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ORIGINAL ARTICLE

Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted *Bd*-inhibitory function

Molly C Bletz[1], RG Bina Perl[1], Bianca TC Bobowski[1], Laura M Japke[1], Christoph C Tebbe[2], Anja B Dohrmann[2], Sabin Bhuju[3], Robert Geffers[3], Michael Jarek[3] and Miguel Vences[1]
[1]*Zoologisches Institut, Technische Universität Braunschweig, Braunschweig, Germany;* [2]*Institut für Biodiversität, Thünen Institut für Ländliche Räume, Wald und Fischerei, Braunschweig, Germany and* [3]*Genomanalytik, Helmholtz Zentrum für Infektionsforschung, Braunschweig, Germany*

Host-associated microbiomes are increasingly recognized to contribute to host disease resistance; the temporal dynamics of their community structure and function, however, are poorly understood. We investigated the cutaneous bacterial communities of three newt species, *Ichthyosaura alpestris, Lissotriton vulgaris* and *Triturus cristatus,* at approximately weekly intervals for 3 months using 16S ribosomal RNA amplicon sequencing. We hypothesized cutaneous microbiota would vary across time, and that such variation would be linked to changes in predicted fungal-inhibitory function. We observed significant temporal variation within the aquatic phase, and also between aquatic and terrestrial phase newts. By keeping *T. cristatus* in mesocosms, we demonstrated that structural changes occurred similarly across individuals, highlighting the non-stochastic nature of the bacterial community succession. Temporal changes were mainly associated with fluctuations in relative abundance rather than full turnover of bacterial operational taxonomic units (OTUs). Newt skin microbe fluctuations were not correlated with that of pond microbiota; however, a portion of community variation was explained by environmental temperature. Using a database of amphibian skin bacteria that inhibit the pathogen *Batrachochytrium dendrobatidis* (*Bd*), we found that the proportion of reads associated with 'potentially' *Bd*-inhibitory OTUs did not vary temporally for two of three newt species, suggesting that protective function may be maintained despite temporal variation in community structure.
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Introduction

Host-associated microbial communities have vital roles for the hosts on which they reside (for example, Stappenbeck *et al.*, 2002; Rakoff-Nahoum *et al.*, 2004; Dethlefsen *et al.*, 2007; Engel and Moran, 2013). In particular, symbiotic bacterial communities are increasingly recognized in mediating protection against pathogens in multiple hosts (Rosenberg *et al.*, 2007; Woodhams *et al.*, 2007; Khosravi and Mazmanian, 2013; Fraune *et al.*, 2014), by modulating and contributing to host immunity (Eberl, 2010; Chung *et al.*, 2012; Gallo and Hooper, 2012; Krediet *et al.*, 2013). Changes in microbiota have also been linked to disease (Stecher *et al.*, 2007; Stecher and Hardt, 2008; Becker *et al.*, 2015). Although in the framework of wildlife and human diseases, disease risk, infection prevalence and infection intensity are known to vary seasonally and across time (Altizer *et al.*, 2006; Grassly and Fraser, 2006; Savage *et al.*, 2011; Langwig *et al.*, 2015), the impact of temporal fluctuations in host microbiota on disease risk has been studied in comparatively few systems.

In amphibians, cutaneous microbes are an important first line of defense against skin pathogens and can reduce disease susceptibility (for example, Becker and Harris, 2010; Bletz *et al.*, 2013). Bacterial symbionts isolated from amphibian skin can inhibit growth of the fungus, *Batrachochytrium dendrobatidis* (*Bd*), *in vitro* (Harris *et al.*, 2006; Flechas *et al.*, 2012; Antwis *et al.*, 2015; Woodhams *et al.*, 2015), and can reduce detrimental effects associated with chytridiomycosis, the disease caused by *Bd* (Harris *et al.*, 2009a, b; Vredenburg *et al.*, 2011). Furthermore, such bacterial protection has been associated with production of particular bacterial metabolites, such as violacein (Becker *et al.*, 2009). Chytridiomycosis has caused drastic declines of anuran populations in Central America and Australia (Berger *et al.*, 1998; Lips *et al.*, 2006). More recently, a new

Correspondence: MC Bletz, Zoologisches Institut, Technische Universität Braunschweig, Mendelssohnstr. 4, Braunschweig 38106, Germany.
E-mail: molly.bletz@gmail.com
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amphibian chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*) (Martel *et al*., 2013) has been found to pose a significant threat to salamander diversity in Europe and North America (Martel *et al*., 2014). With respect to *Bd*, infection prevalence and infection burden exhibits temporal variation (Kinney *et al*., 2011; Phillott *et al*., 2013; Longo *et al*., 2010, 2015), and such variation likely characterizes *Bsal* disease dynamics as well. One important factor driving this variation is temperature (Rohr *et al*., 2008; Bustamante *et al*., 2010), but overall the drivers of this variation are not well understood. Taking into consideration the defensive role of cutaneous microbiota, temporal variability of these communities may also be an important factor in such disease dynamics, especially considering population survival was linked to the proportion of amphibians with *Bd*-inhibitory bacteria in the western USA (Lam *et al*., 2010).

Similar to most other animal-associated microbiotas (Costello *et al*., 2009; Caporaso *et al*., 2011), limited data on temporal dynamics of amphibian skin communities exists (Longo *et al*., 2015), despite recent advances in our understanding of the ecology of these microbiomes. Amphibian microbial communities have been characterized for multiple species at single time-points, illustrating that they vary among species (McKenzie *et al*., 2012; Kueneman *et al*., 2014; Belden *et al*., 2015), across locations (Kueneman *et al*., 2014; Rebollar *et al*., 2016), among developmental stages (Kueneman *et al*., 2015; Sanchez *et al*., 2016) and depending on *Bd* presence and host susceptibility (Rebollar *et al*., 2016). Host properties of the mucosal environment in which bacteria reside, such as antimicrobial peptides, alkaloids, lysozymes, mucopolysaccharides and glycoproteins, (Rollins-Smith, 2009; Woodhams *et al*., 2014), and environmental microbiota of the surrounding habitat (Loudon *et al*., 2014; Walke *et al*., 2014; Fitzpatrick and Allison, 2014), influence host-associated microbial communities.

For amphibians, as well as for other hosts, if and to what extent symbiotic microbes change temporally is important given their defensive role against pathogens (Bletz *et al*., 2013), and for understanding the larger role they have in amphibian health. Their temporal dynamics is necessarily linked to the structure–function relationship. The prominent hypothesis in microbial community ecology is that community composition determines function (Robinson *et al*., 2010; Nemergut *et al*., 2013). Some studies support this hypothesis (Waldrop and Firestone, 2006; Kaiser *et al*., 2010; Fukami *et al*., 2010), whereas others support that structure and function are not inherently linked (Burke *et al*., 2011; Frossard *et al*., 2011; Belden *et al*., 2015; Louca *et al*., 2016). These alternative findings necessitate the following questions: if a bacterial community exhibits structural variation temporally, what are the functional implications? Does functional potential of the community parallel these structural changes or remain unchanged despite altered community structure? To determine whether temporal variation in amphibian skin microbiota influences protective function, we must study these communities across a time-series.

For amphibian microbiota, much of the available knowledge comes from isolated sampling events, providing snapshots of community structure and important insights into the factors structuring these communities. It remains unclear, however, how microbial communities on amphibian skin change through time, what factors drive this variation and how such variation may influence microbial function with respect to protection against pathogens. In this context, we test two main hypotheses: (1) amphibian cutaneous bacterial communities vary across time, and (2) changes in *Bd*-inhibitory predicted function are linked to temporal variation in the community structure, that is, functional changes will result from structural changes. Addressing these two hypotheses provides new insight into the structure–function relationship occurring in skin microbiomes.

To address our first hypothesis, we repeatedly sampled two biphasic newt species, *Lissotriton vulgaris* and *Ichthyosaura alpestris*, in a field survey, and also performed a semi-natural experiment, housing a third newt species, *Triturus cristatus*, in enclosures within field habitats. We characterize and compare newt cutaneous bacterial community diversity and structure through time within their aquatic phase. We test for associations of these communities with environmental temperature and environmental bacterial communities through time to determine the drivers of temporal variation. In addition, we characterize bacterial community structure across the aquatic-to-terrestrial transition. Newts develop as aquatic larvae, metamorphosing into adults which then maintain a biphasic life, spending part of each year within ponds (breeding season: March–May) and part within terrestrial habitats (June–February). This transition is particularly relevant as it applies a drastic restructuring of the skin in some species (Perrotta *et al*., 2012), including our focal species, the salamandrid newts. Such changes in skin morphology may result in temporal changes in cutaneous microbiota. To address the second hypothesis, we predict *Bd*-inhibitory function of operational taxonomic units (OTUs) with a bioinformatic approach, using known functional information from a database of culture isolates tested *in vitro* for the ability to inhibit *Bd* growth (Woodhams *et al*., 2015). We subsequently determine whether this estimated function varies through time.

Materials and methods

Sample collection

Sampling of three newt species was performed at semi-regular intervals (approximately once per

Table 1 Sample sizes of amphibian cutaneous bacterial communities and pond water samples included in this study

Date	*Aquatic phase temporal sampling (16 March–29 May)*			
	Kleiwiesen			*Elm*
	L. vulgaris	*T. cristatus*	*Environment*	*I. alpestris*
16 March				6 (8)
17 March	16 (20)			
18 March		4 (6)		10 (11)
20 March	11(19)	6 (11)	3 (3)	
22 March				10 (10)
24 March	18 (20)		3 (3)	
25 March		17 (17)		
28 March				7 (7)
1 April	18 (19)	15 (17)	0 (1)	
8 April		16 (17)	2 (2)	15 (15)
14 April	12 (12)	16 (17)	2 (2)	
22 April		16 (17)	2 (2)	16 (16)
29 April	8 (8)	13 (17)	1 (2)	
6 May		10 (15)	1 (2)	
29 May	9 (10)		3 (3)	

	Terrestrial–aquatic comparison *Kleiwiesen: 29 May; Elm: 22 April (Aq), 3 June (Ter)*	
	Kleiwiesen (*L. vulgaris* and *I. alpestris*)	Elm (*I. alpestris*)
Aquatic	9 (9 Lv, 0 Ia)	16 (16)
Terrestrial	9 (3 Lv, 6 Ia)	9 (12)

Abbreviations: Aq, aquatic; Ia, *Ichthyosaura alpestris*; Lv, *Lissotriton vulgaris*; Ter, terrestrial.
These sample sizes represent post sequence filtering values. Numbers in parentheses indicate the respective number of individuals sampled in the field. The difference in these numbers is a result of the exclusion of samples because of low sequence coverage (<1000 reads).

week) between March and June 2015 at two locations, Kleiwiesen and Elm (Lower Saxony, Germany). *L. vulgaris* and *T. cristatus*, were sampled at Kleiwiesen, and *I. alpestris*, was sampled at Elm. These amphibian species were selected because of the likelihood of finding adequate sample sizes through time. To monitor temporal changes throughout their aquatic life phase we sampled (1) *L. vulgaris* free-swimming individuals on 17 March, 20 March, 24 March, 1 April, 14 April, 29 April and 29 May, (2) *I. alpestris* free-swimming individuals on 16 March, 18 March, 22 March, 28 March, 8 April and 22 April and (3) conducted a field mesocosm experiment with *T. cristatus,* allowing individuals to be tracked through time; sampling of mesocosm-housed newts occurred on 18/20 March, 25 March, 1 April, 8 April, 14 April, 22 April, 29 April and 6 May (see Mesocosm experiment section). To compare newt microbiota across the aquatic-to-terrestrial transition, we sampled terrestrial phase *L. vulgaris* and *I. alpestris* on 29 May at Kleiwiesen and 3 June at Elm. Table 1 provides sample sizes of each newt species on each sampling day.

Amphibians were captured in one of three ways: directly by gloved hands (clean nitrile gloves were used for each individual), dip nets or Ortmann's funnel traps (Drechsler *et al*., 2010). Each captured individual was held with unique gloves, rinsed with 50 ml of filtered (0.22 μm) deionized water to remove debris and transient microbes, and swabbed on its ventral surface 10 times (1 time = an up and back stroke) using a sterile MW113 swab (Medical Wire and Equipment, Corsham, UK). Care was taken so the sampled surface did not contact the gloves after rinsing. Swabs were stored in unique sterile vials and transferred into a − 20 °C freezer within 2 h after collection. Amphibians were returned to ponds immediately after sampling of all individuals was complete. Sampling resulted in 82 samples from *I. alpestris*, 111 samples from *L. vulgaris* and 134 samples from *T. cristatus* (see Table 1 for distribution of samples across dates).

iButton dataloggers (Thermochron, San Jose, CA, USA) were placed at both sampling locations to collect water temperature hourly. Loggers were placed at two locations within ponds where the water and base substrate meet and newts are commonly observed: (1) shallow water, approximately 20–30 cm and (2) deep water, approximately, 70–90 cm. At Kleiwiesen, pond water bacterial communities were sampled according to Walke *et al.* (2014) on each day in which newt sampling occurred. Twenty pond water samples were taken in total (see Table 1 for distribution of samples across dates).

Mesocosm experiment

For one newt species, *T. cristatus*, individuals were captured on 18 and 20 March 2015 and placed into wide polyester mesh mesocosms with zippered lids (mesh size: 0.5 cm diameter, height: 40 cm, diameter: 38 cm) to allow exact individuals to be tracked through time. This species was selected because of its large size and long uninterrupted aquatic phase making it less likely to escape and more suitable for long-term observation. The mesh size allowed entrance of prey; zippered lids prevented escape of newts or entry of other individuals. Mesocosms ($n = 9$) were located at least 1 m apart within the ponds. Sediment and debris from the pond where newts were captured were placed in the base of all mesocosms, and a rock was used to submerge approximately 75% of each mesocosm within the pond, aiming to mimic natural conditions. Each mesocosm housed two individuals (a single individual in one case) ($n = 17$) that were sampled weekly (total $n = 134$, two individuals were released from their mesocosm before the final sampling date).

DNA extraction and sequencing

Whole-community DNA was extracted from swabs with the MoBio PowerSoil-htp 96-well DNA isolation kit (MoBio, Carlsbad, CA, USA) following the manufacturer's protocol with minor adjustments. A dual-index approach was used to PCR-amplify the V4 region of the bacterial 16S ribosomal RNA gene

with the 515F and 806R primers (Kozich *et al.*, 2013). Pooled PCR amplicons of all samples were sequenced with paired-end 2 × 250 v2 chemistry on an Illumina MiSeq sequencer at the Helmholtz Centre for Infection Research in Braunschweig, Germany (see Supplementary Methods). An aliquot of DNA extract from selected samples (Kleiwiesen: $n = 171$, Elm: $n = 108$) was used for real-time quantitative PCR to determine the occurrence of *Bd* and *Bsal* within the studied locations following Blooi *et al.* (2013).

Sequence processing

Quantitative Insights Into Microbial Ecology (MacQIIME v1.9.1) was used to process all sequence data unless otherwise stated (Caporaso *et al.*, 2010). Briefly, forward and reverse reads were joined, quality-filtered and assigned to OTUs using an open reference strategy at 97% similarity with SILVA 119 (24 July 2014) as the reference database. OTUs making up $< 0.001\%$ of the total reads were removed (Bokulich *et al.*, 2013). All samples were rarefied at 1000 reads to allow inclusion of most samples and capture the majority of the diversity present within these bacterial communities. Samples with < 1000 reads were therefore removed (see Supplementary Methods for details). After filtering, 287 newt samples remained for analyses along with 17 environmental samples (Table 1).

Sequence analysis

Temporal dynamics of skin-associated bacterial communities of three newt species in their aquatic phase was studied for a 3-month period between March and May 2015. Faith's phylogenetic diversity was calculated as a measure of alpha diversity for all samples. Kruskal–Wallis tests were completed to compare diversity through time for *L. vulgaris* and *I. alpestris* in R (R Core Team, 2016). A mixed linear model was used for *T. cristatus* to account for repeated sampling and co-housing (lme4 package, Douglas et al., 2015). *P*-values were approximated with the KR method (afex package, Singmann *et al.*, 2015). Beta diversity was calculated with the Bray–Curtis, weighted Unifrac and unweighted Unifrac metrics and temporal variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) with sampling date as the main fixed factor. For *T. cristatus*, mesocosm and individual (nested within mesocosm) were also included as factors.

Temporal changes in beta diversity for each species were calculated as the mean Bray–Curtis distance between the first sampling date and each subsequent date. To visualize temporal patterns in beta diversity among newt species, we completed a principal coordinate analysis on the Bray–Curtis distance matrix including all samples from all species and time-points. Axis values for PCo1 and PCo2 were extracted and plotted to visualize the time-series for each species.

To determine which OTUs were most responsible for the observed temporal variation across multiple time-points, a Principal component analysis (PCA) was performed for each newt species, with relative abundances of OTUs as the potential explanatory factors. This analysis was used to identify OTUs that were varying the most across multiple time-points. PCA analysis was completed with a modified OTU table containing only the Core-100 OTUs (see Supplementary Methods). From the PCA results, we identified the OTUs most responsible for the temporal variation as those yielding factor loading values of > 0.1 on PC1 or PC2.

In addition, a hierarchical clustering method, unweighted pair group method with arithmetic mean, along with Similarity Profile Analysis (SIMPROF), was used to evaluate similarity among individual newt communities and among the temporal pattern of PCA-identified OTUs. Bray–Curtis distances and Whittaker's index of association were used for clustering of samples and PCA-identified OTUs, respectively. Unweighted pair group method with arithmetic mean analysis and heat map visualization were completed in Primer 7 (Clarke and Gorley, 2015).

To explore drivers of the observed temporal variation in skin microbiota, we tested for associations between newt microbiota and environmental variables, including water temperature and aquatic bacterial community structure. Mantel tests were performed to test for associations between newt bacterial community structure and temperature, using average temperatures from 2 days before the sampling date to account for a likely lag effect of environmental temperature on host microbiota. Distance-based linear modeling was performed to determine the proportion of variation explained. To test for associations between the temporal patterns of newt and aquatic bacterial communities, Kendall's tau rank correlations were performed for each of the PCA-identified OTUs; that is, for each of these OTUs, we tested for correlation between the temporal pattern in mean relative abundance on each newt species, and its relative abundance in pond water. Environmental microbiota correlations were only performed for newts sampled at Kleiwiesen where aquatic microbial community samples were available.

To investigate community changes across the aquatic-to-terrestrial transition, all terrestrially sampled individuals and aquatic individuals from the closest possible date from both Kleiwiesen (aquatic and terrestrial from the same day) and Elm (aquatic: 22 April; terrestrial: 3 June) were analyzed (Table 1). Terrestrial samples were only collected from *L. vulgaris* and *I. alpestris*. Beta diversity was calculated as described above, visualized with PCo Analysis and a PERMANOVA was completed to statistically test for differences between phases. OTUs that were differentially abundant between terrestrial and aquatic newts across both sampled

locations were identified using the linear discriminant analysis effect size (LEfSe) method (Segata *et al.*, 2011). LEfSe analysis was performed on a modified OTU table that contained only the Core-100 OTUs (see Supplementary Methods).

To assess our second hypothesis, looking at the structure–function relationship of cutaneous bacterial communities, we utilized a recently developed database containing over 1900 16S ribosomal RNA gene sequences from amphibian skin bacteria that have been tested for activity against the pathogen, *Bd* (Woodhams *et al.*, 2015). Using this database, we determined which potentially *Bd*-inhibitory OTUs were present in our data set and calculated proportion of reads associated within these OTUs with respect to the full community (see Supplementary Methods). Kruskal–Wallis tests (*L. vulgaris* and *I. alpestris*) and mixed linear model (*T. cristatus*) were performed to test whether proportion of inhibitory reads varied through time. For the mixed linear model, significance was estimated using a likelihood ratio test. To compare terrestrial and aquatic individuals, we pooled data from both locations and used a linear mixed-effect model with life phase as a fixed effect and location (Kleiwiesen/Elm) as a random factor.

Results

Overall, 304 (287 newt and 17 environmental) samples were analyzed (Table 1). The rarified OTU table of skin microbiota samples contained 3503 unique bacterial OTUs, predominately from five main phyla: Proteobacteria, Bacteriodetes, Actinobacteria, Firmicutes and Verrucomicrobia.

Newt microbiota exhibits variation through time

Phylogenetic diversity varied through time for all three newt species (analysis of variance : $P<0.001$, Figure 1). There was an increase in phylogenetic diversity in the second half of March in all species, followed by a decrease in April for *I. alpestris* and *T. cristatus,* and a subsequent increase in May for *T. cristatus* (Figure 1).

Cutaneous bacterial community structure on newts also significantly varied through time using Bray–Curtis (PERMANOVA: Pseudo-F = 13.722 (*I. alpestris*), 8.3865 (*L. vulgaris*), 9.2361 (*T. cristatus*), $P=0.001$), weighted Unifrac (PERMANOVA: Pseudo-F = 10.638 (*I. alpestris*), 8.747 (*L. vulgaris*), 13.469 (*T. cristatus*), $P=0.001$) and unweighted Unifrac (PERMANOVA: Pseudo-F = 3.7736 (*I. alpestris*), 3.2635 (*L. vulgaris*), 3.5643 (*T. cristatus*), $P=0.001$). Mean relative abundance profiles of newt bacterial OTUs showed that community composition differed across time (Figure 2). For both *I. alpestris* and *L. vulgaris*, pairwise distances from the initial sampling point increased through time, but for *T. cristatus* distances were relatively constant (Figure 2). Unweighted pair group method with arithmetic mean clustering showed that samples from the same or nearby dates had more similar community structures and samples typically clustered chronologically for each study species (Figure 3, Supplementary Figure S3). Similarity profile analysis (SIMPROF) revealed significant grouping among samples for each species (I. alpestris: Pi = 12.864, $P=0.001$, *L.* vulgaris: Pi = 8.86, $P=0.001$, *T.* cristatus: Pi = 8.862, $P=0.001$).

I. alpestris (Elm) and *L. vulgaris* (Kleiwiesen) exhibited similar transitions in bacterial community structure, whereas that of *T. cristatus* followed a different pattern (Figure 4). In late March–early April, there was a marked shift in PCo1 and PCo2 for *L. vulgaris and I. alpestris* and in PCo2 only for *T. cristatus* (Figure 4). For *L. vulgaris and T. cristatus,* there was also an observable shift in late April to May in both PCo axes (Figure 4). For *T. cristatus,* where exact individuals were tracked

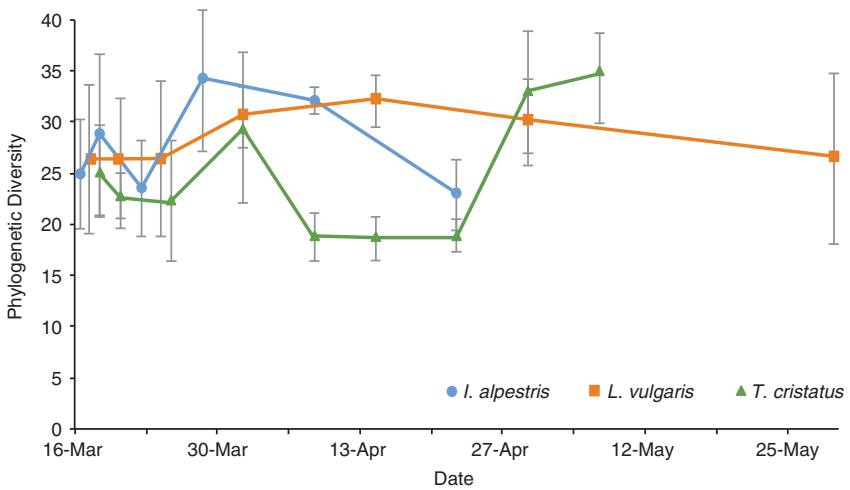

Figure 1 Phylogenetic diversity of cutaneous bacterial communities through time for the three European newt species studied. Points represent the mean diversity for all sampled newts on a given day for the respective newt species. Error bars represent s.e.m. See Supplementary Figure S1 for phylogenetic diversity (PD) of individuals of *T. cristatus* through time.

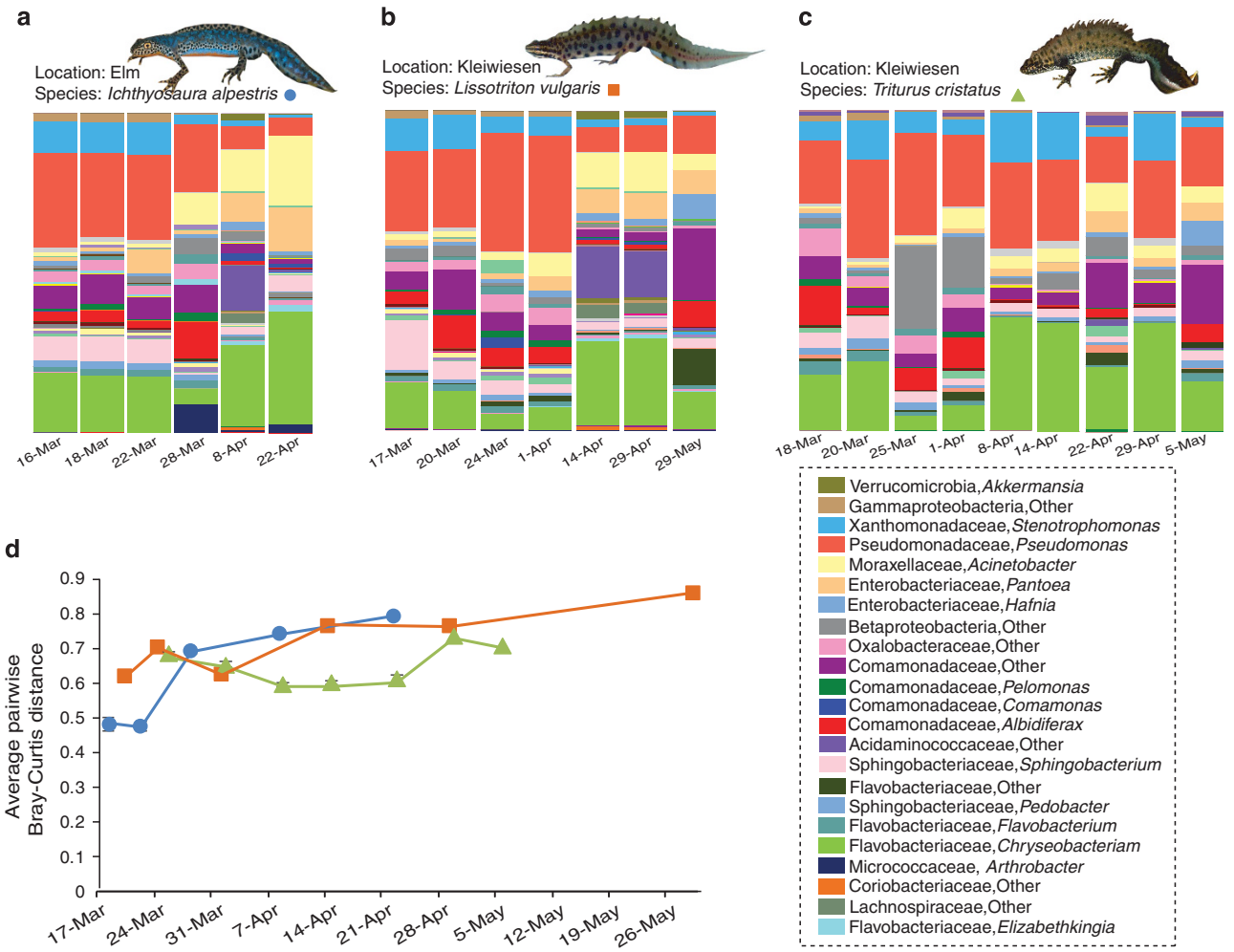

Figure 2 Temporal variation in host-associated skin bacterial communities of three European newt species. Genus-level relative abundance profiles of the Core-100 OTUs through time for (**a**) *I. alpestris*, (**b**) *L. vulgaris*, and (**c**) *T. cristatus*. (**d**) line graph of pairwise distance comparisons between each sampling date and the initial sampling date for each newt species, depicting the continued changes in community structure through time. Points represent the mean pairwise distances for all sampled newts on a given day for the respective newt species. Error bars represent s.e.m. See Supplementary Figure S2 for distance comparisons in individuals of *T. cristatus* through time.

through time, we found that changes in community structure did not differ between mesocosms (date-by-mesocosm interaction Pseudo-F = 1.0108, $P = 0.477$), suggesting cutaneous bacterial communities were temporally changing in a similar manner across individuals, that is, they exhibited synchrony of community changes (Supplementary Figure S4).

To identify OTUs most responsible for temporal variability of skin bacterial communities, we performed a PCA on the Core-100 OTU table for each species. The temporal Core-100 OTUs comprises 79, 68 and 46 OTUs for *I. alpestris, L. vulgaris* and *T. cristatus*, respectively. On average, these OTUs made up 69.6 ± 15.1%, 59.7 ± 6.1% and 70.8 ± 12.5% of the community for the respective species. PC1 accounted for 75.4%, 70.2% and 70% of the variation, and PC2 accounted for 15.6% 15.7% and 19.3%, respectively, for *I. alpestris*, *L. vulgaris* and *T. cristatus*. Fourteen, eleven and nine OTUs were found to strongly influence the temporal variation in community structure for the three species, respectively (Supplementary Table S1). Four of these OTUs were found to strongly influence temporal variation for all three species: *Chryseobacterium* OTU1 (Flavobacteriaceae), *Pantoea* OTU1 (Enterobacteriaceae), *Albidiferax* OTU1 (Comamonadaceae) and *Pseudomonas fluorescens* OTU1 (Pseudomonadaceae) (Supplementary Table S1). *Chryseobacterium* OTU1 increased through time for all three newt species, while *Pantoea* OTU1 increase through time for *I. alpestris* and *L. vulgaris*, but remained more constant for *T. cristatus* (Figure 3). *Pseudomonas fluorescens* OTU1 decreased through time for *I. alpestris* and *L. vulgaris*, and remained more consistent for *T. cristatus* (Figure 3). Temporal patterns in relative abundance of the PCA-identified OTUs are shown in Figure 3 (*I. alpestris*) and Figure S3 (*L. vulgaris* and *T. cristatus*).

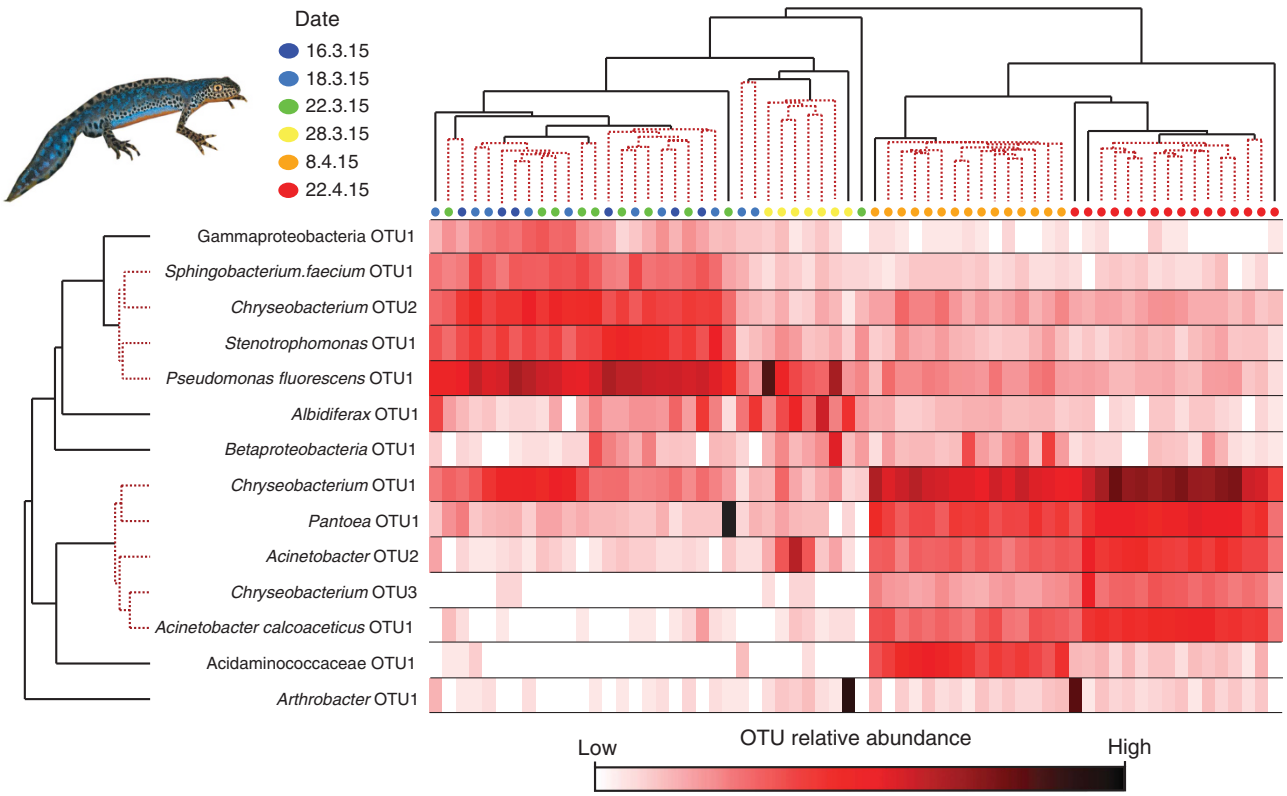

Figure 3 Temporal patterns of bacterial OTUs found to be most responsible for the temporal variation of skin microbial communities on *I. alpestris*. Samples (columns) are clustered by unweighted pair group method with arithmetic mean (UPGMA) clustering of the Bray–Curtis distance matrix and OTUs (rows) are clustered using Whittaker's index of association. UPGMA clustering of samples shows clustering by date and UPGMA clustering of bacterial OTUs showed that some OTUs followed similar temporal trajectories. SIMPROF results are indicated by the red-dotted lines of the UPGMA dendrograms; the transition of black to red-dotted lines indicates the point at which the null hypothesis is no longer rejected, that is, the branches no longer have internal structure and are homogeneous. Heat map displays the relative abundance of the OTUs within the community, with darker shades representing greater values. OTU names are given based on lowest taxonomic assignment with an OTU number specific to the SILVA or *denovo* cluster IDs. UPGMA and heatmaps for *L. vulgaris* and *T. cristatus* are presented in Supplementary Figure S3.

Multiple OTUs remained detectable through time. We observed seven, five and five OTUs, respectively, for *I. alpestris, L. vulgaris* and *T. cristatus* that were present on a minimum of 90% of individuals on all sampling days (Supplementary Table S2). Three of these OTUs were identified for all three newt species: *Chryseobacterium* OTU1 (Flavobacteriaceae), and two pseudomonad OTUs, *Pseudomonas fluorescens* OTU1 and *Pseudomonas* OTU2 (Pseudomonadaceae) (Supplementary Table S2).

Environmental drivers of community variation

Mantel tests showed a significant correlation between water temperature and newt bacterial community structure (P-values = 0.001 for all species). Based on distance-based linear modeling, water temperature explained 24% of the variation for *I. alpestris*, 23% for *L. vulgaris* and 9% for *T. cristatus* (Supplementary Table S3). Pond bacterial community composition also varied through time (Supplementary Figure S5). We tested for correlations between average relative abundances of selected OTUs on newts and in the pond water. For *L. vulgaris*, no OTUs exhibited significant positive correlations, and there was one OTU (Flavobacteriaceae OTU1) that had a significant negative correlation (Kendall's tau: $z = -2.2738$, $P = 0.023$, $\tau = -0.949$). For *T. cristatus*, only one OTU (*Chryseobacterium* OTU4) showed a significant positive correlation (Kendall's tau: $z = 2.1954$, $P = 0.028$, $\tau = 0.72$). All correlation results are provided in Supplementary Table S4.

Cutaneous microbiota differs between life phases

Bacterial community structure differed between terrestrial and aquatic individuals at both locations (Figure 5, PERMANOVA: Pseudo-F = 7.5428 (Kleiwiesen), 10.447 (Elm), $P = 0.001$). Terrestrial individuals exhibited greater relative abundances of Pseudomonadaceae (terrestrial: 12.5/aquatic: 4.3%), Enterobacteriaceae (20.3/8.8%) and Rhodobacteriaceae (2.2/0.4%), whereas aquatic individuals showed greater relative abundances of Moraxellaceae (5.3/10.6%), Comamonadaceae (4.7/13.2%), Rhizobaceae (0.7/2.5%) and Flavobacteriaceae (13.8/23.8%) (Supplementary Figure S6). LEfSe analysis at the OTU-level revealed two

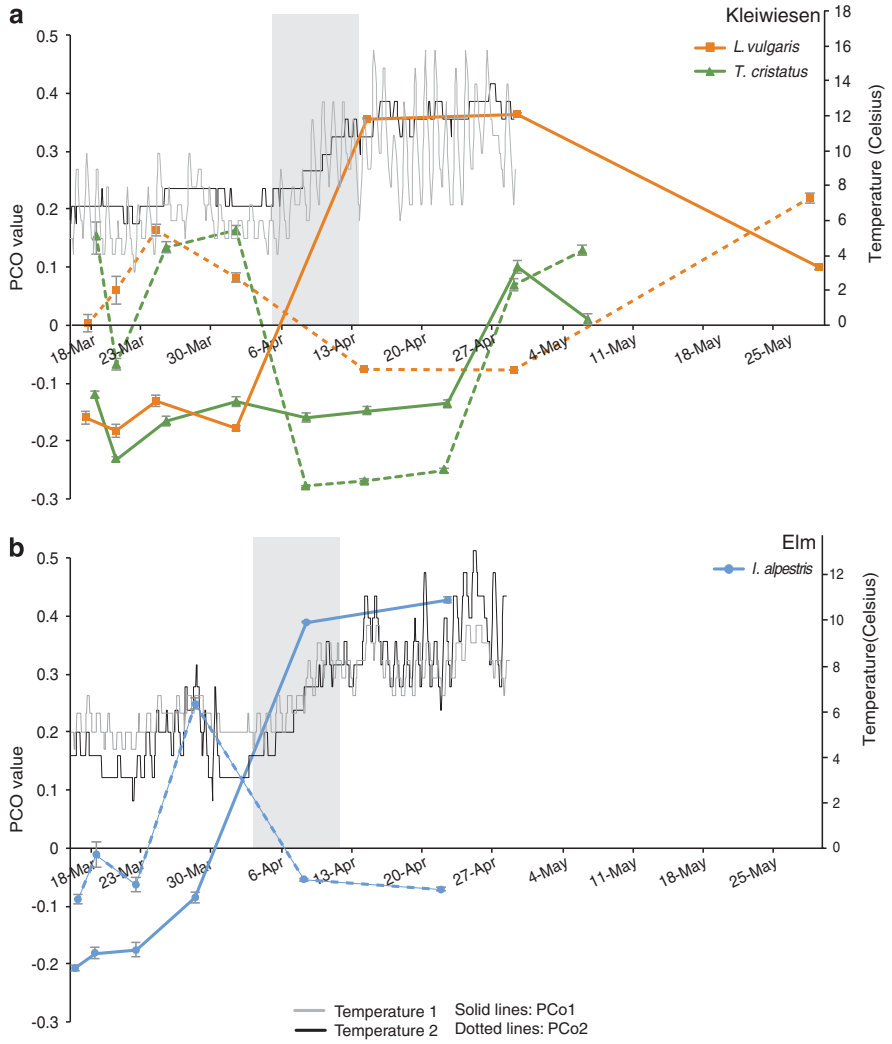

Figure 4 Temporal patterns in host-associated skin bacterial communities of three European newt species and water temperature patterns. (**a**) Principal coordinate analysis (PCoA) axis 1 and 2 values through time for *L. vulgaris* and *T. cristatus* at Kleiwiesen. (**b**) PCoA axis 1 and 2 values through time for *I. alpestris* at Elm. Note the similar pattern in the PCO trends through time for *L. vulgaris* and *I. alpestris*. Water temperature data are only available until 29 April. Temperature 1 is from a logger placed in shallow water of the pond, and temperature 2 is from a logger placed in deep water. Gray boxes highlight the window in time where there was a steady increase in water temperature. Error bars represent s.e.m.

OTUs from the 43 Core-100 OTUs that were significantly more abundant on aquatic newts and 12 OTUs that were more abundant on terrestrial newts across both locations (Supplementary Figure S6).

Pathogen presence and stability of Bd-inhibitory function through time

Bd was present at both Kleiwiesen and Elm, however, *Bsal* was not detected. Of the 273 samples tested, 15 were positive for *Bd* (2 *I. alpestris*, 10 *L. vulgaris* and 3 *T. cristatus*) (Supplementary Table S5).

One hundred thirty-four OTUs from the newt temporal data set matched inhibitory OTUs from the amphibian bacteria database (Woodhams *et al.*, 2015). Relative abundance of *Bd*-inhibitory reads (derived from the summation of rarified reads associated with all 'potentially inhibitory' OTUs, $n = 134$) did not differ across time for *L. vulgaris* (Kruskal–Wallis chi-squared = 6.6038, $P = 0.359$) and *T. cristatus* (mixed linear model comparison: L. ratio = 1.8709, $P = 0.1707$) (Figure 6). *I. alpestris* did show significant differences among dates (Kruskal–Wallis chi-squared = 29.599, $P < 0.001$), and this was due to the proportion of inhibitory reads being greater on 22 April in comparison with all other dates (Wilcoxon post-hoc: P(fdr) < 0.01). Proportion of *Bd*-inhibitory reads also did not differ between terrestrial and aquatic individuals (GLM: t = – 1.1533, $P = 0.2529$).

The observed functional stability resulted both from constancy of particular OTUs early in the

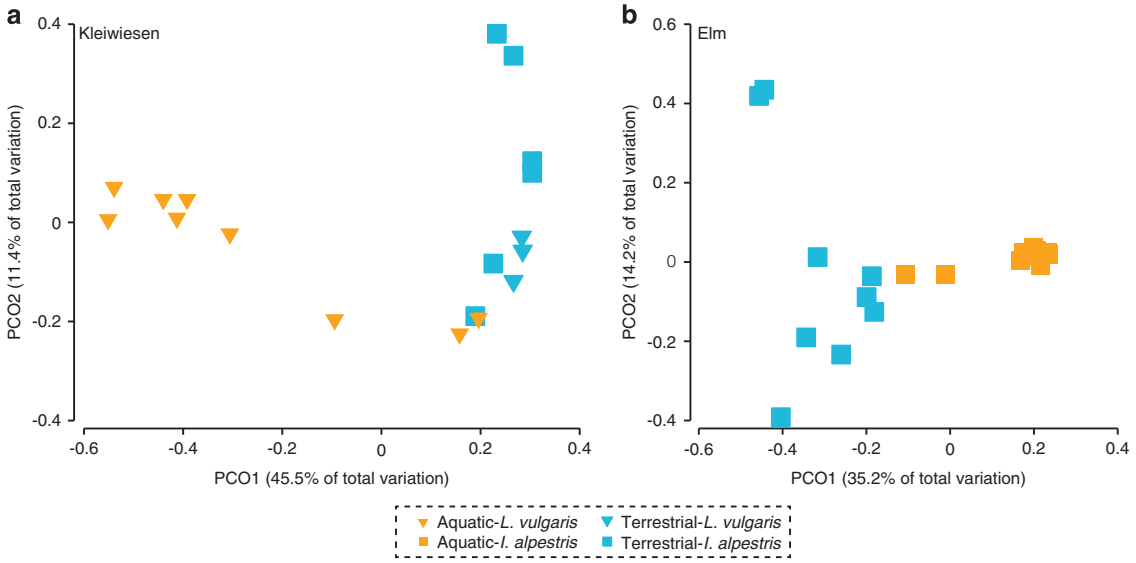

Figure 5 Community structure of terrestrial and aquatic phase newts. PCO analysis of Bray–Curtis distance matrix of terrestrial and aquatic newt skin bacterial communities at Kleiwiesen (**a**) and Elm (**b**). Mean relative abundance profiles of terrestrial and aquatic individuals and LEfSe-identified differentially abundant OTUs are presented in Supplementary Figure S5.

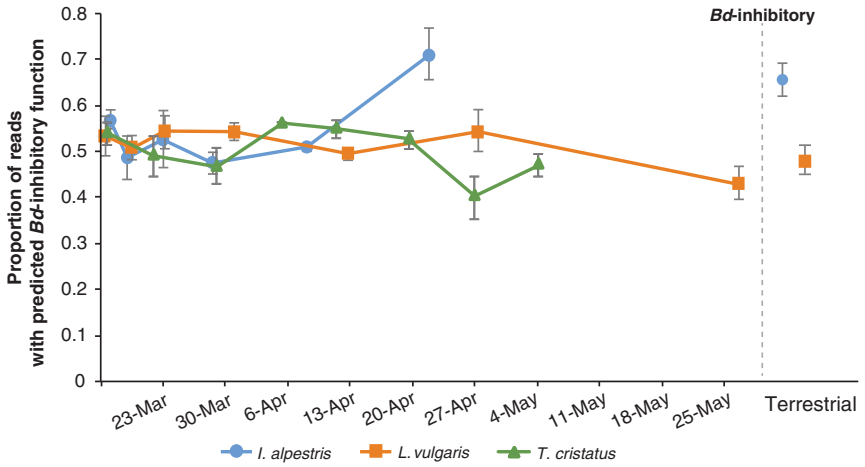

Figure 6 Predicted *Bd*-inhibitory function is maintained through time for two of the three sampled newt species. Proportion of reads assigned to OTUs with known '*Bd-inhibitory*' function for each sampled newt species. For the terrestrial data, Elm and Kleiwiesen are pooled together. Error bars represent s.e.m.

season, and replacement by different OTUs later in the season (Supplementary Figure S5). For example, a *Bd*-inhibitory *Pseudomonas fluorescens* OTU (JQ691706.1.1490) is consistently present during the early time-points, whereas a *Bd*-inhibitory *Chyrseobacterium* OTU (KC734243.1.1310) increases in relative abundance at the later time-points for all three newt species. The top four OTUs, which constitute a large portion of the *Bd*-inhibitory reads, match with database OTUs with strong *Bd*-inhibitory signals (see Supplementary Methods): a *Pseudomonas fluorescens* OTU-JQ691706.1.1490 (80%; 28/35 isolates), a *Chryseobacterium* OTU-KC734243.1.1310 (90%; 28/31 isolates), a *Stenotrophomonas* OTU-HQ224659.1.1422 (100%; 2/2 isolates) and a *Pantoea* OTU-HQ728209.1.1508 (62%; 55/89 isolates) (Supplementary Figure S7).

Discussion

Temporal dynamics of host-associated microbiota has been identified as a gap in our ecological understanding of these communities (Grice and Segre, 2011; Rosenthal *et al.*, 2011; Walter and Ley, 2011), and for many organisms, including amphibians, such dynamics are a foundational piece of knowledge for developing a thorough understanding of the functional role these communities have in host health (Rosenberg *et al.*, 2007; Robinson *et al.*, 2010; Fierer *et al.*, 2012).

We hypothesized that amphibian skin microbiota would vary temporally within the aquatic phase. Our results show that, for all species, date had a significant effect on cutaneous bacterial community structure. *L. vulgaris* and *I. alpestris* showed strikingly similar temporal patterns in community

structure despite being from different locations. However, the patterns observed in *T. cristatus* were different. Whether this observation reflects a true biological difference or was at least partly caused by environmental conditions within mesocosms, or repeated sampling of individuals, requires further study. Despite this restriction, these data from *T. cristatus* conclusively demonstrate that temporal changes observed in cutaneous microbiota are not caused by different individuals captured at different time-points. The consistent temporal trajectory in bacterial community structure among *T. cristatus* individuals suggests that non-stochastic, selective processes of community assembly and succession are at play.

The observed community variation is likely associated with fluctuations in external environmental conditions, including abiotic factors and environmental microbiota, as well as changes in host skin morphology and mucosal characteristics. The marked shift in community structure seen in *L. vulgaris* and *I. alpestris* in early April coincides directly with a steady increase in water temperatures at both locations. Water temperature was found to explain up to 24% of observed microbial variation depending on the host species, suggesting that external environmental parameters are in part dictating the temporal trajectory of these skin microbiotas. As amphibians are ectothermic, their body temperature matches that of the surrounding habitat. Temperature can directly influence growth of host-associated bacterial taxa (Daskin *et al*., 2014) and also influence community member interactions and antifungal capacity (Woodhams *et al*., 2014). Environmental factors, such as temperature, have been found to influence amphibian gut microbiota (Kohl and Yahn, 2016), and also affect microbial communities in surrounding habitats. For aquatic amphibians, such as aquatic phase newts, bacterioplankton is likely an important reservoir from which the skin is colonized, and such freshwater bacterial communities are known to vary temporally (Crump and Hobbie, 2005; rivers—Kent *et al*., 2007; Portillo *et al*., 2012; lakes—Jones *et al*., 2012; Shade *et al*., 2013; temporary pools—Carrino-Kyker and Swanson, 2008). Therefore, seasonal shifts in environmental microbiota could dictate host-associated microbiota variations. For terrestrial salamanders, soil provides a rich bacterial reservoir competing with established cutaneous communities, and such competition may explain the diverse skin microbiota observed (Loudon *et al*., 2014) and possibly its variation over time. In this study, however, temporal patterns of bacterial taxa on newts were not correlated with dynamics of the respective OTUs within the environment. This finding makes sense in the context of other studies that have found amphibian microbial communities are dominated by bacterial taxa that are rare in the environment (Walke *et al*., 2014; Rebollar *et al*., 2016); thus, we can expect different dynamics between these two communities.

The lack of association between the temporal dynamics of environmental microbiota and that of newts, suggests a heightened role of host-associated factors, such as changes in skin morphology and mucosal characteristics, in driving temporal patterns of newt skin microbiota. At the start of sampling newts had just migrated into breeding ponds; the period from mid-March to early April likely coincided with changes in skin morphology from the terrestrial to aquatic phase (Perrotta *et al*., 2012). This transitional process also involves increased skin sloughing, which is known to affect skin microbiota (Meyer *et al*., 2012; Cramp *et al*., 2014). Microbial changes may also be in part related to host immune system differences (Longo *et al*., 2015). Furthermore, skin restructuring may include changes in innate immune system function, skin pH or other components of the mucosal layer, similar to changes at metamorphosis (Kueneman *et al*., 2015), which have a role in structuring host microbiota (Franzenburg *et al*., 2013; Küng *et al*., 2014; Colombo *et al*., 2015).

The influence of skin morphology in temporal variation of skin microbiota is further exemplified by the distinct communities characterizing terrestrial and aquatic newts. Terrestrial phase skin bacterial communities were enriched for 12 OTUs consistently across both Kleiwiesen and Elm. Interestingly, Enterobacteriaceae was more abundant on terrestrial phase newts, and also increased on aquatic individuals at later time-points. As newts prepare for the transition to terrestrial life their skin structure begins to change (Perrotta *et al*., 2012), and this change could drive shifts in community members. Similarly, in humans, temporal shifts in skin, oral and gut microbial communities tend to be associated with transitional events (for example, skin oil production, tooth eruption, milk-to-solid food) that may impose environmental filters that exert a selective effect (Marsh, 2000; Robinson *et al*., 2010; Costello *et al*., 2012).

In this context of temporal changes in host-associated bacterial communities, we hypothesized that such changes would be coupled with significant variation in predicted *Bd*-inhibitory function. Hosts may temporally lose important protective bacterial taxa, which could have drastic consequences with respect to disease. Furthermore, the aquatic-to-terrestrial transition potentially could result in dysbiotic transitional states as has been hypothesized with sloughing (Cramp *et al*., 2014) and metamorphosis (Kueneman *et al*., 2015). We used a bioinformatics approach to identify OTUs that can 'potentially' inhibit *Bd* (Kueneman *et al*., 2015, 2016). Given that we found this pathogen on newts and that it has measurable sublethal effects on a related European newt species (Cheatsazan *et al*., 2013), it is likely exerting selective pressures on the studied hosts and their microbiota. Our results

indicate that the proportion of predicted *Bd*-inhibitory reads did not vary temporally for two of three newt species, and aquatic and terrestrial individuals did not differ, suggesting that this *Bd*-inhibitory function was largely maintained despite structural variation. It is a common view that changes in microbial composition are linked to alterations in functional capabilities (Strickland *et al.*, 2009; Shade *et al.*, 2013). However, more and more studies provide evidence that structure and function can be de-coupled (Burke *et al.*, 2011; Purahong *et al.*, 2014; Louca *et al.*, 2016). Stability of predicted *Bd*-inhibitory function herein appears to be associated with both constancy of particular OTUs and replacement of function by different OTUs. Functional redundancy among bacterial OTUs can make this possible (Allison and Martiny, 2006; Shade *et al.*, 2012). In fact, Burke *et al.* (2011) proposed that microbial communities on algae assemble based on functional genes following a competitive lottery mechanism. More specifically, this model suggests bacteria with similar ecological properties are able to occupy the same niche but the particular taxon occupying that space is stochastically recruited. Perhaps in the newt system, microbes are assembling in a manner similar to the model of Burke *et al.* (2011). As the environmental reservoir shifts temporally and the newt skin environment changes, it recruits different taxa, independent of phylogenetic identity, which are best able to reside within existing physiochemical parameters of the skin; at the same time, selective pressure on the host may lead to evolution of a mucosal environment making it particularly suitable for some protective bacteria (that is, fungal-inhibiting taxa), thereby explaining maintenance of *Bd*-inhibitory function amidst temporal phylogenetic variation in the underlying OTUs within this study. If protection provided by these bacteria can persist temporally, such protection may be able to fill the gaps when host-mediated defenses (for example, antimicrobial peptides) may be reduced (Woodhams *et al.*, 2012; Holden *et al.*, 2015; Kueneman *et al.*, 2015). It is important to acknowledge that our functional inference is from predictions via a bioinformatic approach and therefore depend on assumptions of functional equivalence of 16S gene matches. Our functional predictions serve as a preliminary indication that function may be maintained amidst structural variation and require further testing via metagenomic or metatranscriptomic approaches.

The results of our study support our first hypothesis that newt bacterial communities would exhibit strong temporal variation with respect to their taxonomic structure within the aquatic phase, as well as across the aquatic-to-terrestrial transition. However, the relative stability with respect to their predicted *Bd*-inhibitory function fails to support our second hypothesis that structural and functional variation would be linked. In the context of disease dynamics, these data suggest that protection from disease may be maintained despite taxonomic variability in microbiota, highlighting the potential un-coupling of community structure and function (Purahong *et al.*, 2014) and complexity of host microbial community dynamics. Owing to the importance of beneficial microbes in pathogen defense, maintenance of protective microbial function through time is likely essential for continuous defense against pathogens (Rosenberg *et al.*, 2007; Bletz *et al.*, 2013; Fraune *et al.*, 2014). Perhaps this maintenance of function allows some amphibian species, like those studied here, to resist cutaneous fungal pathogens. For salamandrid newts and North American salamanders, in particular, continuous protection from the emerging pathogen, *Bsal*, (Martel *et al.*, 2014; Spitzen-van der Sluijs *et al.*, 2016) may be critical for population survival. Understanding dynamics of amphibian skin microbiota can facilitate development of probiotic bioaugmentation strategies that can afford protection for these threatened vertebrates. Future studies using metagenomics and metatranscriptomics will further elucidate our understanding of host–microbe interactions and the structure–function relationship within amphibian skin microbiomes, as well as other wildlife hosts threatened by disease.

Data accessibility

Sequence data are deposited in the Sequence Read Database (SRP074714; Bioproject PRJNA320969).

Conflict of Interest

The authors declare no conflict of interest.

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