments (Fig. 1c).

256 Overall, the sex ratio of animals that metamorphosed was not significantly different from 1:1
257 (ratio of males to females; 129:134 in total; no drying 46:47, moderate 49:39, fast 34:48). However,
258 males metamorphosed earlier (75.82±9.6d) than females (85.2±15.86d) in the fast drying treatment
259 (GLMM: β=-0.10, p=0.014, Fig. 2, Table S6), with males emerging 9.77d faster than females on
260 average. The larval period was 84.16±10.23d for males and 88.48±13.48d for females in the moderate
261 drying treatment, and 83.24±13.22d for males and 84.30±14.86d for females in the no drying
262 treatment.
263
264 *Immune function.*
265 There was no difference in BKA across the three drying treatments (LMM: moderate drying:
266 β=-0.02, p=0.90, no drying: β=0.20, p=0.15), but there was a positive association between larval period
267 and the bactericidal ability of whole blood, where BKA was greater in animals that had longer larval
268 periods (LMM: β=0.11, p=0.004, R^2=0.18, Fig. 3a, Table S7).
269 In the lavage assay, there were no differences among drying treatments in the number of live
270 leukocytes per injection volume (LMM: no drying, β=-0.15, p=0.41; moderate drying, β=-0.213,
271 p=0.28), or the number of neutrophils containing fluorescent beads (quasi-binomial GLMM: no drying,
272 β=0.45, p=0.22; moderate drying, β=0.26, p=0.51, Table S8).
273 In our gland morphology analysis, dorsal granular glands were larger in area than ventral
274 granular glands across all treatments (LMM: β=-0.16, $p < 0.001$), and gland size was marginally
275 associated with SVL (β=0.041, p=0.055), but there was no difference in gland size (LMM, no drying:
276 β=0.21, p=0.86; moderate drying: β=0.14, p=0.24) or gland fullness among drying treatments (no
277 drying: β=-0.08, p=0.73; moderate drying: β=0.062, p=0.80; Table S9).
278 We found significantly less swelling (87.14% less swelling) in animals that experienced the fast
279 drying treatment compared to the no drying treatment 24h after PHA injection (pairwise comparison:
280 β=-6.15, t=-2.5, SE=2.45, p=0.03, Fig. 3b, see Table S10), where the no drying treatment animals had
281 a swelling response of 7.06%, and the fast drying animals had swelling response of only 0.91%. There
282 was no difference in swelling of the saline injected leg among the treatments (pairwise comparison:
283 β=1.25, t=0.63, SE=1.99, p=0.80, Fig. S3, Table S10).
284 Relative antibody abundance (both IgY and IgM) was positively correlated with frog size
285 (SVL) (LMM: IgY, β=0.027, p=0.013, R^2=0.15, IgM: β=0.029, p=0.006, R^2=0.22, Fig. 3c, d).
286 However, there was no effect of drying treatment on relative antibody abundance (LMM: IgY, no

drying, β=-0.024, p=0.67; moderate drying, β=0.065, p=0.26; IgM, no drying, β=-0.052, p=0.33; moderate drying, β=0.027, p=0.62, Table S11).

Discussion.

Our experiment indicates that early pond drying as a result of anthropogenic environmental change can not only reduce survival and impact growth, but can also alter post-metamorphic immune function. In this study, we found that larvae of *R. pipiens* developing in artificial ponds with shortened hydroperiods were smaller and had lower overall survival after metamorphosis. While *R. pipiens* did not respond to pond drying by increasing developmental rate overall, males did metamorphose more quickly than females under fast drying conditions, indicating potential sex-specific differences in developmental plasticity. Rapid drying also resulted in a less robust adaptive immune response in post-metamorphic animals. Irrespective of hydroperiod treatment, the blood of animals with shorter larval periods also had reduced bactericidal ability, and smaller animals had a lower relative abundance of antibodies indicating the existence of additional interactions between growth, development and immune function. Taken together, our results indicate that early pond drying might negatively impact the condition and immune function of pond breeding amphibians, potentially reducing resilience to pathogens later in life.

Larval period, size and survival.

We saw large individual variation in larval period (60-120 d) among *R. pipiens* from all drying treatments. Individual variation in larval period might have been driven by differences in foraging behavior, conspecific competition within the mesocosms (Wilbur & Collins, 1973), and/or a bet-hedging strategy to deal with unpredictable environmental conditions (Lane & Mahony, 2002). While previous studies indicate that larval period generally shortens under experimental drying, the magnitude of the response varies within and across anuran families (Edge, Houlahan, Jackson, & Fortin, 2016; Gervasi & Foufopoulos, 2008; O'Regan et al., 2013; Richter-Boix et al., 2011), and permanent pond breeders tend to have longer developmental times than ephemeral pond breeders (Richter-Boix et al., 2011). Amphibians accelerate metamorphosis through the synthesis of thyroxine and corticosterone, which are released after activation of the neuroendocrine stress axis (Denver, 2009). Because they generally breed in wetlands with longer hydroperiods, *R. pipiens* might require stronger stimuli to activate the stress axis (Belden, Rubbo, Wingfield, & Kiesecker, 2007) or might be less developmentally responsive to corticosterone (Glennemeier & Denver, 2002). Such a dampened physiological response might limit their ability to survive to metamorphosis under drying conditions.

Our results indicate that size at and survival after metamorphosis can be negatively impacted by drying, which is also supported by previous research (Crump, 1989; O'Regan et al., 2013). Small size at metamorphosis can have long-term negative consequences. For example, juvenile size is often positively correlated with adult survival (Berven, 1990; Cabrera-Guzmán et al., 2013). Larger juveniles often grow to be larger adults, and smaller juveniles can take longer to reach sexual maturity (Berven, 1990). Animals developing under drying conditions in this study were smaller, on average, at metamorphosis, regardless of the length of their larval period. Furthermore, animals developing under drying conditions had lower survival that those in the no drying treatment, indicating that changes in hydroperiod can negatively impact *R. pipiens* larvae and later life stages.

Sex differences in larval period.

Under more favorable developmental conditions, male and female amphibian larvae typically develop at similar rates (Vorburger, 2001), and this was evident in our no drying treatment group. Surprisingly, we found that males were more likely to metamorphose earlier than females in the fast drying treatment. Because we were only able to sex animals that were old enough to have differentiated sex organs (~12w post metamorphosis or older), it is possible that the animals that died early in the experiment could have represented one sex more than the other. However, our overall sex ratio was close to 1:1, indicating that mortality was similar between the sexes soon after metamorphosis. The sex-bias we observed in the timing of metamorphosis in response to pond drying mimics a facultatively paedomorphic salamander species, where males metamorphose earlier and in greater frequency under drying regimes while females are more likely to remain in the pond as paedomorphic adults, or metamorphose later (Denoël, Mathiron, Lena, & Baouch, 2017). The observation that male leopard frogs from our experiment would be able to escape more easily from a drying pond indicates that males might be more plastic in their response to environmental stressors during development than females. Whether this pattern holds true for other anurans remains unseen because, to our knowledge, no previous study has addressed this.

Differential development rates for males and females under stressful conditions might be due to differences in energy requirements for development. One hypothesis for differential development rates in a drying pond is that females require a longer larval period to build up the required resources for egg development and to reach maturity (Denoël et al., 2017). Male frogs tend to reach sexual maturity faster than females (Brannelly et al., 2016; Dare & Forbes, 2008), and in *R. pipiens*, males reach sexual maturity one year earlier and at a smaller size than females (Christensen, 1930). Male *R. pipiens* might maximize their fitness by minimizing time in a stressful aquatic environment, especially if they can

reproduce during their first year of life. In females, size at metamorphosis can directly impact reproductive success. In marbled salamanders, for example, females that were smaller at metamorphosis produced smaller clutches (Scott, 1990, 1994). Therefore, for females, the risk of metamorphosing at a smaller size might outweigh the risk of increased mortality due to desiccation under a shortened hydroperiod. Differences in metamorphic plasticity in response to developmental stressors might skew the sex ratio toward males, such that the effective sex ratio of breeding adults could also shift. Therefore, the differential developmental rates we observed between the sexes under fast drying has the potential to impact population parameters such as recruitment and population size.

Immune function.

In this experiment we examined multiple measures of both the innate and adaptive immune function in animals that developed under different pond drying regimes. We found that there was a significantly reduced swelling response after PHA injection (adaptive immune response) in animals that experienced fast drying. In a closely related species, *R. sylvatica*, development under drying conditions also led to a reduced swelling response (Gervasi & Foufopoulos, 2008). Taken together, these studies indicate that a reduced hydroperiod during the larval stage negatively impacts this aspect of the adaptive immune response in frogs.

We found that relative IgY and IgM antibody abundance was positively correlated with body size for similarly-aged juvenile *R. pipiens*. Smaller animals had lower relative immunoglobulin abundance, indicating that for frogs of a given age post-metamorphosis, size impacts the degree of development of the general antibody reserve. The relative abundance of both antibodies followed a similar pattern, indicating that variation in body size impacts multiple facets of the adaptive immune response. While drying did not have a direct effect on relative antibody abundance, animals from the no drying treatment were larger at metamorphosis. Overall, we found evidence for a less robust adaptive immune response in animals that experienced drying during their development (reduced swelling response to PHA) and in smaller animals (fewer antibodies produced). A reduction in adaptive immune defenses could impact frogs' abilities to combat certain pathogens, including *Batrachochytrium* pathogens (Ramsey, Reinert, Harper, Woodhams, & Rollins-Smith, 2010) and ranavirus (Robert et al., 2005).

In assessing the innate immune response, we found that bacterial killing ability (BKA) of whole blood increased with larval period. In frogs specifically, BKA predicts susceptibility to disease, such as chytridiomycosis (Savage et al., 2016). Our findings indicate that post-metamorphic amphibians might have a stronger innate immune system if they spend more time as a tadpole. Thus,

larvae that take longer to metamorphose might be more likely to survive an immune challenge post-metamorphosis. While we found no direct effect of hydroperiod on BKA, we found reduced immune function in animals with the shortest larval periods (the same individuals that would have the greatest chance of escaping a drying pond), which might cause indirect negative impacts of pond drying on the overall health of frog populations.

We did not find any effects of hydroperiod, larval period, or body size on the other aspects of innate immune function we measured, including granular gland size and fullness, and leukocyte proliferation and phagocytosis. Accurate assessment of the impact of a stressor on immune function is best achieved when multiple immune measures are considered (Demas et al., 2011). The amphibian immune system is complex (Robert & Ohta, 2009; Rollins-Smith, 1998; Rollins-Smith & Woodhams, 2012); therefore, the impacts of stressors on some immune elements might be more pronounced than others. Our results indicate that some, but not all aspects of immune function in post-metamorphic *R. pipiens* are impacted by a drying pond. Environmental stress such as a reduced hydroperiod during development can impact post-metamorphic size and survival and might result in surviving animals that are less equipped to deal with pathogen pressures.

Broader impact.

Using a multifaceted approach, we demonstrate that amphibians developing under fast drying conditions exhibit lower survival, a reduced adaptive immune response, and smaller size at metamorphosis. Our results demonstrate that hydroperiod, juvenile body size, and larval period can interact to influence the immune system in amphibians. Therefore, changes in water availability could affect the viability of aquatic and semi-aquatic populations. Because human-induced environmental change is predicted to result in shorter and more variable hydroperiods, our results support the idea that future populations of freshwater organisms might incur similar threats to population health and viability (Bunnell & Ciraolo, 2010), particularly those that rely on long larval periods for optimal development. Our results demonstrate that pond drying might impact males and females differently, as male *R. pipiens* developed faster than females under drying conditions. If such accelerated drying occurs frequently, the difference in time to metamorphosis between the sexes might affect population dynamics. While our study investigated one population of a widely distributed species, the clear effect of pond drying indicates that changes in hydroperiod might have negative implications for the species as a whole, and for other species with similar ecologies.

Ethics and permits.

This research was conducted according to the University of Pittsburgh Institutional Animal Care and Use Committee protocol IML-17091291-9. Permission to collect animals was granted by the Pennsylvania Fish and Boat Commission, scientific collection permit number 2016-01-0206.

Data availability.

Data has been uploaded to Dryad, with provisional doi:10.5061/dryad.k3t3432.

Author contributions.

LAB, MEBO, CLRZ conceived and designed the experiment; LAB, MEBO, VES conducted the data collection; MEBO, LAB analysed the data; CLRZ provided material and financial support; LAB, MEBO wrote the manuscript. All authors edited and give final approval of the submitted manuscript.

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615
616 **Figure Legend**

Figure 1. Metamorphic timing, post-metamorphic survival, and frog size across the three drying treatments. (a) The proportion of individuals that successfully metamorphosed from the mesocosms. The shortest time to tail-absorption was 60d after tadpoles were placed in the mesocosms. (b) Size adjusted survival rate for post-metamorphic animals in each treatment up to 45d after tail absorption. Day zero is the day of metamorphosis for each individual. The (c) SVL (mm) of each frog at tail-absorption from the three drying treatments. Shaded areas are 95% confidence intervals.

Figure 2. The continuous distribution of larval periods for males and females in each of the three drying treatments (n=263), expressed as kernel density plots. Larval period is the time between entering the mesocosm and tail-absorption.

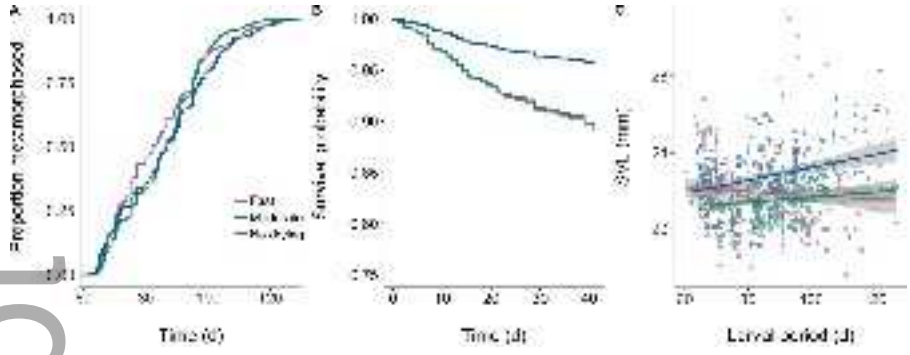
Figure 3. Results of immune function assays. (a) The bacterial killing ability (BKA) of whole blood from frogs that were 6-7w post-tail absorption. BKA was calculated as 1 - (sample absorbance - sample blank absorbance)/(control absorbance - control blank absorbance), negative values indicate enhancement of bacterial growth. Larval period is the time from entry into the mesocosm to tail-absorption. Each point represents one individual, and the linear relationship between BKA and larval period is presented with the 95% confidence interval (shaded area). (b) Skin swelling response 24h after injection of PHA into the thigh. The y-axis indicates the percent change in thigh width 24h after injection. The centre line is the 50^{th} percentile, top and bottom of box represent 75^{th} and 25^{th} percentiles, respectively, and whiskers extend to extreme data points (no more than 1.5 times the interquartile range). (c) The relationship between total IgM, and (d) IgY antibodies and SVL at 19-23w post-metamorphosis. The linear relationship between BKA and larval period is presented with the 95% confidence intervals (shaded area).

Supporting Information Legend.

Appendix S1. Additional details to the study methods, project design and technical protocols

Tables S1-S11. Additional tables to provide clarity on statistical tests performed.

Figures S1-S4. Additional figures to demonstrate the results of the study.



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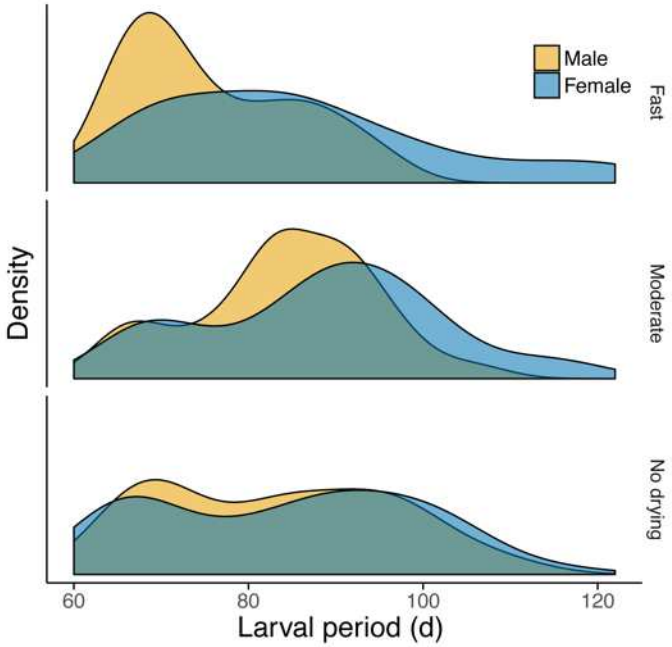

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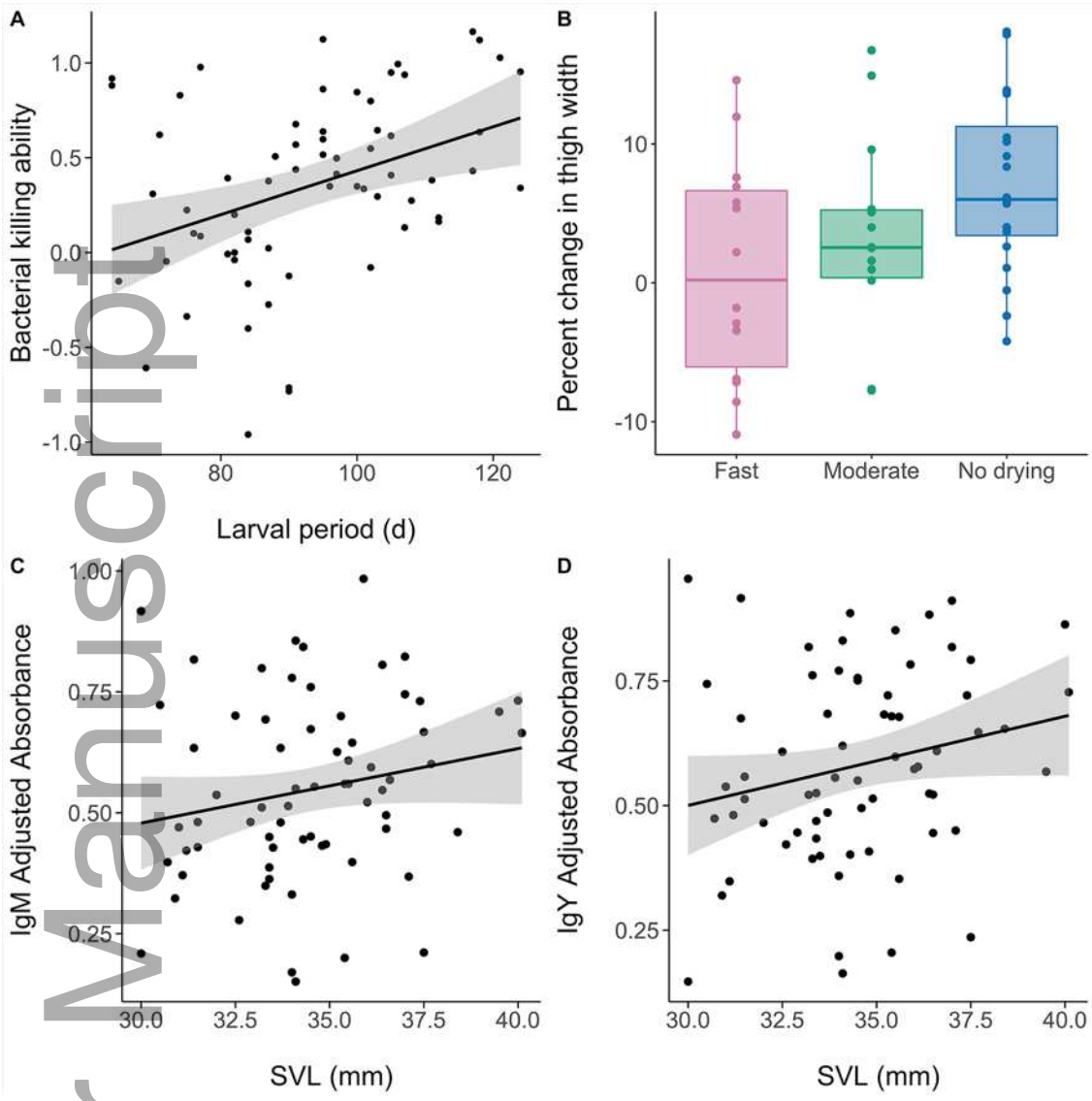

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