Ex situ Diet Influences the Bacterial Community Associated with the Skin of Red-Eyed Tree Frogs (Agalychnis callidryas)

Rachael E. Antwis1*, Rachel L. Haworth1, Daniel J. P. Engelmoer1,2, Victoria Ogilvy1,3, Andrea L. Fidgett4, Richard F. Preziosi1

1 Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom, 2 Department of Ecological Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands, 3 FrogLife, Peterborough, United Kingdom, 4 Chester Zoo, Chester, United Kingdom

Abstract

Amphibians support symbiotic bacterial communities on their skin that protect against a range of infectious pathogens, including the amphibian chytrid fungus. The conditions under which amphibians are maintained in captivity (e.g. diet, substrate, enrichment) in ex situ conservation programmes may affect the composition of the bacterial community. In addition, ex situ amphibian populations may support different bacterial communities in comparison to in situ populations of the same species. This could have implications for the suitability of populations intended for reintroduction, as well as the success of probiotic bacterial inoculations intended to provide amphibians with a bacterial community that resists invasion by the chytrid fungus. We aimed to investigate the effect of a carotenoid-enriched diet on the culturable bacterial community associated with captive red-eyed tree frogs (Agalychnis callidryas) and make comparisons to bacteria isolated from a wild population from the Chiquibul Rainforest in Belize. We successfully showed carotenoid availability influences the overall community composition, species richness and abundance of the bacterial community associated with the skin of captive frogs, with A. callidryas fed a carotenoid-enriched diet supporting a greater species richness and abundance of bacteria than those fed a carotenoid-free diet. Our results suggest that availability of carotenoids in the diet of captive frogs is likely to be beneficial for the bacterial community associated with the skin. We also found wild A. callidryas hosted more than double the number of different bacterial species than captive frogs with very little commonality between species. This suggests frogs in captivity may support a reduced and diverged bacterial community in comparison to wild populations of the same species, which could have particular relevance for ex situ conservation projects.

Introduction

Symbiotic bacterial communities are commonly found in association with both animals and plants. Often the bacterial community provides some advantage to the host in return for a reciprocal benefit, such as nutrients and a suitable microhabitat in which to live and reproduce [1,2]. The diversity of bacteria plays an important role in determining the stability and resilience of the community to intrinsic or extrinsic perturbation, such as stress, environmental change, or invasion by pathogens [3–6]. For example, Dillon et al. [6] demonstrated a significant inverse relationship between the species richness of the gut of locusts (Schistocerca gregaria) and the growth of pathogenic bacteria (Serratia marcescens), as well as a significant decrease in the number of infected individuals with an increase in bacterial community diversity.

There is a growing body of research to show the bacterial community associated with the skin of amphibians can influence host susceptibility to a range of infectious diseases (e.g. refs [1,2,7–12]). Bacteria associated with the skin of amphibians may protect the host from pathogenic infection by: a) increasing competition for space and resources; b) altering the microenvironment of the amphibian skin to prevent colonisation of pathogens, and c) the production of anti-microbials that kill or inhibit the growth of pathogens [1,11,13]. For example, Woodhams et al. [9] found that a population of Rana sierrae persisting with Batrachochytrium dendrobatidis (Bd, the amphibian chytrid fungus) in Yosemite National Park (USA) had significantly more individuals with at least one anti-Bd bacterial strain associated with their skin than a nearby population of Rana muscosa that was naive to the chytrid fungus and subsequently became extinct when Bd spread to the area. Lam et al. [12] compared the same population of R. sierrae to another Bd-naive population of R. muscosa in the area and found similar proportions of individuals in each population that hosted at least one anti-Bd bacterial strain, and this population of R. muscosa managed to persist when the chytrid fungus arrived a year later.

The use of anti-Bd bacteria as a potential probiotic defense against the chytrid fungus is currently being investigated [14]. It has been shown that potential probiotic bacteria can successfully establish on the skin of amphibians, both directly and via transfer
from the environment, with subsequent increases in anti-\textit{Bd}
metabolites on the skin, and significantly increased growth and
survival on exposure to \textit{Bd} zoospores [15–17]. In addition, a range
of lactic acid producing bacteria that naturally occur on American
bullfrogs (\textit{Lithobates catesbeianus}) have been isolated, and \textit{in vitro}
studies suggest inoculating captive populations with these bacteria
has the potential to prevent and/or treat bacterial septicemia,
either individually or as a multi-strain probiotic [18–21].
Therefore the presence of the correct assemblage of bacteria on
the skin of amphibians is likely to be important for protecting them
from infectious diseases.

Amphibians in the wild have relatively high exposure to bacteria
through environmental transmission (e.g. plants, soil, water) and
through interactions with both conspecifics (e.g. maternal or
paternal transfer, and transfer during mating) and other species
[2,22–24]. In contrast, captive amphibians almost certainly
interact with fewer individuals, as well as experiencing a less
diverse and heterogeneous environment through which to gain
bacteria. As a result, captive amphibians are likely to receive lower
exposure to a variety of bacteria, and therefore support a simpler
cutaneous bacterial community structure in comparison to wild
counterparts, making them less resistant to disease on reintroduc-
tion to the wild. To the author’s knowledge, there are currently no
published studies that compare the native microflora of captive
and wild populations of the same species.

Different husbandry conditions in captivity (diet, substrate,
enrichment, etc.) are also likely to influence the microbial
community. Meyer et al. [25] found that cooler temperatures
(10–20°C) led to increased skin sloughing frequency in cane toads
(\textit{Rhinella marina}) when compared with warmer temperatures (20–
30°C), and that skin sloughing led to a decreased abundance of
bacteria on the skin. This indicates that conditions in captivity can
indirectly influence the bacterial community, although the direct
effects of different husbandry practices are yet to be investigated.
For probiotic treatments to be effective, a greater knowledge of the
potential influence of captive conditions of the cutaneous bacterial
communities is required.

The nutritional status of the host may influence the bacterial
community present for two reasons; a) bacteria are likely to utilise
the nutrients in the skin mucus of amphibians; and b) there is
evidence to suggest the gut of an amphibian acts as reservoir for
skin bacteria [2,26,27]. Many amphibian species ingest their skin
on shedding, thereby also ingesting the cutaneous bacteria, which
can then be re-inoculated onto the skin via the cloaca [27]. Diet
has been shown to influence the bacterial community associated
with the gut of many insect species as well as other host organisms
including rainbow trout (\textit{Oncorhynchos mykiss}), Galapagos iguanas
(\textit{Amphibius cristatus}, \textit{Conolophus subserif) and \textit{Conolophus palli-
dus}), and humans [28–32]. The diet of amphibians is also likely to
influence the survival of bacteria in the intestinal tract, and
therefore influence the community that is re-inoculated onto the
skin. This may alter the success of probiotic treatments, as well as
potentially making amphibians in captivity and those released
back into the wild more vulnerable or resistant to infection from
pathogens.

One nutritional aspect of interest to amphibian health is dietary
carotenoids [33,34]. In vertebrates, carotenoids are obtained solely
from the diet and act as anti-oxidants in the body as well as
precursors for vitamin A [35]. In addition, carotenoids confer red,
orange and yellow colouration in many organisms including frogs
[36,37]. In the wild, amphibians have access to a broad range of
feeder insects from which to gain carotenoids, whereas captive
amphibians are generally fed invertebrates deficient in carotenoids
[34]. Gut loading of feeder insects in captivity can be used to
increase the availability of carotenoids to amphibians. Red-eyed
tree frogs (\textit{Agalychnis callidryas}) fed a carotenoid-enriched diet had
significantly greater red colouration in their skin than those fed a
carotenoid-free diet [34], indicating that dietary nutrients are
deposited in the skin and may therefore have a direct effect on the
cutaneous bacterial community.

Here we used culturing methods to compare the cutaneous
bacterial community associated with the skin of red-eyed tree frogs
(\textit{Agalychnis callidryas}) fed on a carotenoid-enriched diet and a
carotenoid-free diet. We also identified bacteria to genus/species
level using 16S ribosomal DNA sequencing, and compared these
to cultured bacteria collected from a wild population of \textit{A. callidryas}
at Las Cuevas Research Station, Chiquibul Rainforest, Belize. We
hypothesised that wild \textit{A. callidryas} would support a greater number
of bacterial species than captive \textit{A. callidryas}, and that there would be
differences in the bacterial species isolated. We also
hypothesised that captive \textit{A. callidryas} fed a high carotenoid diet
would support a significantly greater bacterial diversity than those
fed a carotenoid-free diet.

Methods

Ethics Statement

This study was approved by the University of Manchester
Ethics Committee and The North of England Zoological Society
(Chester Zoo) Ethics Committee, and all methods were non-
invasive. Bacteria was collected and exported from the wild
population of \textit{Agalychnis callidryas} by permission of Belize Forest
Department (Research and Export Permit Number CD/60/3/12)
and imported to the UK by permission of DEFRA (Authorisation
Number TARP/2012/224).

Bacterial Sampling from Captive Frogs

Seventeen captive bred \textit{A. callidryas} (F1, originated from pet
trade) were fed black crickets (\textit{Gryllus bimaculatus}) gut-loaded with
either a high carotenoid diet (5.0 mg/g; n = 10; 5 males, 5 females)
or a carotenoid-free diet (0.0 mg/g; n = 7; 4 males, 3 females) (see
[34] for details of diets). All frogs were from the same clutch of eggs
and were randomly assigned to dietary treatment group at
metamorphosis. Thus, observed differences in bacterial commu-
nities can be attributed to differences in diet alone. We note that
this design is testing for differences between bacterial communities
at a given sampling point rather than how bacterial communities
change over time in response to the addition of carotenoids to the
diet.

Sterile gloves were worn throughout handling and changed for
each frog to minimise cross-contamination. The dorsal and ventral
regions of the body were swabbed separately to increase the
sampling coverage. Frogs were rinsed twice on their dorsal surface
using ~200 ml sterile bottled water to remove any transient
bacteria from their skin (as described by [2]). Frogs were then
swabbed all over their dorsum to collect cutaneous bacterial
communities using sterile Eurotubo\textsuperscript{®} collection swabs (Deltalab,
Spain), and the rinsing and swabbing process was then repeated
for the ventral surface of each frog. Care was taken to ensure frogs
were not harmed during this process, and individuals were
monitored for 2 weeks post-swabbing for signs of distress or injury
in response to the swabbing, of which none were observed. Swabs
were placed into 1.5 ml sterile screw-top tubes containing 1 ml of
1 M NaCl\textsubscript{2} solution for plating out immediately after swabbing.
Tubes containing swabs were vortexed to dissociate bacteria from
the swab, then the swab was removed and samples were diluted by
pipetting 100 µl into 900 µl of 1 M NaCl\textsubscript{2}. Pilot studies showed
plating a dilution of $10^{-1}$ produced plates with an intermediate
obtained.

broth media. Tubes were then shipped to the University of
were used to pick representative colonies of each morphotype,
unlikely this had significant effects on the results. Sterile swabs
samples were not diluted to 10
were covered in parafilm and bacteria left to grow at ambient
time for eight days. Samples were not diluted to 10
were rinsed and swabbed as described for the carotenoid diet study
Morphologically distinct bacterial colonies ('morphotypes') were
represented colonies of each morphotype were then
Morphologically distinct bacterial colonies ('morphotypes') were
were counted at days 6 and 12, after which no new colony growth was

Data Conversion and Statistical Analysis

Bacterial counts for the carotenoid study animals were summed
for the two sampling points (days 6 and 12), multiplied by the
dilution factor of 10, and the dorsal and ventral surfaces summed
for each frog to give a total bacterial community associated with
each individual. Data was checked for normality and bacterial
abundance was log normalised before analysis. Gender had no
statistically significant effect on the bacterial communities associated
with frogs, and so males and females were combined for
subsequent analyses. Overall bacterial community composition
was analysed for differences based on dietary treatment using the
Adonis function of the vegan package in RStudio®. Adonis is a
permutational multivariate analysis that uses a Bray-Curtis
distance matrix based on the abundance of each morphotype to
analyse the variation in the overall bacterial community structure.
The effect of diet on species richness (the number of different
morphotypes isolated from each individual) and total abundance
(number of bacterial colonies isolated from each individual)
were analysed using t tests in JMP 10® (data for bacterial
abundance were log transformed to achieve a normal distribution).

Bacterial Sampling from Wild Frogs

Eight A. callidryas (four males, four females) were collected from
Elegans Pond at Las Cuevas Research Station, Chiquibul Rainforest,
Belize (16˚43'N, 88˚59'W), placed individually in plastic
drums, and returned to the research station. Each frog was then
rinsed and swabbed as described for the carotenoid diet study
animals. Tubes containing swabs were shaken vigorously for 30
seconds, and the contents poured on to R2A agar plates, which
were covered in paraffin and bacteria left to grow at ambient
temperature for eight days. Samples were not diluted to 10
(as for the captive frogs) to minimise the number of steps required to
grow bacteria in the field. As the purpose of this was to compare
the identity of bacteria associated with wild and captive frogs, it is
unlikely this had significant effects on the results. Sterile swabs
were used to pick representative colonies of each morphotype,
which were placed into screw-top tubes containing 1 ml R2A
broom media. Tubes were then shipped to the University of
Manchester (UK), where the contents of the tubes were poured on
to fresh R2A agar plates and incubated at 25°C until bacteria
grew. These were then re-streaked to ensure a pure culture was
defined.

Molecular Methods and Sequencing Analyses

Bacterial species isolated from captive and wild populations of
A. callidryas were identified using 16S rDNA sequencing with
universal primers 27F (5'-GTTGCTGCAGAGTGTAG
GATCCTGCGCTCAG-3') and 1492R (5'-CAGGATCC
TACGGGTACCTTGTGACT-3') (Webster 2003). 16S
rDNA fragments were obtained through direct colony PCR
amplification (no DNA extraction required) using the GoTaq®
Colourless Master Mix (Promega Corp., USA) according to the
manufacturers instructions. 16S rDNA was amplified using direct
colony PCR with the following programme: 95°C for 2 min
followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for
90 s, with a final extension step of 5 minutes at 72°C. PCR
products were checked for the correct length with gel electrophoresis (~1500 bp), and then purified with the QIAquick® PCR
Purification Kit (Qiagen, UK) and sent to Eurofins MWG Operon, Germany for sequencing. A consensus sequence was
obtained by combining the forward and reverse sequences in DNA
Dynamo Sequence Analysis Software® (BluTractorSoftware Ltd., UK). Consensus sequences were then blasted against the
NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to iden-
tify each morphotype to genus level (fragment size of ~1500 bp).
Morphotypes with sequence similarity of 99% or greater were
considered the same species (reviewed in [38]).

Results

Effects of Dietary Carotenoid Availability on the
Cutaneous Bacterial Community

A range of 8–13 morphotypes were isolated per individual from
carotenoid-enriched diet, and 6–11 morphotypes were
isolated per individual from carotenoid-free diet. The Adonis model for diet, gender and the diet × gender interaction showed diet had a significant effect on the overall bacterial
community associated with frogs (F1,13 = 2.868, p = 0.024), but
gender (F1,13 = 0.815, p = 0.529) and diet × gender (F1,13 = 0.733,
p = 0.618) did not. Gender was subsequently removed from the
model, leaving a significant effect of diet on the total bacterial
community (F1,15 = 2.841, p = 0.014).

Gender did not have a significant effect on the bacterial
abundance (t16 = 0.465, p = 0.324) or species richness (t16 = 0.515,
p = 0.308) of the bacterial community associated with frogs, and
there was no significant interaction between gender and diet, and
therefore genders were combined for subsequent analyses. Diet
had a significant effect on the bacterial abundance (t16 = 2.413,
p = 0.027) of the bacterial community associated with frogs, and
carotenoid-enriched diet supporting a greater abundance and
species richness of bacteria on their skin (Figures 1 and 2). The
total abundance (CFU counts) and relative abundance (number of
CFUs of each morphotype relative to total number of CFUs
isolated from a given individual) of each bacterial morphotype
isolated from captive frogs fed the two different diets are shown in
Figures 3 and 4. Of the six most abundant morphotypes, frogs fed
carotenoid-enriched diet produced double or more than double
the number of colonies for five of those (unidentified A, KC853162, KC853165, KC853155, unidentified C) in compar-
isolation. The relative abundance of these five morphotypes was:

[Table 1 shows the individual bacterial species isolated from
captive and wild A. callidryas. Twenty-one morphotypes from 12
families were isolated from the wild populations of A. callidryas.]
Thirteen different bacterial morphotypes (six families) were isolated from frogs fed on a carotenoid-enriched diet, and eleven (five families) were isolated from frogs fed a carotenoid-free diet. Only one bacterial species was isolated from both wild and captive populations (*Stenotrophomonas* sp.; isolated from captive *A. callidryas* fed the carotenoid diet), although three bacterial species isolated from captive frogs could not be identified due to poor sequence data, and colonies were no longer available to resequence. Two dominant families of bacteria were isolated from wild and captive *A. callidryas*; Enterobacteriaceae, and Staphylococcaceae (Table 1). For these two families, more species were isolated from wild frogs than captive (eight vs. two and three vs. two respectively).

**Discussion**

**Effects of Dietary Carotenoid Availability on the Cutaneous Bacterial Community**

Frogs fed a carotenoid-enriched diet had a significantly different bacterial community composition to those fed a carotenoid-free diet. The provision of dietary carotenoids to captive frogs leads to significantly greater number of different bacterial isolates on individual frogs, and a significantly greater abundance of bacteria (Figures 1 and 2). Although the number of different morphotypes isolated from frogs fed a carotenoid-enriched diet were similar to those fed a carotenoid-free diet (13 and 11 respectively), not all frogs supported all these different bacterial morphotypes, and individual frogs fed a carotenoid-enriched diet supported a significantly greater richness of these bacteria compared to individuals fed a carotenoid free diet (Figure 1). Increased species richness and bacterial abundance in a community leads to increased productivity and stability, making the community more resilient to perturbation (e.g. pathogens, stress) and more able to respond to environmental change [1,3–5,39]. High species richness also increases the likelihood of host individuals harboring at least one bacterial species that confers some protection from infectious pathogens. Therefore frogs fed a high carotenoid diet may possess a bacterial community that is more capable of protecting the host from infectious diseases. Further studies are required to determine the susceptibility to infectious disease of captive amphibians fed different diets.

The increased richness of bacteria on the skin of carotenoid-fed frogs compared to frogs on a carotenoid-free diet suggests the presence of carotenoids could improve the growth and/or survival of bacteria both in the gut and on the skin. *Agalychnis callidryas* ingest their skin on shedding (Antwis, pers. obs.), and greater availability of carotenoids in the diet may also protect the bacteria as they pass through the intestine, thereby allowing them to be re-inoculated on to the skin. For example, some bacteria use carotenoids to protect DNA, proteins and cell membranes from damage by reactive oxygen species that are produced during metabolic processes in the host body [35,40]. Wiggins et al. [27] isolated the same bacterial species from the gut and skin of *P. cinereus*, and similar genera of bacteria are found in the gut of *R. catesbiana* and *R. pipsiens* as are found on the skin of other amphibian species, including *A. callidryas* in this study (e.g. species from the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Citrobacter*, *Enterobacter*, *Flavobacteria*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, and *Serratia*) [41–43]. This suggests bacterial communities associated with the skin and guts of amphibians are of similar origin, and have the potential to influence the composition of one another.

The mucus on the skin of frogs is also likely to be a major source of nutrients for bacteria [2,26]. The nutritional status of the host may influence the quantity and nutritional composition of the mucus released by mucous glands, as well as the abiotic environment of the skin, which may in turn affect on the bacterial community associated with its skin. In humans, a higher vitamin A content in the blood serum leads to a reduction in sebum content on the skin of humans, and vitamin A and calcium intake has a significant effect on the pH and hydration of the skin, although the impact of this on the bacterial communities has not been investigated [44]. Carotenoids are a precursor to vitamin A, and so the availability of carotenoids in the diet of *A. callidryas* may influence mucus production and/or the microenvironment of the skin. Indeed, short tongue syndrome in amphibians and reptiles is associated with a lack of vitamin A, leading to keratinisation of the glandular epithelium in the tongue and a subsequent reduction in mucus production [18,33,39]. It is possible that a similar phenomenon is occurring in the epithelial cells of the skin of *A. callidryas* receiving a carotenoid-free diet, leading to a reduction in mucus availability for subsequent bacterial proliferation.

Two morphotypes isolated from carotenoid-fed frogs were not isolated at all from those fed a carotenoid-free diet (*Staphylococcus* sp. KC353166 and *Stenotrophomonas* sp. KC853167; Table 1), although these were found at very low abundance on carotenoid-fed frogs (Figure 3), and so may only play a small role in the bacterial community. For 5 of the 6 most common bacterial morphotypes, frogs fed a carotenoid-enriched diet supported a substantially greater abundance (Figure 3). This may be important for disease prevention in the context of quorum sensing. Quorum sensing is...
the communication between bacterial cells, via production of signal molecules by the bacteria themselves, which allows communities to initiate physiological processes such as gene transfer, bioluminescence, and anti-microbial peptide production [45–47]. For this to occur, a particular density or abundance of bacterial cells is required to obtain a high enough concentration of signal molecules (reviewed in [45]). The role of quorum sensing, or the necessary abundance thresholds of bacteria, has not yet been studied in the context of amphibian defence or probiotic treatment for chytrid disease. Figure 4 shows the relative abundance of each morphotype does not change linearly in bacterial communities of frogs as a result of differences in diet; some morphotypes show an increase in relative abundance with a carotenoid diet, while others decrease or are unaffected. This may also affect community dynamics and responses to pathogenic infection. More work is required to determine the effects of diet, and other ex situ environmental conditions, on disease susceptibility of amphibians.

### Differences in Bacterial Species between Wild and Captive Frogs

A much greater number of bacteria were isolated from wild *Agalychnis callidryas* than those in captivity. There were taxonomic similarities between the bacterial species isolated from the wild and captive populations at the family and genus level. However, only one

<table>
<thead>
<tr>
<th>Family</th>
<th>Species (GenBank accession number; % match to reference strain)</th>
<th>Wild <em>A. callidryas</em></th>
<th>Captive <em>A. callidryas</em> fed a carotenoid-enriched diet</th>
<th>Captive <em>A. callidryas</em> fed a carotenoid-free diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillaceae</td>
<td>Bacillus sp. (KC853209; 99%)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Burkholderiaceae</td>
<td>Cupriavidus sp. (KC853214; 98%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dermabacteriaceae</td>
<td>Brachybacterium sp. (KC853155; 99%)</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Deinococcaceae</td>
<td>Deinococcus sp. (KC853204; 98%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Citrobacter sp. (KC853165; 99%)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Citrobacter sp. (KC853213; 98%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enterobacter sp. (KC853199; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enterobacter sp. (KC853217; 99%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enterobacter sp. (KC853221; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enterobacter sp. (KC853224; 99%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enterobacter sp. (KC853205; 96%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Erwinia sp. (KC853200; 98%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Klebsiella sp. (KC853162; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Serratia sp. (KC853196; 98%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td>Chryseobacterium sp. (KC853218; 97%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td>Chryseobacterium sp. (KC853202; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td>Elizabethkingia sp. (KC853158; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td>Elizabethkingia sp. (KC853163; 99%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td>Flavobacterium sp. (KC853159; 98%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrococcaceae</td>
<td>Arthrobacter sp. (KC853208; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moraxellaceae</td>
<td>Acinetobacter sp. (KC853216; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td>Pseudomonas sp. (KC853219; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobiaceae</td>
<td>Agrobacterium sp. (KC853210; 98%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphingomonadaceae</td>
<td>Novosphingobium sp. (KC853222; 97%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococaceae</td>
<td>Staphylococcus sp. (KC853195; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococaceae</td>
<td>Staphylococcus sp. (KC853206; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococaceae</td>
<td>Staphylococcus sp. (KC853223; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococaceae</td>
<td>Staphylococcus sp. (KC853160; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococaceae</td>
<td>Staphylococcus sp. (KC853161; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococaceae</td>
<td>Staphylococcus sp. (KC853166; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthomonadaceae</td>
<td>Stenotrophomonas sp. (KC853167/KC853198; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthomonadaceae</td>
<td>Stenotrophomonas sp. (KC853220; 99%)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0085563.t001

The table above shows the bacteria isolated from wild *Agalychnis callidryas* and captive *A. callidryas* fed on a carotenoid-enriched and a carotenoid-free diet.
Figure 3. Abundance of each bacterial morphotype (number of colony forming units (CFUs) isolated from each individual) isolated from the skin of Agalychnis callidryas fed on a carotenoid-enriched and carotenoid-free diet. Morphotype identities are indicated by GenBank accession number; see Table 1 for details. Error bars show ±1 S.E.M. doi:10.1371/journal.pone.0085563.g003

Figure 4. Relative abundance of each bacterial morphotype (number of colony forming units (CFUs) isolated from each individual) isolated from the skin of Agalychnis callidryas fed on a carotenoid-enriched and carotenoid-free diet. Morphotype identities are indicated by GenBank accession number; see Table 1 for details. Error bars show ±1 S.E.M. doi:10.1371/journal.pone.0085563.g004
bacterial species was isolated from both populations (*Stenotrophomonas* sp.), although three morphotypes from the captive frogs could not be identified due to poor sequence data. Interestingly, the one species in common between captive and wild frogs was only isolated from captive frogs fed a carotenoid-enriched diet, albeit at relatively low abundance. Given that both the common as well as some bacterial species in particular, are likely to be involved in protecting the host from invasive pathogens, this may have implications for the ability of captive frogs to protect themselves from pathogens, both in captivity and on reintroduction to the wild, and could have particular relevance for long-term conservation projects involving ex situ conservation of amphibians.

Differences in bacterial species between captive and wild *A. callidryas* may be attributed to differences in environment, habitat and diet (as well as many other factors) [1,13,51,52]. Kueneman et al. [50] show that amphibian bacterial assemblage within a species is site specific, with different populations of the same host species supporting different bacterial assemblages. Moreover, 45–76% of *environmental* taxonomic units were shared between the environment (lake water/soil) and the host amphibian [50], indicating the amphibian environment is likely to have a strong effect on the associated bacterial community. This is also likely to be true of the captive environment, particularly in comparison to the wild. The actual species richness is likely to be much higher on both the wild and captive populations of *A. callidryas* as culturing methods only allow a small proportion of the total community to be identified [48]. Greater investigation into the bacteria associated with *A. callidryas* using next-generation sequencing is required to fully understand the true diversity of the community. Individuals from the captive population in this study did not originate from the wild population of *A. callidryas*, and this may explain the lack of shared bacterial species between the two. However, there is growing evidence that shows host species is one of the predominant factors influencing bacterial community assemblage [49,50], and so we may expect more similarity between bacterial communities isolated from wild and captive populations, irrespective of origin. More work is required to look at how bacterial communities change when host organisms are brought into captivity, and how this may affect their susceptibility to disease.

**Conclusions**

This study shows that a diet enriched with carotenoids significantly increases the richness and abundance of bacteria associated with the skin of *A. callidryas*. Given the role of cutaneous bacteria in disease prevention in amphibians, evidence that conditions in captivity have the potential to significantly affect the bacterial community may have implications for the success of probiotic treatments, as well as influence the suitability of amphibians for reintroduction. We also show that captive frogs support a diverged and reduced bacterial community in comparison to wild frogs of the same species. More work is required to determine the effects of other captive conditions on the bacterial communities associated with amphibians, as well as changes in microbiota in response to transfer from the wild to captivity, particularly with regard to the implications for disease resistance and probiotic success.

**Acknowledgments**

The authors would like to thank David Spraggle and John Fitzpatrick for their assistance with analyses in *R*. We are particularly grateful to Paul Johnston and Andres Arce for providing invaluable advice on the initial planning of this study, and to Christopher Michaels who provided comments on drafts of this paper. Lisa Andrezejak provided daily care for the frogs.

**Author Contributions**

Conceived and designed the experiments: REA RLH DJPE VO RFP. Performed the experiments: REA RLH. Analyzed the data: REA RFP. Wrote the paper: REA.

**References**


20. Mendoza GM, Pasteris SE, Ale CE, Otero MC, Buhler MI, et al. (2012) Cultivatable microbiota of *Lithobates catesbeianus* and advances in the selection of...
lactic acid bacteria as biological control agents in rainforest. Research in Veterinary Science, 93, 1160–7. DOI:10.1016/j.vres.2012.05.007.


