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Landscape and water characteristics correlate with immune defense traits across Blanchard's cricket frog (*Acris blanchardi*) populations

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ABSTRACT

Due to ease of global transportation, disease threats to amphibians are expected to increase. Therefore it is crucial that we improve our understanding of factors which may depress disease resistance so that we can incorporate this information into long-term conservation planning. Amphibians are protected from disease-causing pathogens by two skin-associated immune defense traits: the skin microbiome and the antimicrobial peptides found within natural peptide secretions (NPS) produced by the skin. Particular environmental characteristics may alter these amphibian immune defense traits and potentially affect disease resistance. We surveyed the skin-associated microbial communities (microbiome) and natural peptide secretions (NPS) of Blanchard's cricket frogs (*Acris blanchardi*), at each of eleven sites across the species' declining range. We utilized an AICc model selection and model averaging approach to test for potential environmental influence on these traits. We found that populations differed in microbiomes and NPS production, but not NPS bioactivity against Bd (*Batrachochytrium dendrobatidis*). The microbiome was associated with water conductivity, ratio of natural to managed land, and latitude. Additionally the microbiome was affected by interactions between frog sex and latitude, between frog sex and water surface area, and between the ratio of natural to managed land and water surface area. NPS production was influenced by an interaction between water surface area and conductivity. We found no evidence that NPS influence the microbiome; however, Bd growth rate in culture was positively associated with NPS production. This study indicates that environmental characteristics influence amphibian immune defense traits and may explain population differences in pathogen resistance.

1. Introduction

Amphibian populations have experienced large declines over the last several decades as a result of anthropogenic disturbance including habitat destruction, environmental contamination and the introduction of invasive pathogens (Daszak et al., 2003). Due to the ease of global transportation, the introduction of disease-causing pathogens is expected to increase. Therefore it is crucial to understand which factors affect disease resistance so that this information can be incorporated into conservation planning. Amphibians are protected from numerous pathogens in the environment via two skin-associated immune defense traits: the microbial communities (microbiome) inhabiting the skin surface (Belden and Harris, 2007) and the antimicrobial peptides, found within the natural peptide secretions (NPS) produced by granular glands within the host's skin (Rollins-Smith et al., 2005). While there is some evidence of adaptive immunity in amphibians (McMahon et al., 2014), these two skin-associated traits act as a first line of defense against pathogen invasion (Rollins-Smith, 2009); therefore understanding environmental factors which cause differences in these traits between populations is important for understanding disease resistance and susceptibility.

It is known that the structure of the amphibian skin microbiome is strongly associated with host species (Kueneman et al., 2014; McKenzie et al., 2012; Walke et al., 2014) and there is also evidence that microbiome structure changes with host ontogeny (Krynak et al., 2015; Kueneman et al., 2014). In contrast, few studies test for differences in microbiome structure across amphibian populations and little is known about what components of the environment influence interpopulation variation in the amphibian microbiome (Becker et al., 2014; Fitzpatrick and Allison, 2014; Krynak et al., 2015). Similarly, there are few tests for intraspecific variation in NPS (Tennessen

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et al., 2009) and little information regarding potential host or environmental characteristics which may account for these population level differences (Groner et al., 2013; Groner et al., 2014; Krynak et al., 2015; Woodhams et al., 2007). While Conlon (2011) found that antimicrobial peptides within *Acris crepitans* natural peptide secretions were not bioactive against Bd, it is not clear whether the study was conducted with animals from a single population. It is important to evaluate how generalizable peptide bioactivity is across populations. Even common environmental variation, such as small shifts in pH (7 to 6) and degree of pond shading, can alter amphibian skin microbiome and NPS production (Krynak et al., 2015). Studies which assess the influence of environmental characteristics on these traits across populations can improve our understanding of differential disease resistance, and provide rationale for altering land-management practices to better protect wildlife health.

Variation in water characteristics including pond pH, alkalinity, total phosphate levels, and conductivity, may explain skin-associated immune-defense trait differences across amphibian populations. Environmental pollutants which alter these water characteristics have been associated with increased rates of amphibian skeletal deformities and parasitic infections (Hopkins et al., 2013; Hopkins et al., 2000). Water quality characteristics have also been associated with effects on other more traditional fitness correlates including survival (Dobbs et al., 2012; Karraker and Ruthig, 2009), larval duration (Ling et al., 1986), and post-metamorphic mass (Brand et al., 2010; Rowe et al., 1992; Smith and Burgett, 2012). Landscape-level environmental characteristics such as amount of residential and agricultural habitat are also associated with effects on these traditional fitness correlates. Land management practices are associated with changes in amphibian abundance, growth rate and body size (Barrett et al., 2010; Gray and Smith, 2005; Gray et al., 2004). Although growth and development are correlated with amphibian fitness (Semlitsch et al., 1988; Stephens et al., 2013), their assessment alone may give an incomplete picture of the effect of environmental change on amphibian population persistence and disease resistance capabilities (Gervasi and Foufopoulos, 2008).

Amphibians with small effective population size and limited dispersal capabilities may be particularly vulnerable to disease-associated mortality and subsequent decline if changes in their environment depress immune function. The Blanchard's cricket frog, *Acris blanchardi*, is one such species (Gray, 1983, Burkett 1984). This species has undergone dramatic declines over the past four decades (Beauclerc et al., 2010; Gray and Brown, 2005) and a variety of anthropogenic environmental alterations including habitat loss, fragmentation, acidification, and chemical contamination have been hypothesized to have caused these declines (Lehtinen and Skinner, 2006; Reeder et al., 2005; Russell et al., 2002). In addition, disease outbreaks, including those caused by *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen associated with global amphibian declines and extinctions, have been suspected as having a potential role in these declines (Gray et al., 2009; Steiner and Lehtinen, 2008). However, synergistic interactions between environmental change and disease are likely (Hayes et al., 2010). *A. blanchardi* also have highly vascularized skin, which may enhance the effects of chemical contamination and disease susceptibility (Beasley et al., 2005). This potential sensitivity, suspected disease susceptibility and declining status make *A. blanchardi* an excellent model for examining environmental influence on skin-associated immune defense traits.

To determine the effect of water quality and landscape characteristics on *A. blanchardi* skin-associated immune defense traits, we conducted a field survey across pond sites in Ohio and Michigan. Our sites extended in a latitudinal transect across the northern edge of the species' geographic range (Fig. A.1). We surveyed the skin-associated microbiome and NPS of multiple individuals at each site. We hypothesized that 1) environmental variation across sites correlate with differences in immune defense traits among populations 2) that pond site would explain differences in microbiome structure, NPS production, and NPS bioactivity and 3) that trait differences would correlate to differences between sites in terms of water and landscape characteristics.

2. Materials and methods

2.1. Site selection

Between May 30 and June 28 of 2012, we assessed 52 potential sampling sites, including a mix of historic and predicted (based on habitat type) *A. blanchardi* populations (Lehtinen, 2002). We chose sampling sites based on *A. blanchardi* population size and accessibility. We assumed populations to be independent if they were greater than 2 km from other sites based on the low dispersal distance in *Acris* sp. (Gray, 1983; Gray and Brown, 2005). Since many *A. blanchardi* populations are experiencing dramatic declines, if a population was deemed small (<100 calling males), we did not include the site in the immune defense trait survey. Only 11 sites had large enough populations and occurred in terrain conducive for animal capture (Fig. A.1). We sampled sites after the main breeding period to avoid removing animals from the populations before they had reproduced (Gray, 1983). High temperatures (up to 36.6 °C air temperature) over our sampling period resulted in animals moving considerable distances off shore into the deeper, cooler, waters onto emergent vegetation. This movement offshore limited the number of captured animals for our study. These sites varied in water and landscape characteristics (Table A.1) and some sites may be highly influenced by anthropogenic factors. Consequently, our study sites span a range from relatively undisturbed habitat to habitat greatly affected by human activities including chemical treatment.

2.2 Data collection

With the exception of a single site, we hand-captured six-10 adult *A. blanchardi*, targeting five males and five females, during daylight hours, at each of the 11 sites (Table A.1). We maintained frogs in individual air-filled plastic bags, in a cooler until sample collection (within 6 h of capture). We collected skin-associated microbiome samples from individual pre-rinsed animals via a standardized swabbing technique. We rinsed animals thoroughly by submerging them in sterile water. We swabbed the dorsum and venter of each animal 12 times (rotating the swab during the process) using sterile synthetic swabs (Advantage Bundling: MW-113), stored in 95% ETOH in 2 ml cryovials on ice until transferred to a −80 °C freezer (within 4 days of sample collection). Preserved swab samples remained frozen at −80 °C until DNA extraction. We collected natural peptide secretions from individuals immediately after microbial community samples utilizing a 0.01% norepinephrine (20 mM norepinephrine hydrochloride) bath to elicit the secretion of the proteins (Krynak et al., 2015; Sheafor et al., 2008). We euthanized animals in MS-222 immediately after NPS sample collection, weighed each frog, collected a tissue sample which was preserved in 95% ETOH, and formalin fixed the body of each frog for museum donation. Sampled frogs were euthanized to avoid reintroducing immunocompromised animals back into the populations. We acidified NPS samples with 100% trifluoroacetic acid (TFA) and purified samples using C-18 SepPak Classic Cartridge (Waters Corporation), saving the acidified collection buffer for a second NPS purification event at the time of sample elution. We stored NPS samples on ice until transferred to the −80 °C freezer.

We measured pH, alkalinity (methyl orange), conductivity, and total phosphate at each pond site using a HACH Stream Survey test kit (Table A.1). Water samples for analysis were collected at the frog collection site (pond edge). We collected data on landscape

characteristics including latitude, the ratio of natural (prairie and forest) to managed (agricultural and residential land) terrestrial cover (referred to as N:M hereafter), and water surface area (m^2; "water SA" hereafter) within a 200 m buffer of each pond site digitizing open layers Google satellite imagery in Qgis (Quantum GIS Development Team, 2015). We chose a 200 m buffer size based on the limited dispersal capabilities of the species and the desire to assess immediate environmental influences at each collection site (Gray, 1983). We chose these environmental characteristics because they are typically expected to affect the distribution of microbial communities (Carrino-Kyker et al., 2012; Carrino-Kyker and Swanson, 2008).

2.3 Microbiome analysis

We extracted microbial DNA from the skin swabs using a bead beating (2 × 40 s) and phenol chloroform extraction method (Burke et al., 2008; Burke et al., 2006). Negative PCR results using two different primer sets (58A2F and NLB4, 58A2F and ITS4) targeting the ITS-2 gene region of fungal DNA suggested that fungal communities did not contribute significantly to the microbial community on the skin of the animals used in this study; therefore further fungal community analyses were not performed (Krynak et al., 2015). These negative PCR results also suggested that Bd was either not present in our frog skin samples, or was not present in a high enough abundance for detection. We amplified bacterial DNA using 16S rRNA gene primers: 338f and 926r (Muyzer et al., 1993) according to the Carrino-Kyker et al. (2012) protocol. Using terminal restriction fragment length polymorphism profiling (TRFLP), we examined microbiome structure across sites (Burke et al., 2008; Carrino-Kyker et al., 2012; Krynak et al., 2015). This profiling procedure provides results comparable to high-throughput sequencing when sampling across local spatial scales such as in this study (van Dorst et al., 2014). We used the restriction enzyme *MboI* (Promega) to prepare samples for TRFLP profile analyses subsequently generated at the Life Sciences Core Laboratory Center (Cornell University) using a GS600 LIZ size standard (Applied Biosystems). We used Peak Scanner ™ Software (version 1.0, Applied Biosystems, 2006) and R (R version 3.0.2, 2013) for our analyses. TRFLP profiles were processed using the TRFLPR package in R (Petersen et al., 2015; R version 3.0.2, 2013). Only peaks which accounted for >1% of the relative peak area were included in sample analyses (Burke et al., 2008). We used nonmetric multi-dimensional scaling analyses (NMDS) and multi-response permutation procedures (MRPP) to assess bacterial community structure across sites in PC-ORD (Version 5.0; Bruce McCune and MJM Software, 1999). MRPP is a non-parametric discriminant function analysis which tests for differences between two or more groups of entities (McCune et al., 2002). TRFLP profiles were arcsine-square root transformed prior to analysis (McCune et al., 2002). We used axis scores from resulting NMDS ordination solution to assess influence of environmental and host characteristics on the variation across each NMDS axis independently (see analysis description below). We utilized a cloning and sequencing approach to identify dominant members of skin-associated microbiome (Qiagen PCR Cloning Plus) constructing a single clone library (N = 169 clones produced). Clones were created utilizing PCR product from DNA template pooled by site (N = 11 PCR reactions). Clones were picked randomly, and as such, the dominant members of the community are at greater probability of being chosen. We archived resulting cloned sequences in the European Bioinformatics Institute (EMBL; Cambridge, UK), DNA DataBank of Japan (DDBJ), and GenBank (Table A.6; LN794355–LN794520). We performed TRFLP on the clones to determine actual TRF size for each clone, again using the *MboI* restriction enzyme (Promega). We conducted indicator species analyses on terminal restriction fragments from the microbiome profiles and identified taxa using TRFs from the clone library. We completed indicator species analysis (a Monte Carlo test for group prediction) using PC-ORD (version 5.0) to examine relative proportions of bacterial taxa from *A. blanchardi* skin ($p < 0.05$) by site.

2.2. Natural peptide secretions (NPS)

We eluted NPS from the C-18 SepPaks, and subsequently passed the saved, acidified collection buffer through the SepPaks for a second collection attempt (Sheafor et al., 2008). This second pass of NPS was then immediately eluted from the SepPaks. We dried eluted samples at 15 °C in an Eppendorf Vacufuge™ and reconstituted samples in 500 μl of sterile water (HPLC grade) and syringe filtered them (13 mm Pall Acrodisc with Tuffryn® membrane and 0.2 μm pore size) prior to analysis. We utilized a Micro BCA™ Protein Assay Kit (product # 23235) for analysis of total protein concentration from our NPS sampling. We used 100 μl reactions to measure optical density at 562 nm (absorbance) with a BioTek Synergy HT plate reader. We used absorbance measures to estimate concentration of the protein (μg/ml) using Bradykinen as the protein standard (referred to as NPS production). Each sample and standard was run in triplicate and we standardized NPS production by frog mass (μg/ml per gram body weight). Site influence on NPS production was assessed via ANOVA

We measured NPS bioactivity by determining pathogen growth rate in culture when challenged by NPS from individuals across sites. We conducted assays against *B. dendrobatidis* (Bd strain JEL 404, originally isolated from a *Rana catesbeiana* larva in Oxford Co., Maine) in culture. Based upon the BCA assay results, a standardized concentration (100 μg/ml stock, 50 μg/ml in assay) of each NPS sample was made. 50 μl of Bd zoospore solution at a concentration of approximately 2×10^6 zoospores/ml (in 1% tryptone broth) was added to each well of a 96 well flat-bottom sterile plate. 50 μl of NPS at the aforementioned concentration was then added to each well (each sample replicated 3 times). We prepared positive and negative controls on each 96 well plate (three replicates per control on each plate). Positive controls consisted of 50 μl of 2×10^6 Bd zoospores/ml and 50 μl of sterile PCR grade water. Negative controls contained 50 μl of heat-killed Bd zoospores of the same concentration and 50 μl of sterile PCR grade water (Gibble and Baer, 2011; Gibble et al., 2008). We read optical density (OD; BioTek Synergy HT) of wells at 490 nm on day 0 (immediately after plating), day 1(13 h post plating), day 2, day 3, day 4, day 6, day 7, and day 8. A logistic growth model was fit to data using a self-starting nls logistic model function (R version 3.0.2, *stats* package, José Pinheiro and Douglas Bates), and the growth rate (r) of Bd was determined (Krynak et al., 2015). Site influence on Bd growth rate (called NPS bioactivity hereafter) was assessed via ANOVA.

2.3. Model selection and model averaging

We used variance inflation factor (VIF) to assess collinearity between explanatory variables and we excluded variables if their VIF was greater than five. pH was the only variable which was excluded from our statistical analyses as having a VIF greater than five. We used an AICc model selection approach to compare linear mixed models, with site held as the random factor in every model to assess 1) environmental factors influencing the immune defense traits ((microbial community variation along NMDS axes (axis 1, 2, and 3 scores); Beals, 2006; Gotelli and Ellison, 2004; Quinn and Keough, 2002), NPS production, and NPS bioactivity) and 2) host factors (NPS production and NPS bioactivity) influencing microbial community NMDS axis scores (Burnham and Anderson, 2002). Environmental models included main effects (alkalinity, total phosphate, conductivity, N:M, water SA, latitude, and sex of the frog) and interactions perceived to be biologically important: water SA × N:M, water SA × conductivity, water SA × alkalinity, N:M × alkalinity, and latitude × alkalinity as well as interactions between the sex of the animal sampled and each of the main

environmental predictors for a total of 23 environmental models (Table A.2). We included all 23 environmental models in assessment of each of the response variables (microbial community NMDS axis 1, 2, and 3 scores, NPS production, and NPS bioactivity). Host models included those examining potential main effects of NPS production and NPS bioactivity (r), their additive effects, and their interaction effects on microbial community NMDS axis scores, for a total of four models for each response variable (NMDS axis 1, 2, and 3 scores). Model fit for environmental and host models was assessed using conditional R^2, which describes the proportion of variance explained by both the fixed and random factors (Nakagawa and Schielzeth, 2013). We used a model averaging (Burnham and Anderson, 2002) approach to assess predictor influence on every response variable examining both environmental and host influences on these immune defense traits. The influence of NPS production on NPS bioactivity (r) was assessed separately via linear regression mixed-model analysis; NPS bioactivity (r) as a function of NPS production. All analyses, unless otherwise stated, were conducted in R (R version 3.0.2, 2013).

3. Results

3.1. Microbiome structure and composition

A three-dimensional ordination solution for NMDS analysis of *A. blanchardi* microbiome revealed a significant site effect on microbiome structure (MRPP: A = 0.146, $p < 0.0001$; Fig. 1).The variation observed across each of the NMDS axes was explained by environmental parameters. AICc model selection found multiple environmental models which had similar model weights and ΔAICc ≤ 4 (Table A.3) to explain the variation across each NMDS axis. Model averaged parameter estimates on the variation observed across NMDS axis 1 indicated a main effect of N:M, and interaction effects of frog sex × latitude, and frog sex × water SA (Table 1). As N:M increased, axis 1 scores also increased (conditional $R^2 = 0.44$). Female frogs from the northern latitudes had different microbial communities than females from southern latitudes, while male frogs' microbial communities did not differ with latitude (conditional $R^2 = 0.46$; Fig. 2).The microbial communities of males and females responded in opposite ways to water SA but only when surface area was large (≥50,000 m^2); under conditions of small water SA (≤10,000 $m^{2)}$, the microbial communities on the skin of males and females were similar (conditional $R^2 = 0.48$; Fig. 2). Model averaged estimates of parameter influence on the variation in microbial communities across axis 2 revealed significant main effects of conductivity, water SA, and latitude (Table 1). Axis 2 scores increased with latitude and conductivity, but decreased as water SA increased (conditional $R^2 = 0.22$, 0.25, and 0.25 respectively).Variation in microbial communities across axis 3 was associated with an interaction effect of N:M and water SA across sites (conditional $R^2 = 0.34$; Table 1). Axis 3 scores were similar under conditions of high N:M and small water SA and also when N:M was low but water SA was large. Those microbial communities differed from those where N:M was high and water SA was large; however, this later condition was only represented by a single site (Fig. A.2).

Cloning and sequencing of microbial communities across *A. blanchardi* populations revealed that Betaproteobacteria (51.8%) make up the major division of bacteria found on the frogs' skin, followed by Gammaproteobacteria (15.7%; Fig. A.3). Of the 51.8% of Betaproteobacteria sequenced from the clone library, 65% of these were significant indicators of a single site, Ypsilanti, Michigan (J; Table A.6). These Betaproteobacteria were largely represented by members of the order Burkholderiales, including the genera *Acidovorax*, *Aquabacterium*, *Polynucleobacter* and *Pelamonas*, and the genus *Vogesella* of the order Neisseriale. Multiple other indicators of site included *Microbacterium* as an indicator of The Nature Conservancy site (I). *Cloacibacterium* and *Hymenobacter* of the class Flavobacteria and *Zoogloea* of the order Rhodocyclales were indicators of Madison Township Park (C). *Pedobacter* of the class Sphingobacteriia was an indicator of a residential Butler County, Ohio site (A). *Rhizobium*, *Methylobacterium*, and *Ochrobactrum* of the order Rhizobiales (division Alphaproteobacteria), were indicators of another residential Butler Co., Ohio site (B). *Porphyrobacter* of the order Sphingomonadales (division Alphaproteobacteria), was an indicator of residential Butler Co., Ohio site (A) and St. Mary's fish hatchery in Auglaize Co., Ohio (E).

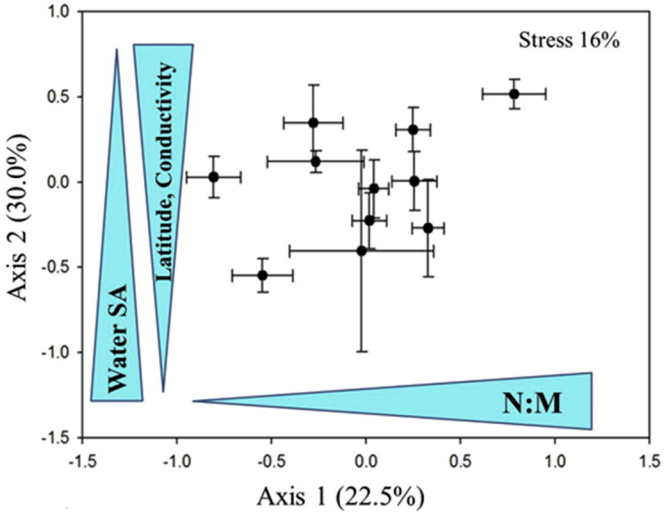

Fig. 1. NMDS ordination of *A. blanchardi* skin-associated microbial communities; two axes of a three-dimensional ordination solution. Goodness of fit, stress = 16%. Points represent site averages with standard error (MRPP site: A = 0.146, $p < 0.0001$). Water surface area ("SA", m^2), latitude, conductivity and the ratio of natural to managed land (N:M, m^2) were predictive of microbial community axis scores of the NMDS ordination (eg. high N:M ratios are correlated with higher axis 1 scores).

3.2. Natural peptide secretions (NPS)

Site significantly predicted NPS production ($F_{(10,76)} = 3.377$, $p = 0.001$; Fig. A.4). We found a single best environmental model to explain the variation in NPS production across sites (Table A.3; AICcω = 0.94). We found an interaction effect of water SA × conductivity on the amount of NPS produced across sites (conditional $R^2 = 0.24$; Table 1; Fig. 3). NPS production was highest from frogs at sites with larger water SA and high conductivity, and NPS production was lower from frogs at sites with smaller water SA and low conductivity. Site did not significantly predict NPS bioactivity (r) ($F_{(10,76)} = 0.593$, $p = 0.815$), and we did not find any water or landscape characteristics that predicted NPS bioactivity (r) (Table A.3; Table 1). Host characteristics, NPS production and NPS bioactivity (r), did not predict microbial community NMDS axis scores (Table A.4 and Table A.5).

A linear regression which examined the influence of NPS production on NPS bioactivity (r) (i.e. Bd growth rate) indicated a marginal positive relationship, meaning as more NPS were produced by the frogs, the faster Bd grew in vitro (Estimate = 4.0×10^{-04}, SE = 2.0×10^{-04}, df = 75, t value = 1.979, $p = 0.051$; conditional $R^2 = 0.04$; Fig. A.5).

4. Discussion

Amphibians have undergone dramatic disease-associated declines in recent years and these declines are expected to increase due to the ease of global transportation and introduction of novel diseases

Table 1
Model averaged parameter estimates, unconditional standard error (SE) of the estimate, and 95% unconditional confidence intervals (CI) of landscape and water characteristics on *Acris blanchardi* immune defense traits across sites in Ohio and Michigan. Only parameters from top models (ΔAICc ≤ 4)are included.

Response	Predictor	Est.	SE	95% CI
Microbial community Axis 1	**N:M**	**0.067**	**0.028**	**0.011** to **0.122**
	Total phosphate	−0.020	0.017	−0.054 to 0.014
	Sex * latitude	**−0.103**	**0.048**	**−0.196** to **−0.001**
	N:M * water SA	-2.0×10^{-06}	1.1×10^{-06}	-4.2×10^{-06} to 2.0×10^{-06}
	Latitude	0.201	0.109	−0.013 to 0.415
	Water	-1.0×10^{-06}	4×10^{-06}	-9.0×10^{-06} to 8.0×10^{-06}
	N:M * total phosphate	−0.005	0.009	−0.023 to 0.013
	Alkalinity * latitude	−0.002	0.002	−0.007 to 0.002
	Sex * water SA	$\mathbf{-4.9 \times 10^{-06}}$	$\mathbf{2.1 \times 10^{-06}}$	$\mathbf{-9.0 \times 10^{-06}}$ to $\mathbf{-8.0 \times 10^{-06}}$
	Sex * N:M	−0.006	0.012	−0.032 to 0.019
Microbial community Axis 2	**Water SA**	$\mathbf{-1.1 \times 10^{-05}}$	$\mathbf{4.8 \times 10^{-06}}$	$\mathbf{-2.0 \times 10^{-05}}$ to $\mathbf{-1.6 \times 10^{-06}}$
	Conductivity	**0.002**	$\mathbf{7.8 \times 10^{-04}}$	$\mathbf{8 \times 10^{-05}}$ to **0.003**
	Water SA × conductivity	3.0×10^{-08}	3.0×10^{-08}	-3.0×10^{-08} to 8.0×10^{-08}
	Total phosphate	−0.012	0.013	−0.038 to 0.015
	Latitude	**0.167**	**0.078**	**0.014** to **0.321**
	N:M	0.024	0.023	−0.020 to 0.068
Microbial community Axis 3	**N:M * water SA**	$\mathbf{-4.8 \times 10^{-06}}$	$\mathbf{9.0 \times 10^{-07}}$	$\mathbf{-6.5 \times 10^{-06}}$ to $\mathbf{-3.0 \times 10^{-06}}$
NPS production (μg/ml per gbw)	**Water * conductivity**	$\mathbf{4.69 \times 10^{-05}}$	$\mathbf{1.38 \times 10^{-05}}$	$\mathbf{1.99 \times 10^{-05}}$ to $\mathbf{7.4 \times 10^{-05}}$
Bd growth rate in vitro[a]	Conductivity	-7.4×10^{-04}	5.7×10^{-04}	-0.002 to 3.8×10^{-04}
	Alkalinity	−0.002	0.002	-0.006 to 8.9×10^{-04}
	Total phosphate	−0.013	0.011	−0.035 to 0.008
	Sex	0.079	0.071	−0.06 to 0.219
	Water SA	-2.0×10^{-06}	4.0×10^{-06}	-9.0×10^{-06} to 5.0×10^{-06}
	N:M	0.012	0.019	−0.026 to 0.050
	Latitude	0.058	0.071	−0.082 to 0.198

[a] Indicates that only the top 10 models are represented and are all ΔAICc ≤ 4. Based on 95% CI, influential parameters are in bold.

(Daszak et al., 2003). This hypothesized increase in pathogen introduction, coupled with changing climate and other anthropogenic environmental stressors make understanding how amphibian immune defense traits are altered by changing environments crucial for successful long-term conservation efforts (Lips et al., 2008; Rohr et al., 2008).This is particularly important for species with small populations which are restricted in their ability to disperse to new habitats, like *A. blanchardi* (Gray and Brown, 2005). Our study has shown that multiple environmental factors including the ratio of natural to managed land, water conductivity, water surface area, and latitude can influence the skin-associated microbiome of *A. blanchardi*. Additionally, we found interactions between frog sex and latitude, frog sex and water surface area, as well as the ratio of natural to managed land and water surface area can all influence the microbiome of this species. These results are in accordance with previous work which has shown inter-population differences in skin microbiome of amphibians (Kueneman et al., 2014), including an experimental study in which

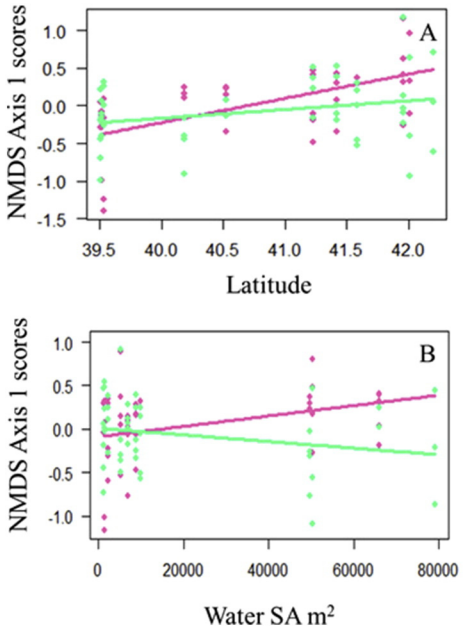

Fig. 2. A. Interaction effect of frog sex and latitude on microbial community NMDS axis 1 scores of *Acris blanchardi* across sites in Ohio and Michigan (conditional $R^2 = 0.46$). Females = pink. Males = aquamarine. B. Interaction effect of frog sex and water surface area ("SA", m^2) on microbial community NMDS axis 1 scores of *Acris blanchardi* across sites in Ohio and Michigan (conditional $R^2 = 0.48$). Females = pink. Males = aquamarine. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

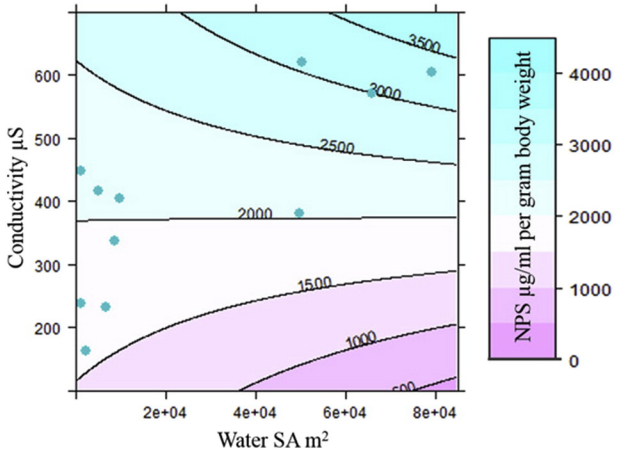

Fig. 3. Interaction effect of water surface area ("SA", m^2) and Conductivity (μS) on NPS production (shading; NPS μg/ml per gram body weight) in *Acris blanchardi* across sites in Ohio and Michigan (conditional $R^2 = 0.24$).

we found that environmental characteristics can drive those differences (Krynak et al., 2015). We also found that the environment altered another important component of immune defenses; the peptides produced by granular glands in the frog's skin (Rollins-Smith et al., 2005). Water surface area and conductivity interacted to influence the amount of NPS produced. We did not find evidence that host characteristics, NPS production and bioactivity, influenced the microbiome which may suggest co-evolution between the microbiome and the host (Bordenstein and Theis, 2015), or it may be that certain unidentified peptides within the natural peptide secretions do affect certain bacteria found within our samples, shaping the microbiome structure in a way we cannot detect with our methodology (Kung et al., 2014). We did find some evidence for a positive relationship between NPS production and growth rate of Bd challenged with NPS from *A. blanchardi*. Across sites, as *A. blanchardi* produced more NPS, Bd growth rate (NPS bioactivity (r)) increased. We found that *A. blanchardi* NPS, regardless of the amount produced, were not able to depress growth of Bd based on our in vitro analysis of bioactivity, which is in agreement with previously published findings (Conlon, 2011).

The hypothesis that the environment may alter microbial community structure is not new; however, few have tested whether the environment alters the skin-associated microbiome of amphibians (Kueneman et al., 2014; Loudon et al., 2014a; McKenzie et al., 2012). Microbial studies conducted in culture have shown that environment affects which bacterial species can persist on a particular media, at differing temperatures, pH, and nutrient concentrations (Vartoukian et al., 2010). Bacterial species compete for space and nutrients in these environments and this in turn can shift the relative proportions of species present (Nichols et al., 2008; Vartoukian et al., 2010). In nature, habitat disruption could cause a change in the local pool of microbial colonists, thereby affecting the microbiome of the amphibian skin (Fitzpatrick and Allison, 2014), or habitat disruption may elicit selection pressure on the relative proportions of the host's microbial colonists. Alternatively, physiological changes in the frog skin could be associated with biological stress resulting from habitat disruption (e.g. mowing of lawns and plowing of fields in more managed lands) and could result in microbiome shifts. Biological stress associated with habitat disruption causes immune suppression across many taxa (Morimoto et al., 2011) and stress from habitat disruption, which can include habitat degradation or other changes in the habitat, such as competitor and predator abundance, can alter physiological traits like corticosterone levels (Homan et al., 2003; Liesenjohann et al., 2013).These physiological changes may make the skin less habitable for some bacterial species, but more habitable for others, shifting the microbiome structure.

We found that frogs from similar habitats had similar microbiome structure; furthermore, environmental conditions of the habitat correlated with microbiome structure. For example, the ratio of natural to managed land influenced the variation in frog microbiome structure across NMDS axis 1. The microbiome on frogs from populations in more natural habitats was most similar to the microbiome on frogs from other populations in more natural habitats; however, these microbiomes differed from the microbiomes of frogs from populations in more managed habitats. The observed relationship between land use and amphibian microbiome agrees with studies which have found land use influences the microbial communities in soil and water (Carrino-Kyker et al., 2011; Yao et al., 2000), suggesting that the differences in frog skin microbial communities could be due to differences in available colonizing microbes, rather than differences in frog physiology. Alternatively the environment external to the frog host may select for particular bacterial taxa persistence on the host frog's skin (Vartoukian et al., 2010).

We also found water conductivity was associated with variation in the amphibian microbiome across sites; frogs from ponds with similar conductivity had similarly structured microbiomes. Pond conductivity is affected by both natural and anthropogenic factors (Carrino-Kyker et al., 2011). Furthermore, residential and agricultural runoff can alter microbial communities in vernal pools (Carrino-Kyker et al., 2011). Water conductivity could therefore be directly altering the relative proportions of bacterial taxa on the frogs' skin though selective pressures or indirectly by altering the bacterial taxa available in the habitat to colonize the amphibian. Residential and agricultural run-off alter traditional measures of amphibian fitness (Gallagher et al., 2014; Hua and Pierce, 2013), but our results indicate that additional measures of amphibian health, including the immune defense traits need be examined.

The relationship between water surface area and microbiome variation indicate that the size of the pond can affect microbiome structure (Fig. 1). We found that water surface area also interacted with the ratio of natural to managed land to affect the *A. blanchardi* skin microbiome (Fig. A.2). We found greater inter-pond variation in frog microbiome structure between large water bodies than between small water bodies. This leads us to suggest that this variability is influenced by surrounding terrestrial land use or differences in relative spatial heterogeneity of pond water chemistry. Large ponds may display greater habitat heterogeneity and localized differences may exist in water chemistry, which could affect within pond variability in frog skin microbial communities. Small ponds may display lesser habitat heterogeneity, and therefore less within-site variability in skin microbiome. Differences in surrounding land use and within pond spatial heterogeneity between large and small ponds could influence differences in variability in frog microbiome structure. Although the cause of differences in variability between small and large ponds is unknown, our data suggest that surrounding land use, which is known to affect water chemical quality, may be partly responsible for these differences.

The microbiome structure of *A. blanchardi* skin also changed with latitude. The latitudinal differences in microbiome of *A. blanchardi* may reflect differences in pathogen resistance among populations across the species' range, particularly in northern latitudes (Gray and Brown, 2005). Declines have resulted in *A. blanchardi* being listed as a species of concern in Michigan, while declines have lessened in Ohio in recent years (Lehtinen and Witter, 2014). If microbiome structural differences caused depressed immune function, this may have led to the declines observed in the northern latitudes including Michigan and Ohio.

We also observed an interaction between the frogs' sex and latitude and frogs' sex and water surface area indicating that the microbiome response is partially dependent on the sex of the individual animal. This differential response in microbiome structure across environments between the sexes may help to explain the sex ratio differences that have been documented across populations; males largely outnumbering females or females largely outnumbering males at particular locations (Gray, 1983; Reeder et al., 1998). Previous studies have linked amphibian sex ratio shifts to chemical contamination of the habitat (Boegi et al., 2003; Hayes et al., 2010; Reeder et al., 2005). However, our results suggest an alternative hypothesis for interpopulation variation in sex ratios. If the differences in microbiome observed in our study do affect frog immune defense (Harris et al., 2009), then males and females may differ in pathogen resistance at different latitudes and among different-sized ponds. Further, differential mortality of the sexes due to differences in pathogen resistance could cause interpopulation variation in sex ratio.

Although microbiome structure differs between populations, it is possible that the function of different microbial communities is the same (Lear et al., 2014). In the present study, we documented the structure of microbial communities, but did not conduct functional experiments to determine if particular skin microbiome structures confer stronger immune defense than other skin microbiome structures. Culture-based studies have found that particular microbial

taxa produce metabolites which are capable of providing resistance to amphibian pathogens (Becker et al., 2009; Brucker et al., 2008; Harris et al., 2006). However, relative to the number of taxa estimated to be associated with amphibian skin from studies utilizing sequencing approaches (McKenzie et al., 2012), few taxa have been investigated in pure culture in terms of disease resistance due to limitations of culture-based techniques. Additionally, it has been discovered that once microbial taxa are incorporated into a community, emergent metabolites can be produced, which are not produced by individual microbial taxa as found in pure culture (Loudon et al., 2014b; Raes and Bork, 2008; Xavier, 2011); therefore microbial taxa functionality needs to be investigated on a community basis. Our study provides evidence that the relative proportions of bacterial taxa present on the skin of *A. blanchardi* are affected by environmental characteristics; however, functional properties of these communities across environments, as related to pathogen resistance, will require meta-transcriptomic techniques and will be an important next step in amphibian conservation research.

Our study also found that the environment influenced other components of the *A. blanchardi* immune defense system: the production of NPS. This is similar to what we found during an experimental study which showed environmental variation in larval habitat pH and degree of pond shading had long-term (post-metamorphic) effects on antimicrobial peptide production in *Rana catesbeiana* (Krynak et al., 2015). Predators and competitors also alter antimicrobial peptide production in amphibians (Groner et al., 2013; Groner et al., 2014). We found that environmental variation in conductivity and water surface area interacted to affect NPS production in *A. blanchardi*. Specifically, NPS production increased with water surface area and conductivity. The cause of this pattern is unknown, however, it is possible that larger water bodies have a larger surface water catchment within the surrounding landscape, and this leads to greater surface water runoff into these ponds. This would increase the concentration of chemical constituents within the pond, leading to greater stress on individual animals and possibly higher NPS production. This pattern may also reflect other unmeasured factors which may influence NPS production, such as disease presence or unmeasured chemical contamination that may be interacting with these landscape characteristics (Rollins-Smith, 2009).

Surprisingly, we found that NPS production was positively associated with Bd growth rate in vitro, though this effect is marginal. Other studies have found species which produce more antimicrobial peptides, or particular types of antimicrobial peptides, are more protected from Bd (Rollins-Smith and Conlon, 2005; Tennessen et al., 2009); however, in the case of *A. blanchardi*, Bd growth was not inhibited by the NPS (Conlon, 2011), regardless of the amount of NPS produced. The effect size of the relationship between NPS production and NPS bioactivity in our study is small; however, the importance of this potential relationship gives cause for attention. A positive relationship between NPS production and Bd growth rate may be particularly detrimental to amphibian populations if NPS production, which has presumably evolved to provide broad pathogen resistance, instead stimulates the growth of this non-native pathogen (Rollins-Smith et al., 2005; Weldon et al., 2004). Our study indicates that NPS of some amphibian species or populations may actually promote an increase in Bd zoospore formation. Though our study suggests that NPS from *A. blanchardi* do not provide effective protection against Bd, they may reduce growth rate or cure other pathogen infections of the skin, and therefore understanding the influence of environmental conditions on NPS production is important for understanding the role of these proteins on disease resistance of *A. blanchardi* populations.

Lastly, the lack of latitudinal effect on *A. blanchardi* NPS production and bioactivity along the species' declining range can be explained by multiple hypotheses. This may suggest that NPS in this species are not bioactive against any pathogens which may be associated with latitudinal declines in the species and therefore, we do not see evidence of selection on these traits. It also could be that historic *A. blanchardi* declines in the northern regions of the species' geographic range are not related to disease (Steiner and Lehtinen, 2008). It may also be that these traits are not genetically determined, but are instead environmentally induced by factors not associated with latitude, or it could be that environmental characteristics interact with the genetic expression of these immune defense traits. An interaction between a population's genes and the environment could lower heritability of traits (Dutilleul et al., 2015) and thereby reduce heritable expression of disease resistance by NPS. In other words, environmental factors may limit a population's ability to evolve resistance to pathogens.

5. Conservation implications

In light of the fact that future pathogen introduction is inevitable due to the ease of global transportation (Daszak et al., 2003), it is imperative that we improve our understanding of what environmental conditions affect traits known to provide amphibians with broad pathogen resistance. By knowing what aspects of the environment may alter these traits, we can modify our land management practices to better protect amphibian health. Our results indicate that particular landscape and water characteristics are correlated with between-population differences in the skin-associated immune defense traits of *A. blanchardi*. While our results do not provide causal links, they do provide informed motivation for experimental manipulations to further tease apart potentially important indirect effects of anthropogenic change on amphibian populations. Future studies are also needed to assess how other potentially less benign anthropogenic environmental characteristics such as eutrophication, pesticide usage, and climate change may be altering these immune defense traits. We must first understand the direct effects of the environment on amphibian immune defense traits across species, but then we must also examine potential interactions between environmental and genetic factors on the expression of immune defense traits to protect amphibians from disease threats in the future.

Role of the funding source

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Appendix A

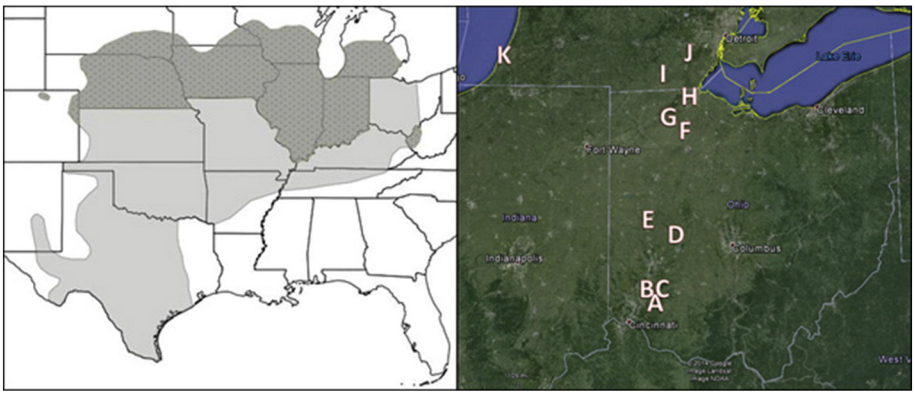

Fig. A.1. Left panel: Geographic range of *Acris blanchardi* and areas of documented decline are shown in dotted dark gray (Gamble et al. 2008); additionally, a range contraction has been reported from east to west in the state of Ohio (Lehtinen and Skinner, 2006). Right panel: Survey site locations in Ohio and Michigan across a portion of *Acris blanchardi*'s declining range (source: lat 40.405760 long −82.930501. *Google Earth*. May 9 2013. February 11, 2015). Site identification by county: A–C: Butler Co.; D: Champaign Co.; E: Auglaize Co.; F–H: Wood Co.; I: Lenawee Co.; J: Washtenaw Co.; and K: Berrien Co.

Table A.1
Survey site water characteristics and number of individual *Acris blanchardi* sampled.

Site	Co., State	Animals sampled (N = ♂,♀)	pH	$CaCO_3$ (mg/L)	Conductivity (μS)	Total phosphate (mg/L)	N:M	Water (m^2)
A. Mynheir Site	Butler Co., OH	5,3	9.75	100	239	5	0.2	1191.4
B. Williamson Site	Butler Co., OH	5,5	9.74	100	232	0	0	6871.1
C. Madison Township Park	Butler Co., OH	5,3	8.06	180	448	19	0.7	1371.4
D. Kiser Lake	Champaign Co., OH	5,5	9.12	200	380	10	2.5	49,753.6
E. St. Mary's	Auglaize Co., OH	2,5	9.45	105	570	3	0	65,983.1
F. Cricket Frog Cove	Wood Co., OH	5,5	8.9	90	163	5	7.7	2270.5
G. Neal's Site	Wood Co., OH	5,5	8.36	180	337	0.1	0.4	8787
H. W.W. Knight Nature Center	Wood Co., OH	5,1	7.9	90	405	15	0.3	9941.6
I. The Nature Conservancy,	Lenawee Co., MI	5,5	9.02	120	417	0	10.7	5242.5
J. Ypsilanti	Washtenaw Co., MI	3,1	8.24	180	604	0	6.3	79,206.2
K. Grand Mere	Berrien Co., MI	3,4	8.03	200	619	0	2.3	50,344.0

Table A.2
Response variables (NMDS axis 1, 2, and 3 scores, NPS production, NPS bioactivity (r) were modeled as a function of each of the following predictors.

Model number	Predictors
1	N:M
2	Alkalinity
3	Conductivity
4	Total phosphate
5	Latitude
6	Water SA
7	Sex
8	N:M + water SA
9	N:M * water SA
10	Water SA + conductivity
11	Water SA * conductivity
12	Water SA + alkalinity
13	Water SA * alkalinity
14	N:M + alkalinity
15	N:M * alkalinity
16	Latitude + alkalinity
17	Latitude * alkalinity
18	Sex * N: M
19	Sex * conductivity
20	Sex * alkalinity
21	Sex * total phosphate
22	Sex * water SA
23	Sex * latitude

Table A.3
Top models explaining environmental influence on *Acris blanchardi* immune defense traits across sites in Ohio and Michigan based on AICc ranking. Microbial community axis scores are based on a three dimensional NMDS ordination solution and describe the variation seen across each axis. Models were capped at six parameters (K = 6) because of the small sample size (N = 11 sites). AICc score, change in AICc (ΔAICc), and the AICc model weight (ω) for each model are shown for the top models (ΔAICc ≤ 4) for each response variable. The top 10 models are shown for Bd growth rate in vitro (r) and are all ΔAICc < 4.

Response	Model	K	AICc	ΔAICc	AICcω
Microbial community axis 1	N:M	4	129.54	0.00	0.20
	N:M + total phosphate	5	130.77	1.23	0.11
	Sex * latitude	6	130.82	1.28	0.11
	N:M * water SA	6	131.32	1.77	0.08
	Latitude	4	131.57	2.03	0.07
	N:M + water	5	131.80	2.25	0.07
	Total phosphate	4	132.42	2.88	0.05
	N:M * total phosphate	6	132.81	3.26	0.04
	Alkalinity * latitude	5	132.89	3.35	0.04
	Sex * water SA	6	132.96	3.41	0.04
	Sex * N:M	6	133.04	3.49	0.04
Microbial community axis 2	Water SA + conductivity	5	132.25	0.00	0.32
	Water SA * conductivity	6	133.62	1.37	0.16
	Water SA	4	134.76	2.51	0.09
	Water SA + total phosphate	5	135.77	3.52	0.06
	Latitude	4	135.81	3.56	0.05
	N:M + water SA	5	135.85	3.60	0.05
Microbial community axis 3	N:M * water SA	6	140.47	0.00	0.98
NPS production (μg/ml per gbw)	Water SA*conductivity	6	1257.01	0.00	0.94
Bd growth rate in vitro	Conductivity	4	183.45	0.00	0.12
	Alkalinity	4	183.46	0.02	0.11
	Total phosphate	4	183.95	0.51	0.09
	Sex	4	184.12	0.67	0.08
	Water SA	4	184.73	1.28	0.06
	N:M	4	184.83	1.38	0.06
	Latitude	4	184.84	1.39	0.06
	Alkalinity + latitude	5	184.89	1.44	0.06
	Water SA + total phosphate	5	185.13	1.68	0.05
	Water + conductivity	5	185.63	2.18	0.04

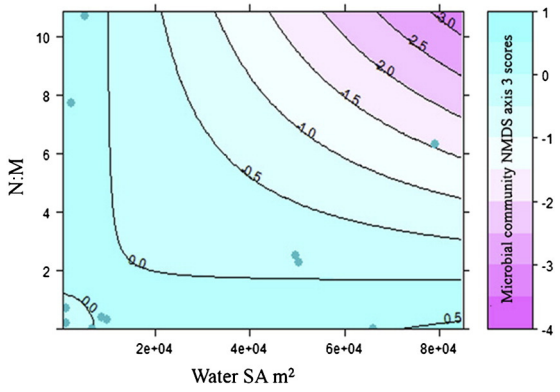

Fig. A.2. Interaction effects of the ratio of natural to managed terrestrial habitat (N:M) and water surface area ("SA", m^2) on microbial community NMDS axis 3 scores (represented by color shading) of *Acris blanchardi* across sites in Ohio and Michigan (conditional $R^2 = 0.34$).

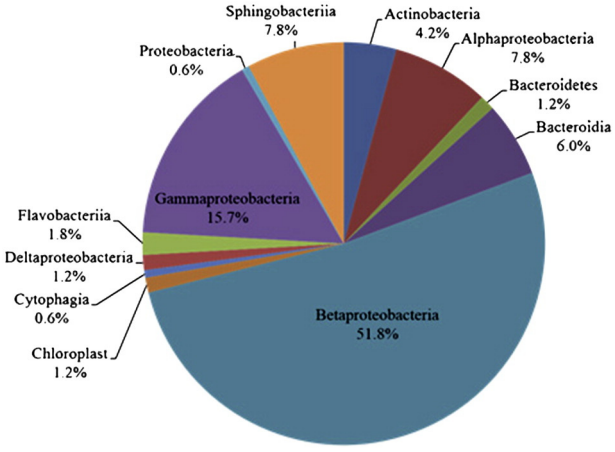

Fig. A.3. Clone library of *Acris blanchardi* skin-associated bacteria. The percent of the clone library represented by each taxonomic group is shown. (N = 169). Of Betaproteobacteria cloned (N = 86 clones), 65.1% were significant indicators of site J. Ypsilanti, MI.

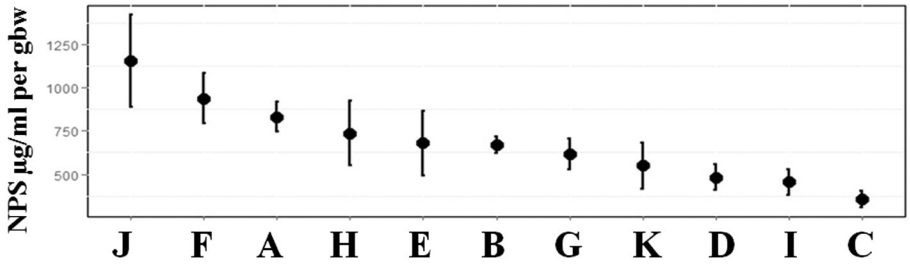

Fig. A.4. NPS production (in the form of natural peptide mixtures) standardized by gram body weight (gbw) of *Acris blanchardi* across sites in Ohio and Michigan. Letters correspond to Fig. A.1 site locations.

Table A.4
Models used to assess host influence (NPS production and NPS bio-activity (r)) on *Acris blanchardi* skin-associated microbial community NMDS axis scores across sites in Ohio and Michigan based on AICc ranking. AICc score, change in AICc (ΔAICc), and the AICc model weight (ω) for each model are shown for each response variable.

Response	Model	K	AICc	ΔAICc	AICcω
Microbial community Axis 1	NPS bioactivity (r)	4	133.99	0.00	0.42
	NPS production	4	134.16	0.18	0.39
	NPS production + r	5	136.17	2.19	0.14
	NPS production * r	6	138.47	4.48	0.05
Microbial community Axis 2	NPS production	4	138.39	0.00	0.45
	NPS bioactivity (r)	4	138.89	0.50	0.35
	NPS production + r	5	140.54	2.15	0.15
	NPS production * r	6	142.78	4.38	0.05
Microbial community Axis 3	NPS bioactivity (r)	4	154.25	0.00	0.41
	NPS production * r	6	154.80	0.55	0.31
	NPS production	4	156.29	2.04	0.15
	NPS production + r	5	156.41	2.16	0.14

Table A.5
Model averaged parameter estimates, unconditional standard error (SE) of the estimate, and 95% unconditional confidence intervals (CI) of host characteristics on *Acris blanchardi* skin-associated microbial community NMDS axis scores across sites in Ohio and Michigan.

Response	Predictor	Est.	SE	95% CI
Microbial community Axis 1	NPS production	5.0×10^{-05}	1.6×10^{-04}	-2.6×10^{-04} to 3.6×10^{-04}
	NPS bioactivity (r)	0.04	0.07	−0.10 to 0.18
	NPS production * r	4.0×10^{-05}	2.7×10^{-04}	-5.7×10^{-04} to 5.0×10^{-04}
Microbial community Axis 2	NPS production	-1.2×10^{-04}	1.6×10^{-04}	-4.4×10^{-04} to 2.0×10^{-04}
	NPS bioactivity (r)	0.018	0.078	−0.14 to 0.17
	NPS production * r	7.7×10^{-05}	2.9×10^{-04}	-4.9×10^{-04} to 6.4×10^{-04}
Microbial community Axis 3	NPS production	-3.2×10^{-05}	1.8×10^{-04}	-3.8×10^{-04} to 3.2×10^{-04}
	NPS bioactivity (r)	0.12	0.08	−0.04 to 0.28
	NPS production * r	6.1×10^{-04}	3.0×10^{-04}	-1.0×10^{-05} to 1.2×10^{-03}

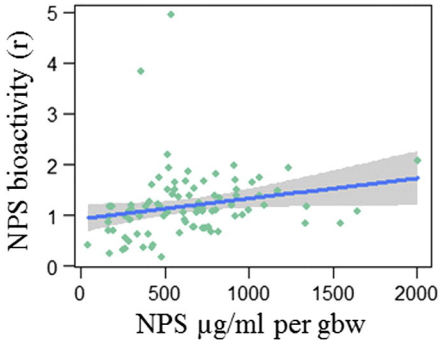

Fig. A.5. NPS bioactivity (r) as a function of NPS produced (standardized by gram body weight) from *Acris blanchardi* across sites in Ohio and Michigan (Estimate = 4.0×10^{-04}, SE = 2.0×10^{-04}, df = 75, p = 0.051; conditional $R^2 = 0.04$). 95% confidence interval is displayed as the shaded region.

Table A.6
The sequence similarity of clones (out of 169 total) created from skin swabs of *Acris blanchardi* using primers 338f and 926r.

Clone ID	Clone accession ID	Best match	% ID	Division/phylum	Fragment size (bp)		Indicator Site ID ($p < 0.05$)
					338f	926r	
A1	LN794355	*Stenotrophomonas*	100	Gammaproteobacteria	44.9	221.1	
A2	LN794356	*Pedobacter*	100	Sphingobacteriia	158.1	381.6	
A3	LN794357	*Pedobacter*	100	Sphingobacteriia	158.4	381.7	
A4	LN794358	*Pedobacter*	100	Sphingobacteriia	158.1	381.5	
A5	LN794359	*Cloacibacterium*	100	Flavobacteriia	578.9	577.0	C
A6	LN794360	Burkholderiales	100	Betaproteobacteria	46.5	534.8	J
A7	LN794361	*Pedobacter*	100	Sphingobacteriia	157.9	381.7	
A8	LN794362	*Vogesella*	100	Betaproteobacteria	45.4	534.5	J
A10	LN794363	Burkholderiales	98	Betaproteobacteria	46.6	534.7	J
A11	LN794364	*Rhizobium*	100	Alphaproteobacteria	45.0	509.9	B
A12	LN794365	*Pseudoxanthomonas*	100	Gammaproteobacteria	45.0	221.0	
A13	LN794366	Burkholderiales	95	Betaproteobacteria	45.7	535.4	
A14	LN794367	*Stenotrophomonas*	100	Gammaproteobacteria	44.9	221.0	
A15	LN794368	*Stenotrophomonas*	99	Gammaproteobacteria	44.9	221.0	
A16	LN794369	Proteobacteria	100	Gammaproteobacteria	46.5	218.7	
A17	LN794370	Actinomycetales	96	Actinobacteria	537.7	17.8	
A18	LN794371	*Sphingobium*	99	Alphaproteobacteria	45.2	511.6	
A19	LN794372	*Microbacterium*	100	Actinobacteria	375.6	192.0	I
A20	LN794373	*Stenotrophomonas*	99	Gammaproteobacteria	45.0	221.0	
A21	LN794374	*Aquabacterium*	100	Betaproteobacteria	45.1	535.0	J
A22	LN794375	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	221.0	
A23	LN794376	Burkholderiales	98	Betaproteobacteria	46.5	534.9	J
A24	LN794377	*Vogesella*	100	Betaproteobacteria	45.5	534.5	J
A25	LN794378	Burkholderiales	99	Betaproteobacteria	46.5	534.8	J
A26	LN794379	*Alistipes*	100	Bacteroidia	158.2	217.8	
A27	LN794380	Bradyrhizobiaceae	100	Alphaproteobacteria	44.9	274.3	
A28	LN794381	*Bacteroides*	100	Bacteroidia	157.6	381.3	
A29	LN794382	*Aquabacterium*	100	Betaproteobacteria	45.2	535.0	
A30	LN794383	*Vogesella*	100	Betaproteobacteria	45.7	534.6	J
A31	LN794384	*Parabacteroides*	100	Bacteroidia	77.1	145.7	
A32	LN794385	Burkholderiales	100	Betaproteobacteria	46.5	535.0	
A33	LN794386	Burkholderiales	99	Betaproteobacteria	46.6	534.8	J
A34	LN794387	*Aquabacterium*	100	Betaproteobacteria	45.3	534.8	J
A35	LN794388	*Acidovorax*	100	Betaproteobacteria	45.6	535.4	
A36	LN794389	*Vogesella*	92	Betaproteobacteria	45.6	535.0	
A37	LN794390	Burkholderiales	99	Betaproteobacteria	46.5	535.0	J
A39	LN794391	*Pedobacter*	100	Sphingobacteriia	158.2	381.7	
A40	LN794392	*Stenotrophomonas*	90	Gammaproteobacteria	44.9	220.9	
A41	LN794393	Actinomycetales	97	Actinobacteria	581.1	579.9	
A42	LN794394	*Vogesella*	100	Betaproteobacteria	45.6	534.9	J
A43	LN794395	*Stenotrophomonas*	100	Gammaproteobacteria	44.9	221.0	
A44	LN794396	*Stenotrophomonas*	100	Gammaproteobacteria	45.1	221.1	
A45	LN794397	Burkholderiales	98	Betaproteobacteria	47.0	534.9	J
A46	LN794398	Burkholderiales	100	Betaproteobacteria	46.5	534.6	J
A47	LN794399	*Variovorax*	96	Betaproteobacteria	45.7	535.2	
A48	LN794400	*Bradyrhizobium*	92	Alphaproteobacteria	46.8	275.4	
A49	LN794401	*Aquabacterium*	100	Betaproteobacteria	45.2	534.0	J
A50	LN794402	Comamonadaceae	100	Betaproteobacteria	45.6	534.9	J
A51	LN794403	*Aquabacterium*	100	Betaproteobacteria	45.0	534.6	J
A52	LN794404	*Aquabacterium*	93	Betaproteobacteria	45.1	535.1	
A53	LN794405	*Dechloromonas*	98	Betaproteobacteria	46.9	536.7	
A54	LN794406	Burkholderiales	92	Betaproteobacteria	46.5	534.9	J
A55	LN794407	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	221.0	
A56	LN794408	*Bacteroides*	100	Bacteroidia	158.6	381.5	
A57	LN794409	Burkholderiales	97	Betaproteobacteria	46.5	534.8	J
A58	LN794410	*Pelomonas*	100	Betaproteobacteria	45.1	534.9	J
A59	LN794411	*Aquabacterium*	100	Betaproteobacteria	45.1	535.1	
A60	LN794412	Chloroplast	100	Chloroplast	563.4	562.6	
A61	LN794413	*Stenotrophomonas*	99	Gammaproteobacteria	42.8	221.0	
A62	LN794414	Burkholderialesincertaesedis	93	Betaproteobacteria	46.5	534.7	J
A63	LN794415	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	221.2	
A64	LN794416	Desulfobacteraceae	100	Deltaproteobacteria	587.0	585.8	
A65	LN794417	Bacteroidetes	100	Bacteroidia	577.7	577.1	A
A66	LN794418	*Phenylobacterium*	100	Alphaproteobacteria	558.5	557.7	
A67	LN794419	*Comamonas*	100	Betaproteobacteria	45.7	535.4	
A68	LN794420	Comamonadaceae	90	Betaproteobacteria	46.4	534.9	J
A70	LN794421	*Stenotrophomonas*	99	Gammaproteobacteria	44.9	221.0	
A71	LN794422	Deltaproteobacteria	88	Proteobacteria	137.5	448.7	
A72	LN794423	*Acidovorax*	100	Betaproteobacteria	45.6	535.3	
A73	LN794424	Comamonadaceae	100	Betaproteobacteria	45.5	537.3	C
A74	LN794425	*Aeromonas*	100	Gammaproteobacteria	367.6	219.1	
A75	LN794426	Burkholderiales	100	Betaproteobacteria	46.8	534.8	J
A76	LN794427	*Pedobacter*	97	Sphingobacteriia	381.7	158.2	A

(continued on next page)

Table A.6 (*continued*)

Clone ID	Clone accession ID	Best match	% ID	Division/phylum	Fragment size (bp)		Indicator Site ID ($p < 0.05$)
					338f	926r	
A77	LN794428	Burkholderiales	97	Betaproteobacteria	46.4	534.9	J
A78	LN794429	*Sanguibacter*	100	Actinobacteria	566.8	566.0	
A79	LN794430	Burkholderiales	98	Betaproteobacteria	45.0	534.8	J
A81	LN794431	Phyllobacteriaceae	94	Alphaproteobacteria	44.9	74.0	
A82	LN794432	Burkholderiales	97	Betaproteobacteria	46.4	534.8	J
A84	LN794433	*Cloacibacterium*	100	Flavobacteriia	579.1	577.7	
A85	LN794434	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	220.9	
A86	LN794435	Betaproteobacteria	91	Betaproteobacteria	45.5	534.0	
A87	LN794436	*Acidovorax*	100	Betaproteobacteria	45.6	535.5	
A88	LN794437	*Aquabacterium*	100	Betaproteobacteria	44.9	534.9	J
A89	LN794438	*Aquabacterium*	100	Betaproteobacteria	44.8	534.9	J
A90	LN794439	Burkholderiales	100	Betaproteobacteria	46.4	534.5	J
A91	LN794440	*Porphyrobacter*	97	Alphaproteobacteria	46.5	511.1	
A92	LN794441	Burkholderiales*incertaesedis*	92	Betaproteobacteria	45.1	534.7	J
A93	LN794442	*Bacteroides*	100	Bacteroidia	158.8	381.4	
A94	LN794443	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	221.0	
A95	LN794444	*Pedobacter*	100	Sphingobacteriia	158.1	381.7	
A96	LN794445	Comamonadaceae	99	Betaproteobacteria	45.5	534.6	J
A97	LN794446	*Aquabacterium*	94	Betaproteobacteria	45.0	534.7	J
A98	LN794447	Bacteroidetes	95	Bacteroidetes	578.7	578.1	C
A100	LN794448	Comamonadaceae	100	Betaproteobacteria	45.1	448.1	
A101	LN794449	*Aquabacterium*	99	Betaproteobacteria	45.0	534.9	J
A102	LN794450	Burkholderiales	94	Betaproteobacteria	46.5	534.4	J
A103	LN794451	Burkholderiales	93	Betaproteobacteria	46.8	534.7	J
A104	LN794452	*Zoogloea*	100	Betaproteobacteria	45.5	537.2	C
A105	LN794453	Proteobacteria	100	Betaproteobacteria	46.6	534.9	J
A106	LN794454	Burkholderialesincertaesedis	96	Betaproteobacteria	46.6	535.0	
A107	LN794455	*Parabacteroides*	100	Bacteroidia	77.1	145.7	
A108	LN794456	Burkholderiales	90	Betaproteobacteria	46.5	534.8	J
A109	LN794457	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	221.0	
A110	LN794458	*Acidovorax*	100	Betaproteobacteria	584.4	583.8	J
A111	LN794459	Comamonadaceae	100	Betaproteobacteria	44.9	535.3	
A112	LN794460	*Variovorax*	98	Betaproteobacteria	45.6	535.3	
A113	LN794461	Betaproteobacteria	87	Betaproteobacteria	46.5	534.8	J
A114	LN794462	*Stenotrophomonas*	97	Gammaproteobacteria	45.0	221.0	
A115	LN794463	Comamonadaceae	100	Betaproteobacteria	45.2	448.1	
A116	LN794464	Burkholderiales	98	Betaproteobacteria	46.6	535.0	J
A118	LN794465	Deltaproteobacteria	95	Deltaproteobacteria	586.2	585.4	J
A119	LN794466	Burkholderiales	99	Betaproteobacteria	45.1	534.9	J
A120	LN794467	*Dechloromonas*	99	Betaproteobacteria	45.6	535.6	
A122	LN794468	Burkholderiales	99	Betaproteobacteria	46.5	534.7	J
A123	LN794469	Burkholderiales	97	Betaproteobacteria	46.5	534.9	J
A124	LN794470	*Stenotrophomonas*	98	Gammaproteobacteria	45.1	220.9	
A125	LN794471	Burkholderiales	100	Betaproteobacteria	45.6	534.8	J
A126	LN794472	*Ochrobactrum*	100	Alphaproteobacteria	45.0	509.6	B
A127	LN794473	*Vogesella*	99	Betaproteobacteria	45.7	534.8	J
A128	LN794474	Chitinophagaceae	100	Sphingobacteriia	414.7	17.8	
A129	LN794475	Chitinophagaceae	89	Sphingobacteriia	46.0	145.7	
A130	LN794476	*Aquabacterium*	100	Betaproteobacteria	45.0	535.0	
A131	LN794477	*Methylobacterium*	100	Alphaproteobacteria	45.0	509.3	B
A132	LN794478	*Bradyrhizobium*	99	Alphaproteobacteria	46.7	275.5	
A133	LN794479	Erythrobacteraceae	100	Alphaproteobacteria	45.4	510.0	A, E
A134	LN794480	*Bacteroides*	100	Bacteroidia	157.6	381.3	
A135	LN794481	*Stenotrophomonas*	100	Gammaproteobacteria	44.9	221.2	
A136	LN794482	Burkholderiales	99	Betaproteobacteria	46.4	534.8	J
A137	LN794483	*Pedobacter*	100	Sphingobacteriia	158.2	381.7	
A138	LN794484	*Acidovorax*	100	Betaproteobacteria	45.6	535.5	
A139	LN794485	*Aquabacterium*	93	Betaproteobacteria	45.2	535.2	
A140	LN794486	*Vogesella*	98	Betaproteobacteria	85.02	500.19	J
A141	LN794487	Actinomycetales	100	Actinobacteria	98.0[a]	476.0[a]	
A142	LN794488	*Stenotrophomonas*	96	Gammaproteobacteria	45.0	221.0	
A143	LN794489	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	221.1	
A144	LN794490	*Bacteroides*	100	Bacteroidia	158.7	381.6	
A145	LN794491	*Delftia*	90	Betaproteobacteria	45.6	221.0	
A146	LN794492	*Pedobacter*	95	Sphingobacteriia	157.9	381.7	
A147	LN794493	Burkholderiales	100	Betaproteobacteria	46.6	534.8	J
A148	LN794494	Burkholderiales	97	Betaproteobacteria	46.7	534.8	J
A149	LN794495	Burkholderiales	97	Betaproteobacteria	46.5	534.8	J
A150	LN794496	*Aquabacterium*	100	Betaproteobacteria	45.3	535.0	
A151	LN794497	*Hymenobacter*	100	Cytophagia	578.4	577.4	C
A153	LN794498	*Stenotrophomonas*	100	Gammaproteobacteria	45.0	221.1	
A154	LN794499	*Vogesella*	100	Betaproteobacteria	367.0	218.8	
A155	LN794500	Microbacteriaceae	93	Actinobacteria	374.7	192.1	I
A156	LN794501	Comamonadaceae	85	Betaproteobacteria	45.6	535.5	
A157	LN794502	*Stenotrophomonas*	99	Gammaproteobacteria	44.8	220.9	

Table A.6 (*continued*)

Clone ID	Clone accession ID	Best match	% ID	Division/phylum	Fragment size (bp) 338f	Fragment size (bp) 926r	Indicator Site ID ($p < 0.05$)
A158	LN794503	Burkholderiales	98	Betaproteobacteria	46.4	534.8	J
A159	LN794504	*Pedobacter*	99	Sphingobacteriia	158.2	381.7	
A160	LN794505	*Aquabacterium*	100	Betaproteobacteria	45.0	535.0	J
A161	LN794506	*Porphyrobacter*	100	Alphaproteobacteria	46.3	510.9	A, E
A162	LN794507	*Bacteroides*	100	Bacteroidia	158.6	381.6	
A164	LN794508	Burkholderiales	97	Betaproteobacteria	46.4	534.9	J
A165	LN794509	Bacteroidetes	100	Bacteroidetes	549.8	17.2	C
A166	LN794510	*Pedobacter*	100	Sphingobacteriia	158.3	381.6	
A167	LN794511	Bacteroidetes	100	Betaproteobacteria	46.4	535.3	
A168	LN794512	*Aquabacterium*	100	Betaproteobacteria	45.1	535.0	
A170	LN794513	*Flavobacterium*	100	Flavobacteriia	44.4	379.6	
A171	LN794514	Streptophyta	100	Chloroplast	96.0[a]	413.0[a]	
A172	LN794515	*Stenotrophomonas*	99	Gammaproteobacteria	44.9	221.0	
A173	LN794516	*Novosphingobium*	100	Alphaproteobacteria	45.7	511.1	
A175	LN794517	*Rhodococcus*	100	Actinobacteria	492.1	74.3	
A176	LN794518	*Vogesella*	100	Betaproteobacteria	45.5	534.8	J
A177	LN794519	Burkholderiales	100	Betaproteobacteria	46.5	534.7	J
A178	LN794520	*Polynucleobacter*	100	Betaproteobacteria	46.8	534.5	J

Identification is based upon comparison to NCBI database entries using the FASTA program (National Center for Biotechnology Information).The percent identity (% ID) to best match is shown. Fragment size in base pairs (bp) generated using *MboI* restriction enzyme. Indicator species analysis based on community profiles. Letters designate sites with specific bacterial taxa.

[a] Predicted TRF based on *MboI* cut site. Actual TRF not available.

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