Symbiotic skin bacteria as a source for sex-specific scents in frogs

Andrés E. Brunetti[a,1], Mariana L. Lyra[b], Weilan G. P. Melo[a], Laura E. Andrade[a], Pablo Palacios-Rodríguez[c], Bárbara M. Prado[a], Célio F. B. Haddad[b], Mônica T. Pupo[a], and Norberto P. Lopes[a,1]

[a]Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-903 Ribeirão Preto, SP, Brazil; [b]Departamento de Zoologia e Centro de Aquicultura, Instituto de Biociências, Universidade Estadual Paulista, 13506-900 Rio Claro, SP, Brazil; and [c]Departamento de Ciencias Biológicas, Universidad de los Andes, AA4976 Bogota DC, Colombia

Edited by John G. Hildebrand, University of Arizona, Tucson, AZ, and approved December 19, 2018 (received for review April 22, 2018)

Amphibians are known to possess a wide variety of compounds stored in their skin glands. While significant progress has been made in understanding the chemical diversity and biological relevance of alkaloids, amines, steroids, and peptides, most aspects of the odorous secretions are completely unknown. In this study, we examined sexual variations in the volatile profile from the skin of the tree frog *Boana prasina* and combined culture and culture-independent methods to investigate if microorganisms might be a source of these compounds. We found that sesquiterpenes, thioethers, and methoxypyrazines are major contributors to the observed sex differences. We also observed that each sex has a distinct profile of methoxypyrazines, and that the chemical origin of these compounds can be traced to a *Pseudomonas* sp. strain isolated from the frog's skin. This symbiotic bacterium was present in almost all individuals examined from different sites and was maintained in captive conditions, supporting its significance as the source of methoxypyrazines in these frogs. Our results highlight the potential relevance of bacteria as a source of chemical signals in amphibians and contribute to increasing our understanding of the role that symbiotic associations have in animals.

amphibia | Anura | bacterial community diversity | chemical ecology | smells

Chemical signaling is regarded as the most ancient mode of communication (1). Because all living organisms emit, detect, and respond to chemical cues, there is an enormous number and diversity of potential chemical interactions within and between species across all kingdoms, from bacteria and archaea to plants and animals (1–3). Several vertebrate species are known to produce volatile substances that convey information related to species and kin recognition, as well as recognition and assessment in sexual interactions, thus mediating social and sexual behavior (4). Like other substances used in communication, volatiles come from four main sources: (*i*) de novo synthesis, generally in specific secretory glands; (*ii*) metabolic by-products released with excreted material; (*iii*) environmental sequestration; and (*iv*) the products of microbial symbionts (2).

In particular, the "fermentation hypothesis" proposed in the 1970s states that symbiotic bacteria in mammals metabolize proteins and lipids occurring in the scent glands, producing volatile compounds used by their host to communicate (5). Recent advances in various "Omic" technologies (6) have enabled scientists to examine this hypothesis in different mammal species (7–9). However, this hypothesis is not supported by empirical evidence in other vertebrate groups (8), which hinders its generalization, as well as the examination of the coevolution of host–microbe interactions.

In anuran amphibians (frogs and toads), communication has traditionally been assumed to rely almost exclusively on acoustic signals, whereas other sensory modalities were considered minor subjects (10). However, these assumptions have been recently challenged, as we now know that several families use visual signals (10), which are often accompanied by acoustic signals in multimodal (multisensory) displays (11, 12). In addition, behavioral, morphological, and chemical evidence suggest that chemically mediated interactions could actually be much more common and phylogenetically widespread in anuran amphibians than what had traditionally been thought (13–16). In particular, the skin glands distributed along the body in hundreds of species secrete volatile compounds with characteristic odors (17–19). The few studies that have investigated these volatile secretions suggest that compounds likely come from two different sources: de novo synthesis by anuran amphibians and environmental sequestration (20, 21). Alternatively, just as they occur in mammals, volatile compounds may originate from interaction with the rich microbiota that inhabit amphibian skin (22, 23). Unraveling this interaction could help to solve diverse ecological questions, like the impact of symbiotic bacteria in individual recognition and mate choice (8, 24).

To address some of these questions, we used the South American tree frog *Boana prasina* as a biological model (*SI Appendix*, Fig. S1). This species has a prolonged reproductive period with males displaying a rich vocal repertoire in different social contexts (25, 26) and, like in other members of the *B. pulchella* group, emits a strong and characteristic smell (19, 21). In a prior study, we found that the volatile secretion in two other species of this group is formed by a blend of 35–42 compounds from nine different chemical classes. Although no functional study has yet been conducted, the variety of components of the secretion suggests that they may be linked to

Significance

Symbiotic microbes play pivotal roles in different aspects of animal biology. In particular, it has been increasingly recognized that they may produce molecules used by their host in social interactions. Herein, we report that symbiotic bacteria in amphibians can account for some odorous compounds found in the host. We found that sex-specific scents in a common South American tree frog can be traced to a class of compounds with strong odor properties produced by a bacterium isolated from the frog's skin. This insight challenges our appreciation of the role of microorganisms in amphibians and not only reveals exciting perspectives into the analysis of a frog's skin secretion, but also into the association and coevolution of host–microbe interactions in animals.

Author contributions: A.E.B., M.L.L., C.F.B.H., M.T.P., and N.P.L. designed research; A.E.B., M.L.L., W.G.P.M., B.M.P., and C.F.B.H. performed research; A.E.B., M.L.L., L.E.A., P.P.-R., and N.P.L. analyzed data; and A.E.B., M.L.L., and L.E.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the Sequence Read Archive, www.ncbi.nlm.nih.gov/sra (Bioproject ID PRJNA498895). The sequences reported in this paper have been deposited in Genbank, https://www.ncbi.nlm.nih.gov/genbank/ (accession nos. MK100853–MK100897).

[1]To whom correspondence may be addressed. Email: andresbrunetti@gmail.com or npelopes@fcfrp.usp.br.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1806834116/-/DCSupplemental.

different functions, including defense and reproduction (21). Here, we report sex-specific odor profiles in *B. prasina*, identify the chemical classes that account for such differences, and examine through culture and culture-independent methods whether the skin microbiota could be the source of these sex-specific components. The results presented here have implications for the role of the skin microbiome in amphibians and highlight the relevance of symbiosis on chemical communication in less-studied vertebrates.

Results

Diversity of Volatile Components in the Skin of *B. prasina*. Solid-phase microextraction (SPME) and gas chromatography/mass spectrometry (GC/MS) analyses revealed that the volatile skin secretion of adult males and females of *B. prasina* is a multicomponent blend of 60–80 compounds including alcohols, aldehydes, alkenes, ethers, ketones, methoxypyrazines (MOPs), terpenes [hemiterpenes, monoterpenes, and sesquiterpenes (SQTs)], and thioethers (TOEs) (Fig. 1, Table 1, *SI Appendix*, Fig. S2, and Dataset S1 *A–E*). Our results show that regardless of the sex, the mean relative abundance of hemiterpenes, ketones, and alcohols combined make a large contribution (49.9–78.3%) to the overall volatile composition in the species (Table 1). Hemiterpenes and ketones were relatively unaffected by the sampling procedures (i.e., in vivo or skin sampling), whereas alcohols were only abundant when using skin sampling (Table 1).

Sex Differences in the Composition of the Volatile Secretion. Principal component analysis (PCA) revealed that SQTs, TOEs, and MOPs contributed the most to the observed variations between males and females (Fig. 2 *A* and *B*). The created linear discriminant analysis (LDA) function shows that frogs can be discriminated by sex based on their volatile profile using one dimension (Kruskal–Wallis test: DF = 1, $P = 0.001$; Fig. 2*C*). Of these three compound classes, MOPs were, on average, more abundant in females than males, regardless of the sampling procedure [likelihood ratio tests (LRTs): interaction: $\chi^2_1 = 1.202$; $P = 0.2729$; sex: $\chi^2_1 = 12.778$; $P = 0.0004$; Table 1]. However, relative abundance of SQTs were higher in males than in females, but only in in vivo sampling [LRTs: interaction $\chi^2_1 = 23.817$; $P < 0.0001$; $\text{sex}_{(\text{in vivo sampling})}$: $\chi^2_1 = 22.933$; $P < 0.0001$; $\text{sex}_{(\text{skin sampling})}$: $\chi^2_1 = 1.317$; $P = 0.2511$; Table 1]. Similarly, the relative abundance of TOEs was higher in males than in females only in in vivo sampling [LRTs: interaction $\chi^2_1 = 10.618$; $P = 0.0011$; $\text{sex}_{(\text{in vivo sampling})}$: $\chi^2_1 = 10.9505$; $P = 0.0009$; $\text{sex}_{(\text{skin sampling})}$: $\chi^2_1 = 2.0051$; $P = 0.1568$; Table 1]. When considering the chemical composition within SQTs and TOEs, we noticed that only one compound contributed mainly to the total abundance in each of these two classes, namely, dihydroedulan II and (2E)-4-(methylsulfanyl)-2-pentene, respectively (Dataset S1*A*).

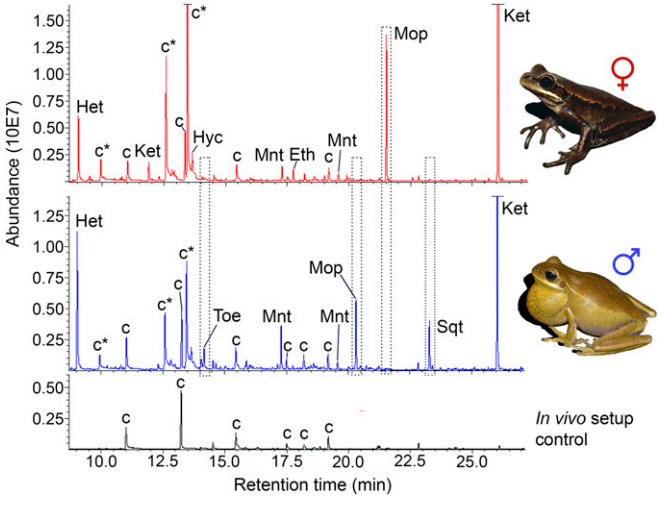


Fig. 1. GC/MS total ion current chromatograms showing volatile profiles for *B. prasina* females (*Top*) and males (*Middle*) after in vivo sampling procedure. The compound classes TOEs (Toe), MOPs (Mop), and SQT (Sqt) (dotted lines/boxes) show significant sex differences. Peaks identified as c depict substances also occurring in control samples (*Bottom*). Peaks identified as c* depict aromatic compounds also occurring in terraria (*SI Appendix*). Eth, ethers; Het, hemiterpenes; Hyc, hydrocarbons; Ket, ketones; Mnt, monoterpenes.

Table 1. Mean relative abundance (±SEM) of the chemical classes identified in the volatile secretion of *B. prasina*

	In vivo procedure (mean % ± SEM)		Skin sampling procedure (mean % ± SEM)	
Compound class	Males ($n = 14$)	Females ($n = 3$)	Males ($n = 6$)	Females ($n = 4$)
Hemiterpenes	27.7 ± 2.9	28.6 ± 18.9	25.4 ± 4.6	16.4 ± 1.5
Ketones	21.1 ± 4.3	34.5 ± 14.4	15.0 ± 3.4	16.9 ± 10.0
Alcohols	1.1 ± 0.3	0.3 ± 0.3	37.8 ± 6.6	29.7 ± 6.6
MOPs	3.3 ± 0.6	26.1 ± 4.9	7.3 ± 0.8	23.3 ± 4.6
SQT	8.8 ± 2.1	0.1 ± 0.1	1.3 ± 0.8	2.7 ± 1.1
TOEs	4.3 ± 1.0	0.4 ± 0.4	0.2 ± 0.1	0.1 ± 0.0
Aldehydes	2.7 ± 0.4	1.2 ± 0.8	1.5 ± 0.3	1.5 ± 0.3
Ethers	1.5 ± 0.3	1.5 ± 0.9	1.4 ± 0.4	1.5 ± 1.3
Hydrocarbons	5.2 ± 1.5	2.4 ± 1.2	3.8 ± 1.8	3.7 ± 0.8
Monoterpenes	21.7 ± 3.3	4.0 ± 2.0	4.2 ± 0.9	3.8 ± 0.8
NIs	2.5 ± 0.6	0.8 ± 0.8	2.0 ± 0.8	0.3 ± 0.2

NIs represents compounds that were not identified and, thus, could not be assigned to any specific chemical class.

Males and Females Exhibit a Distinct Profile of MOPs. In addition to the higher percentage of total MOPs in females, we also detected differences within the four MOPs (MOP **1**: 2-isopropyl-3-methoxypyrazine, MOP **2**: 3-isopropyl-2-methoxy-5-methylpyrazine, MOP **3**: 2-*sec*-butyl-3-methoxypyrazine, and MOP **4**: 3-*sec*-butyl-2-methoxy-5-methylpyrazine) occurring in both males and females (Figs. 3 and 4*A* and Dataset S1 *F–H*). We found that MOP **3** was the major MOP in males and was poorly represented in females (Fig. 3). The relative abundance of this compound was much higher in males than in females, regardless of the sampling procedure [(LRTs: interaction $\chi^2_1 = 0.1219$; $P = 0.727$; sex $\chi^2_1 = 49.4548$; $P < 0.0001$), with no significant differences found between sampling procedures (LRTs: sampling procedure $\chi^2_1 = 1.07$; $P = 0.3$); Fig. 3]. In contrast, MOP **4** was the major MOP in females. The relative abundance of this compound was much higher in females than in males in both sampling procedures (Fig. 3), but the difference between sexes was larger in in vivo samples than in skin samples [LRTs: interaction $\chi^2_1 = 5.3977$; $P = 0.0202$; $\text{sex}_{(\text{in vivo sampling})}$: $\chi^2_1 = 25.5523$; $P < 0.0001$; $\text{sex}_{(\text{skin sampling})}$: $\chi^2_1 = 33.1275$; $P < 0.0001$].

MOP **1** and MOP **2** were less represented in the total abundances of MOPs in both sexes (0–12.8%). MOP **1** showed higher percentages in males than in females [absent in in vivo females; $\text{sex}_{(\text{skin sampling})}$: $\chi^2_1 = 21.766$; $P < 0.0001$; Fig. 3], whereas the relative abundance of MOP **2** was higher in females (LRTs: interaction $\chi^2_1 = 0.0181$; $P = 0.8929$; sex $\chi^2_1 = 9.829$; $P = 0.0017$; Fig. 3). This latter compound showed a higher relative abundance in skin samples than in those obtained by in vivo sampling (LRTs: sampling procedure $\chi^2_1 = 4.621$; $P = 0.0316$; Fig. 3).

A Skin-Associated *Pseudomonas* Produces the Same MOPs Found in Frogs. Of the three components responsible for sex differences, TOEs and MOPs are compounds typically produced by microorganisms. To explore whether this is the case in *B. prasina*, we isolated,

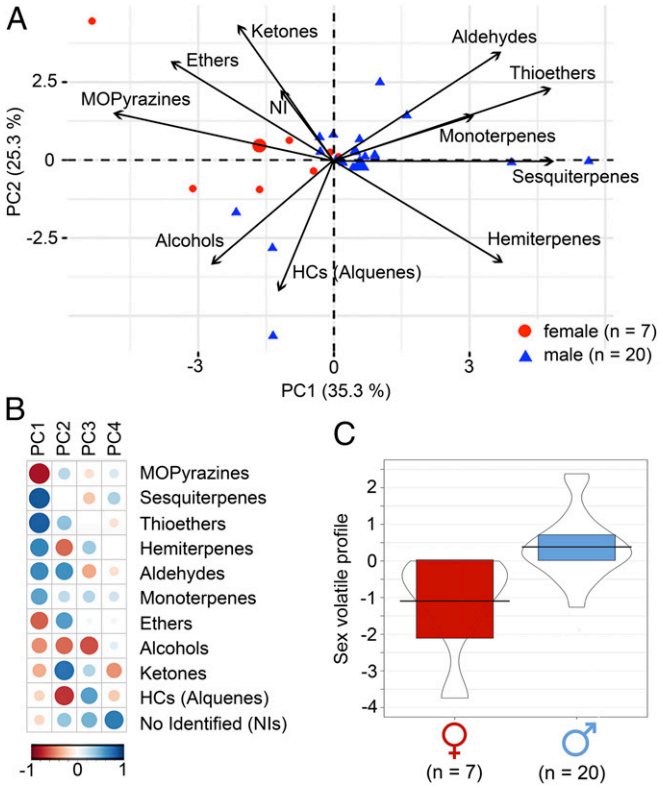


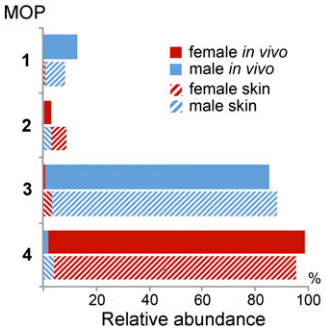
Fig. 2. Differences in the skin volatile profile of females and males of the tree frog *B. prasina*. (*A*) Biplot of PCA performed on relative abundance of volatile compounds. Centroids of specimens' dispersion are depicted as a big circle for females and a triangle for males. (*B*) Relative contribution of each compound class to the first four principal components obtained in the PCA analysis. The absolute value of each contribution is depicted according to the size of the circle, whereas blue and red colors show positive and negative contributions, respectively. (*C*) Discriminant function (=Sex Volatile Profile). Confidence intervals and the medians obtained from Bayesian inferences are depicted as color boxes and horizontal lines, respectively. The shape around the rectangles represents the complete data distribution in each group.

cultured, and identified skin-associated bacteria and analyzed their volatile composition by SPME-GC/MS. Forty-one bacterial morphotypes were obtained from eight males and one female and were identified based on sequence databases (Dataset S2*A*). The analysis of their volatile metabolites revealed the presence of 128 compounds, 16 of which occur in both bacteria and frogs (Dataset S2 *B* and *C*), mainly hemiterpenes (3 compounds) and ketones (4 compounds), which were mostly produced by different bacteria. We also found that five TOEs occurred in several of the isolated morphotypes (Dataset S2*B*), but none of them were analogous to those found in the frogs (Dataset S1*A*).

In contrast, the same four MOPs present in males and females (MOPs **1**–**4**) were found to be produced by a single bacterium isolate (Fig. 4 and Dataset S2 *D*–*F*), which was identified as *Pseudomonas* sp. (Dataset S2*A*). Other major volatile metabolites identified in this bacterium were four additional MOPs (MOPs **5**–**8**, Fig. 4*A*) and other pyrazines, none of which have been detected in frogs (Datasets S1*A* and S2*B*). We also observed that the relative abundances of MOPs in this *Pseudomonas* sp. sample varied in comparison with those detected in the frogs (Dataset S2*F*). MOP **5** (2-methoxy-3,5-dimethylpyrazine) was the major MOP compound (82.4 ± 16.7%) found in this bacterium. When considering the four MOPs shared with the frogs (MOPs **1**–**4**), MOP **2** exhibited higher abundances (5.9 ± 6.9%), while both MOP **3** and MOP **4**, the major MOPs of male and female frogs, respectively, showed lower and similar abundances (MOP **3** = 2.6 ± 5.0%, MOP **4** = 2.7 ± 2.1%).

The MOP-Producing *Pseudomonas* Occurs in All Specimens from Different Sites Surveyed and Is Maintained in Captive Conditions. The general structure of skin bacterial communities varies among sites and lengths of time in captivity (Fig. 5 *A* and *B*, *SI Appendix*, Fig. S3, and Dataset S3 *C* and *D*). Despite this variation, more than half of the relative abundance of operational taxonomic units (OTUs) observed in the skin bacterial communities of four sites (60–63%) and in five different captivity times (56–66%) can be explained by members of seven and nine genera from the orders Enterobacteriales, Pseudomonadales, Methylophilales, Oceanospirillales, Actinomycetales, Bacillales, Alteromonadales, and Burkholderiales, respectively (Dataset S3 *E*–*H*). Among them, the genera *Klebsiella* (Enterobacteriales) and *Pseudomonas* (Pseudomonadales) were the two most prominent OTUs shared by all sites (relative abundance 7–30%) and were also abundant in all captive frogs (relative abundance 4–35%; Fig. 5 *A* and *B*). We found that OTU richness is not very different among the sites surveyed (mean number of observed OTUs site A: 115 ± 31, site B: 113 ± 17, site C: 161 ± 20, and site D: 143 ± 24; Dataset S3*C*). We also found that the OTU richness was larger at the beginning of the captivity, decreasing in subsequent periods (mean no. of OTUs 1-d: 163 ± 26, 3-d: 145 ± 35, 10-d: 100 ± 9, 2-wk: 104 ± 41, 8-mo: 155 ± 34; Dataset S3*D*).

Within the genus *Pseudomonas*, the haplotype network showed that although some OTUs were exclusively found in one site (Fig. 5*C*; e.g., *P. umsongensis*, haplotype 34) or only under captive conditions (Fig. 5*D*; e.g., *P. stutzeri*; haplotypes 32 and 33), four strains occurred in most of the samples and were common to all categories independent of the geographical origin of the frogs and captivity times (Fig. 5 *C* and *D*). These OTUs correspond to the MOP-producing *Pseudomonas* (haplotypes 11 and 12), *Pseudomonas* sp. (occurring also in environmental samples; haplotypes 17 and 18), *P. veronii* (haplotypes 35 and 36), and *P. fragi* (haplotypes 26 and 27). When specifically examining the MOP-producing *Pseudomonas*, we found that this bacterium was present in most field samples from all of the sites (n = 10 of 11 males, n = 1 female) and in samples from all of the different captivity times (n = 6 males) (Fig. 5). The mean relative abundance of this bacterium in male samples collected in the field was 1.7% (CI95% 0.6–2.8; Dataset S3*A*), and although we were only able to analyze one female sample, its relative abundance (2.4%; Dataset S3*A*) was similar to that observed in the males. We also detected variations in the abundance of the MOP-producing *Pseudomonas* during the time in captivity, which was particularly evident in the first 3 d immediately after a frog's collection (1 d = 8.8 ± 4.3%, 3 d = 0.4 ± 0.2%; Dataset S3*B*).



In vivo			
MOP	females (n = 3)	males (n = 14)	Sign. *P* value
1	0	12.8 ± 1.9	-
2	2.8 ± 0.9	0.5 ± 0.3	P = 0.0017
3	0.5 ± 0.3	84.8 ± 2.4	P < 0.0001
4	96.7 ± 0.9	1.9 ± 1.6	P < 0.0001

skin			
MOP	females (n = 4)	males (n = 6)	Sign. *P* value
1	0.4 ± 0.3	7.8 ± 1.2	P < 0.0001
2	5.6 ± 2.0	3.0 ± 1.5	P = 0.0017
3	2.6 ± 1.8	85.2 ± 3.9	P < 0.0001
4	91.4 ± 0.8	4.0 ± 2.6	P < 0.0001

Fig. 3. MOPs identified in females and males of *B. prasina*. Graphical representation (*Left*) and table with mean, SEM, and *P* values (*Right*) of the relative abundance of each MOP (abundance of MOP#/overall abundance of MOPs, where # = 1, 2, 3, or 4 ± SEM). All MOPs have significant sexual differences.

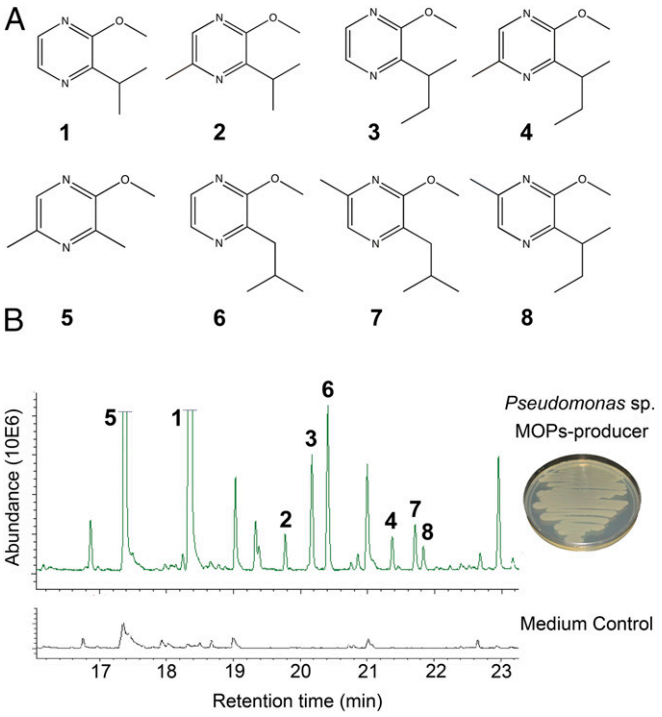


Fig. 4. (*A*) MOPs found in males and females of *B. prasina* (**1**–**4**) and in the skin-associated *Pseudomonas* sp. (**1**–**8**) (*B*) GC/MS total ion current chromatogram showing production of MOPs **1**–**8** (*Left*) by *Pseudomonas* sp. cultivated on Mueller–Hinton agar medium (*Right*). Their relative abundances are shown in Dataset S2 *D*–*F*.

Discussion

Hundreds of amphibian species from different lineages worldwide are known to secrete strong and characteristic smells from their skin, but the biological function and origin of their components remain largely unknown (17, 19). Using a multidisciplinary approach, we examined sex differences in the volatile profile of the tree frog *B. prasina* and explored whether the compounds responsible for these differences have a bacterial origin. Consistent with previous descriptions in two related species of the *B. pulchella* Group (21), our results showed that the skin secretion of males and females of *B. prasina* is rich in volatile chemicals, including at least 10 different compound classes. Our results also showed that the volatile profile of this species varies with sex and, most notably, that some of the compounds responsible for the sex variation are derived from symbiotic bacteria.

The greater abundance of SQTs and TOEs in males than in females may be associated with functions such as female attraction, male-male competition, and aggression (see below in this section). Among terrestrial vertebrates, these compound classes are known to mediate sexual behavior in mammals (27, 28). With regard to their origin, SQTs are likely obtained from environmental sources. A previous study has demonstrated that SQTs in the skin of the Australian tree frog *Ranoidea caerulea* (20) can be sequestered from its diet, thus suggesting that the diet of males and females of *B. prasina* might differ in the content of SQTs. Because sulfur derivatives are widespread across bacterial taxa (29, 30), it is plausible that a frog's skin bacteria may be involved in the synthesis of TOEs. Although we have identified TOEs in the bacterial isolates, none of them was identical to the TOEs found in the frogs. These results can be explained by different (albeit not mutually exclusive) possibilities, including: (*i*) the bacterial production of frog TOEs only under certain substrate conditions; (*ii*) the sequestration of TOEs from the bacteria and subsequent metabolization by the frogs; (*iii*) the production of frog TOEs by unidentified bacteria; or (*iv*) the synthesis of TOEs by the frogs.

MOPs presented marked sex differences in *B. prasina* and were found to be major constituents of a skin symbiotic *Pseudomonas* sp. Pyrazines are widely distributed in nature, possess intense odors, and are some of the major volatile compounds produced by bacteria (29, 30). They likewise mediate communication in different organisms such as bacteria (31), insects (32, 33), and mammals (34, 35). Particularly, MOPs have been described as mate attractants and aposematic signals in insects (32, 36). However, within vertebrates, they had only been reported in two other species of *Boana* (21). The biosynthesis of pyrazines is still an open debate with different pathways proposed (33, 37). Specifically, animals are not known to synthesize MOPs, but instead, their biosynthetic pathways have been described in two species of *Pseudomonas*, namely, *P. perolens* (38) and *P. taetrolens* (39), and in plants (40). In this scenario, it seems likely that symbiotic bacteria are the source of MOPs in frogs, whereas in insects, they could be derived either from symbiotic bacteria or, especially for phytophagous species, sequestered from plants.

Two biosynthetic pathways have been suggested for MOPs, and current evidence indicates that either pathway is possible (38–41). Both involve the condensation of one amino acid (e.g., leucine, isoleucine, and valine) with either another amino acid (e.g., glycine) or glyoxal. Following either pathway, the synthetic origin of MOPs reported here (Fig. 4) could be traced to valine (MOPs **1** and **2**), isoleucine (MOPs **3**, **4**, and **8**), alanine (MOP **5**), and leucine (MOPs **6** and **7**). Since our results showed that the same MOPs found in *B. prasina* are produced by the symbiotic *Pseudomonas* sp.,

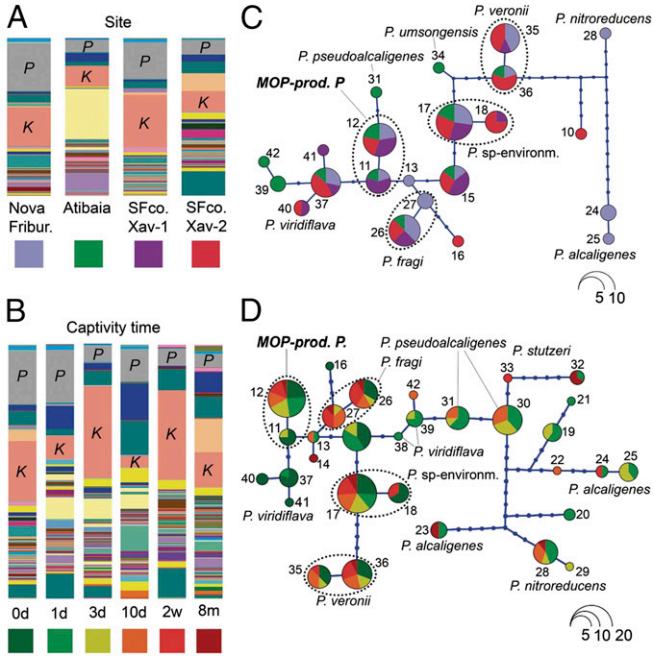


Fig. 5. Skin bacterial community structure of *B. prasina* and haplotype network of OTUs assigned to the *Pseudomonas* genus. (*A*) Bacterial community in frogs from four sites. (*B*) Bacterial community in frogs maintained in captive conditions at the time of arrival in the laboratory (0 d), at days 1, 3, and 10 in the laboratory (1d, 3d, 10d), and after 2 wk (2w) and 8 mo in the laboratory (8m). (*C* and *D*) Networks showing *Pseudomonas* haplotypes that are common (encircled by dashed lines) or unique among sites and captivity times. Circle sizes are proportional to the number of individuals that present the OTU and lines connecting haplotypes represent one mutational step. Small black dots in the network represent additional mutational steps. Most abundant classes of OTUs are indicated in capital letters, *Klebsiella* (K) or *Pseudomonas* (P). Complete OTU tables are available in Dataset S3 *E*–*H*.

we can hypothesize that the characteristic sex profile of MOPs in frogs might be a consequence of distinct microenvironmental and/or metabolic conditions in males and females. This hypothesis is supported by experimental evidence showing that the production of volatile compounds in microorganisms is strongly influenced by environmental variables like nutrient availability and metabolic interactions with the host (33, 42). Furthermore, the variation in the MOP profile of *Pseudomonas* sp. in culture mediums and in the frogs themselves may be due to strong differences in both environments, among them, a high content of nutrients without competition in cultures vs. limited nutrient supply with several competitors in the frogs (42). Similar reasoning applies to the volatile profile of other bacteria isolated from *B. prasina*.

Our study suggests that the environment may affect the structure of the bacterial community and the composition of the *Pseudomonas*. However, it also reveals a similar bacterial composition across samples. In particular, it showed that frogs from all sites and times under captive conditions shared four *Pseudomonas* lineages, which might constitute part of the core microbiome of *B. prasina.* One of them is the MOP-producing *Pseudomonas* sp., whereas the others are symbiotic bacteria that might affect different aspects of their host's biology. It has been proposed that the particular composition of amphibian skin, such as the presence of several classes of biomolecules, acts as a host filtering mechanism, thus creating a unique skin-associated bacterial community (23, 43, 44). Subsequently, natural selection may act on the hosts and their associated microbiota. Indeed, mammalian species would have a strong selection for gland microenvironments favorable to odor-producing bacteria (7). Lastly, the volatile compounds emitted by the MOP-producing *Pseudomonas* sp. in the frog's skin can act as infochemicals mediating interactions with other members of the bacterial community, and reciprocally, the bacterial community may have effects on the production of MOPs. Because our experimental design was not planned to evaluate sex differences in the bacterial community, future studies are crucial to examine the potential effects of other skin microorganisms in the MOP profile of males and females of *B. prasina*.

Although chemical communication in anurans may be favored in species that lack acoustic signals, most species in which communication by chemical signals was suggested based on morphological (10, 15, 16), chemical (13, 14, 45), and/or behavioral (13, 14, 45) evidence also vocalize. Our results, along with a vocalization analysis and field behavioral observations published for *B. prasina* (25, 26), indicate that the reproductive behavior of this species may include a combination of chemical and acoustical signals. Examples of these signal's interactions in anurans could be found in: (*i*) female recognition by satellite and vocalizing males (45), (*ii*) male recognition and assessment by females (45), (*iii*) female mate assessment, and (*iv*) recognition of competing males. Because behavioral studies in amphibians have focused almost exclusively on the acoustic components, our study points out the need to address the role of chemical signaling within a more comprehensive context that includes signals from different sensory modalities.

As previously seen in insects and mammals (6–8), our analyses support the concept that symbiotic bacteria are involved in the production of frog's volatile compounds, which might act as chemical signals in distinct social interactions. Similar cases may also occur in other amphibians. For instance, macrolides, which are polyketide natural products typically produced by microorganisms, have also been reported functioning as sexual pheromones in mantellid frogs (13). In mammals, the host–symbiont interaction leading to the emission of particular odors is called fermentation hypothesis, because the compounds are known products of bacterial fermentation (5). However, to include other microbial metabolites that may participate in host chemical communication, a broader terminology is needed. Our results illustrate that studies on the ecological role of host–microbiome associations in amphibians are in their early infancy and delineate some crucial questions to increase our understanding of this interaction.

Materials and Methods

Full experimental details including citations are provided in the *SI Appendix*.

Specimen Collecting and Handling. Adults of the hylid tree frog *B. prasina* were collected at night from four sites in Brazil: Nova Friburgo, Rio de Janeiro (site A: 22°16′55″S, 42°36′18″W); two sites in São Francisco Xavier, São Paulo (site B: 22°55′29″S, 45°53′14″W; site C: 22°52′35″S, 45°56′7″W), and Atibaia, São Paulo (site D: 23°9′11″S, 46°30′42″W). Males were distinguished from females based on the presence of pigmented vocal sacs. Individuals were transported to the laboratory and were kept in glass terraria and provided with dechlorinated tap water. Various different plant species and rocks that serve as refugees were added. Frogs were kept at 22–26 °C, with a 14:10 h (light/dark) period, and fed with crickets.

Bacterial Cultures of Skin Microbiota. Isolation of microorganisms was performed through swabbing the dorsal skin from two groups of specimens. In the first group, two males and one female were collected in site A and swabbed in the field. Each swab was suspended in 5 mL of TSB medium (Trypticase Soy Broth-BD) and kept at room temperature for 96 h. Then, in the laboratory, a 100-μL aliquot of each tube was transferred to agar plates with TSA (Trypticase Soy Agar-BD) or ISP2 (International *Streptomyces* Project) medium. In the second group, six males were collected in site B and swabbed in the laboratory, 2 d (three individuals) or 2 wk (three individuals) after collection of the specimens. The swabs were directly scrubbed onto ISP2 media plates. All media were supplemented with antifungal agents (nystatin: 0.04 g/L; cycloheximide: 0.05 g/L), and plates were incubated in biochemical oxygen demand (BOD) at 28 °C for 3 d. Different bacterial colonies were identified according to distinct morphotypes and were preserved in a liquid ISP2 medium with 30% glycerol at −80 °C for posterior identification by DNA sequencing and volatile analysis.

Volatile Surveys of Frogs and Bacteria. In vivo sampling and skin sampling procedures were employed for volatile surveys of frogs as described (21). Fourteen males and three females were examined for in vivo sampling, and six males and four females were examined for skin sampling. For volatile surveys of bacteria cultures, a bioassay system consisting of the bottoms of two lidless Petri dishes laid in opposing positions and sealed with two layers of thin plastic film (Parafilm; Bemis NA) was used. After culturing the bacteria for 72 h, the headspace was sampled by inserting a SPME into this system. All volatile analyses were undertaken on a Shimadzu GC coupled to a Mass Spectrometer Detector (GC/MS QP2010 Plus). Data analyses were performed using the Shimadzu GCMS solution software Version 2.53 SU3, which includes the NIST21 and NIST107 Mass Spectral Libraries.

Statistical Analyses of Volatile Compounds in Frogs. For statistical analysis, we used the relative abundances for all compounds. This abundance was calculated as the ratio of the area of each compound over the area of all compounds combined. The area of each chemical class was determined as the sum of all compounds within its category. Multivariate analyses approaches (PCA and LDA) were used to assess whether females and males can be discriminated based on their volatile profile and to identify whether any of the compound classes is most likely to be associated with one sex rather than the other.

Generalized least squares (GLS) models were used to test differences between sexes in those compound classes identified by PCA (i.e., SQTs, TOEs, and MOPs). Two fixed factors (sex and sampling procedure) and the two-way interaction between those factors were tested using a model selection approach that compares nested models with a likelihood ratio test, as suggested by Zuur et al. (46). For each of these three compound classes, we used models that accounted for the observed heterogeneity of variance. In addition, we analyzed differences between sexes in relation to the relative abundance of each of the four MOPs identified in the frogs using GLS models. All analyses were conducted with the help of the software R-studio. See *SI Appendix* for description of the models.

Identification of Bacterial Cultures and Dorsal Skin Bacterial Community Assessment. An aliquot of 100 μL of each isolated bacterium was transferred to a 1.5-mL microcentrifuge tube with ISP2 medium. After 24–36 h, the cells were centrifuged for 1 min at 10,000 × *g* and genomic DNA extraction was conducted using the ammonium acetate precipitation method (47). Bacterial 16S rDNA was amplified and sequenced using the bacterial 16S rDNA primers 27F and 1492R (48). Amplified fragments were purified and sent to Macrogen Inc. for sequencing. The sequences were quality verified and trimmed using Geneious V.6 (49), and the preliminary identification of each bacterium was performed using the online BLASTN 2.7.0 (50) and the 16S ribosomal RNA (Bacteria and Archaea) Database. New sequences were submitted to GenBank (Dataset S2*A* and BioProject ID PRJNA498895).

ECOLOGY

For the skin bacterial community profiling, we followed procedures described in Bletz et al. (51) (see *SI Appendix* for a full description of methods). Since we were interested in exploring the ecological role of the isolated MOP-producing *Pseudomonas* sp. strain in amphibian skin, we used the filtered OTU table (not rarefied) to verify the presence of the *Pseudomonas* sp. strain in each sample. We also explored *Pseudomonas* diversity by constructing a haplotype network with the OTU sequences classified by QIIME. This approach allowed us to access the geographical and temporal diversity and similarities of the *Pseudomonas* strains in amphibian skin and to take into account possible population variation of the isolated MOP-producing *Pseudomonas* sp. strain. For the haplotype network, we used Haploviewer (www.cibiv.at/~greg/haploviewer) and a maximum parsimony tree was inferred using the Phylip 3.695 package (52) with the DNApars extension. Sequences that differed in only one base pair were considered a variation within the same strain.

Animal Use and Care. Collection permits were issued by the Instituto Chico Mendes de Conservação da Biodiversidade/Biodiversity Information and Authorization System (SISBIO)/National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) (Permits 41508-8, 50071-1, 50071-2, A1FC113). Experimental procedures were performed according to the regulations specified by the Conselho Nacional de Controle de Experimentação Animal and Ministério da Ciência, Tecnologia e Inovação, Brazil and were approved by the Ethics Committee on Animal Use (CEUA) of Universidade Estadual Paulista (N#36/2015) and the Pharmaceutical Sciences of Ribeirão Preto-CEUA (N#17.1.1074.60.0). Voucher specimens are housed in the herpetological collection of C.F.B.H., Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil (*B. prasina*: CFBH 41014–41033).

ACKNOWLEDGMENTS. We thank Carlos Taboada, Eduardo Silva-Junior, Lisa Dillman, Eugenia Sanchez, Heidi Parker, Diego Baldo, Julian Faivovich, Javier Pascual, and two anonymous reviewers for comments and discussion; Larissa Rolim, Miriam Vera, Lucas Mariotto, Pedro Taucce, Celeste Luna and Natalia Salles for their support in the field work; and Dr. Hisakatsu Iwabuchi generously provided some authentic reference samples. This research was supported by São Paulo Research Foundation Grants 2013/50741-7, 2014/50265-3, and 2013/50954-0; postdoctoral fellowships 2014/20915-6 and 2017/23725-1 (to A.E.B.), 2017/26162-8 (to M.L.L.), and 2015/01001-6 (to W.G.P.M.); and University of São Paulo Grant 2012.1.17587.1.1. (to N.P.L.). We also acknowledge the Brazilian federal funding agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico for research fellowships and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

1. Wyatt TD (2014) *Pheromones and Animal Behavior* (Cambridge Univ Press, Cambridge, UK), 2nd Ed.
2. Bradbury JW, Vehrencamp SL (1998) *Principles of Animal Communication* (Sinauer Associates, Sunderland, UK).
3. Meinwald J, Eisner T (2008) Chemical ecology in retrospect and prospect. *Proc Natl Acad Sci USA* 105:4539–4540.
4. Johansson BG, Jones TM (2007) The role of chemical communication in mate choice. *Biol Rev Camb Philos Soc* 82:265–289.
5. Albone ES, Perry GC (1976) Anal sac secretion of the red fox, *Vulpes vulpes*; volatile fatty acids and diamines: Implications for a fermentation hypothesis of chemical recognition. *J Chem Ecol* 2:101–111.
6. Brunetti AE, et al. (2018) An integrative omics perspective for the analysis of chemical signals in ecological interactions. *Chem Soc Rev* 47:1574–1591.
7. Theis KR, et al. (2013) Symbiotic bacteria appear to mediate hyena social odors. *Proc Natl Acad Sci USA* 110:19832–19837.
8. Ezenwa VO, Williams AE (2014) Microbes and animal olfactory communication: Where do we go from here? *BioEssays* 36:847–854.
9. Leclaire S, Jacob S, Greene LK, Dubay GR, Drea CM (2017) Social odours covary with bacterial community in the anal secretions of wild meerkats. *Sci Rep* 7:3240.
10. Wells KD (2007) *The Ecology and Behavior of Amphibians* (Univ Chicago Press, Chicago).
11. Stange N, Page RA, Ryan MJ, Taylor RC (2017) Interactions between complex multisensory signal components result in unexpected mate choice responses. *Anim Behav* 134:239–247.
12. Narins PM, Hödl W, Grabul DS (2003) Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proc Natl Acad Sci USA* 100:577–580.
13. Poth D, Wollenberg KC, Vences M, Schulz S (2012) Volatile amphibian pheromones: Macrolides from mantellid frogs from Madagascar. *Angew Chem Int Ed Engl* 51:2187–2190.
14. Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP (1999) Aquatic sex pheromone from a male tree frog. *Nature* 401:444–445.
15. Starnberger I, et al. (2013) Take time to smell the frogs: Vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails. *Biol J Linn Soc Lond* 110:828–838.
16. Brunetti AE, et al. (2015) Diversity and evolution of sexually dimorphic mental and lateral glands in Cophomantini treefrogs (Anura: Hylidae: Hylinae). *Biol J Linn Soc Lond* 114:12–34.
17. Smith B, Williams C, Tyler M, Williams B (2004) A survey of frog odorous secretions, their possible functions and phylogenetic significance. *Appl Herpetol* 2:47–82.
18. Brunetti AE, Hermida GN, Iurman MG, Faivovich J (2016) Odorous secretions in anurans: Morphological and functional assessment of serous glands as a source of volatile compounds in the skin of the treefrog *Hypsiboas pulchellus* (Amphibia: Anura: Hylidae). *J Anat* 228:430–442.
19. Faivovich J, McDiarmid RW, Myers CW (2013) Two new species of *Myersiohyla* (Anura, Hylidae) from Cerro de la Neblina, Venezuela, with comments on other species of the genus. *Am Mus Novit* 3792:1–63.
20. Smith BPC, Hayasaka Y, Tyler MJ, Williams BD (2004) β-caryophyllene in the skin secretion of the Australian green tree frog, *Litoria caerulea*: An investigation of dietary sources. *Aust J Zool* 52:521–530.
21. Brunetti AE, et al. (2015) Frog volatile compounds: Application of *in vivo* SPME for the characterization of the odorous secretions from two species of *Hypsiboas* treefrogs. *J Chem Ecol* 41:360–372.
22. Kueneman JG, et al. (2014) The amphibian skin-associated microbiome across species, space and life history stages. *Mol Ecol* 23:1238–1250.
23. Bletz MC, et al. (2017) Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Front Microbiol* 8:1530.
24. Archie EA, Theis KR (2011) Animal behaviour meets microbial ecology. *Anim Behav* 82:425–436.
25. Delgado DB, Haddad CFB (2015) Calling activity and vocal repertoire of *Hypsiboas prasinus* (Anura, Hylidae), a treefrog from the Atlantic Forest of Brazil. *Herpetologica* 71:88–95.
26. Kiss ACI, de Carvalho JE, Navas CA, Gomes FR (2009) Seasonal metabolic changes in a year-round reproductively active subtropical tree-frog (*Hypsiboas prasinus*). *Comp Biochem Physiol A Mol Integr Physiol* 152:182–188.
27. Ma W, Miao Z, Novotny MV (1999) Induction of estrus in grouped female mice (*Mus domesticus*) by synthetic analogues of preputial gland constituents. *Chem Senses* 24:289–293.
28. Schultz TH, et al. (1988) Coyote estrous urine volatiles. *J Chem Ecol* 14:701–712.
29. Schulz S, Dickschat JS (2007) Bacterial volatiles: The smell of small organisms. *Nat Prod Rep* 24:814–842.
30. Citron CA, Rabe P, Dickschat JS (2012) The scent of bacteria: Headspace analysis for the discovery of natural products. *J Nat Prod* 75:1765–1776.
31. Papenfort K, et al. (2017) A *Vibrio cholerae* autoinducer-receptor pair that controls biofilm formation. *Nat Chem Biol* 13:551–557.
32. Al Abassi S, Birkett MA, Pettersson J, Pickett JA, Woodcock CM (1998) Ladybird beetle odour identified and found to be responsible for attraction between adults. *Cell Mol Life Sci* 54:876–879.
33. Silva-Junior EA, et al. (2018) Pyrazines from bacteria and ants: Convergent chemistry within an ecological niche. *Sci Rep* 8:2595.
34. Jemiolo B, Novotny M (1994) Inhibition of sexual maturation in juvenile female and male mice by a chemosignal of female origin. *Physiol Behav* 55:519–522.
35. Osada K, Miyazono S, Kashiwayanagi M (2015) The scent of wolves: Pyrazine analogs induce avoidance and vigilance behaviors in prey. *Front Neurosci* 9:363.
36. Moore BP, Brown WV, Rothschild M (1990) Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology* 1:43–51.
37. Dickschat JS, et al. (2010) Pyrazine biosynthesis in *Corynebacterium glutamicum*. *Eur J Org Chem* 2010:2687–2695.
38. Cheng TB, Reineccius GA, Bjorklund JA, Leete E (1991) Biosynthesis of 2-methoxy-3-isopropylpyrazine in *Pseudomonas perolens*. *J Agric Food Chem* 39:1009–1012.
39. Gallois A, Kergomard A, Adda J (1988) Study of the biosynthesis of 3-isopropyl-2-methoxypyrazine produced by *Pseudomonas taetrolens*. *Food Chem* 28:299–309.
40. Murray KE, Shipton J, Whitfield FB (1970) 2-methoxypyrazines and the flavour of green peas (*Pisum sativum*). *Chem Ind* 27:897–898.
41. Dunlevy JD, et al. (2013) A methyltransferase essential for the methoxypyrazine-derived flavour of wine. *Plant J* 75:606–617.
42. Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK (2013) Microbial volatile emissions as insect semiochemicals. *J Chem Ecol* 39:840–859.
43. Rebollar EA, et al. (2016) Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J* 10:1682–1695.
44. Walke JB, et al. (2014) Amphibian skin may select for rare environmental microbes. *ISME J* 8:2207–2217.
45. Byrne PG, Keogh JS (2007) Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. *Anim Behav* 74:1155–1162.
46. Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) *Mixed Effects Models and Extensions in Ecology with R* (Springer, New York).
47. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, New York).
48. Lane DJ (1991) *Nucleic Acid Techniques in Bacterial Systematics*, eds Stackebrandt E, Goodfellow M (John Wiley and Sons, New York).
49. Kearse M, et al. (2012) Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649.
50. Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. *J Comput Biol* 7:203–214.
51. Bletz MC, et al. (2016) Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. *Nat Commun* 7:13699.
52. Felsenstein J (1989) Mathematics vs. evolution: Mathematical evolutionary theory. *Science* 246:941–942.