

The Rufford Foundation

Final Report

Congratulations on the completion of your project that was supported by The Rufford Foundation.

We ask all grant recipients to complete a Final Report Form that helps us to gauge the success of our grant giving. The Final Report must be sent in **word format** and not PDF format or any other format. We understand that projects often do not follow the predicted course but knowledge of your experiences is valuable to us and others who may be undertaking similar work. Please be as honest as you can in answering the questions – remember that negative experiences are just as valuable as positive ones if they help others to learn from them.

Please complete the form in English and be as clear and concise as you can. Please note that the information may be edited for clarity. We will ask for further information if required. If you have any other materials produced by the project, particularly a few relevant photographs, please send these to us separately.

Please submit your final report to jane@rufford.org.

Thank you for your help.

Josh Cole, Grants Director

Grant Recipient Details	
Your name	Sandra Victoria Flechas
Project title	Fighting <i>Batrachochytrium dendrobatidis</i> : seeking for the mechanism allowing <i>Bd</i> and host co-existence in two Andean frogs from Colombia
RSG reference	15304-1
Reporting period	
Amount of grant	£5500
Your email address	vickyflechas@gmail.com
Date of this report	December 10, 2015

1. Please indicate the level of achievement of the project's original objectives and include any relevant comments on factors affecting this.

Objective	Not achieved	Partially achieved	Fully achieved	Comments
To confirm the presence of the fungus and to accurately quantify the number of <i>Bd</i> zoospores in the sample			X	To determine infection status and to avoid cross contamination, animals were collected using clean, decontaminated equipment, individually handled with fresh disposable gloves, and placed in individual bags prior to obtaining the skin swab samples. Each animal was sampled by running a sterile synthetic cotton swab (Medical Wire Equipment MW100) over the ventral surface, the inner thigh area and the plantar surface of the hind feet webbing for a total of 50 strokes. Skin swabs were preserved dry and stored at -20°C until processed. Infection intensity (as zoospores equivalents) was determined through Real-time TaqMan PCR – qPCR
To perform antagonism assays where each isolated morphotype will be tested against <i>Bd</i> .			X	In order to characterise the culturable portion of the skin microbiota and identify candidate bacteria with anti- <i>Bd</i> properties, we took samples from 1 to 5 individuals per stage and species, in total we collected samples from 24 individuals. Samples were preserved in 2 mL cryovials containing 1 mL DS solution. We performed serial dilutions (until 10 ⁻⁴) and plated it in three different media: R2A, Avena and PDA (Potato Dextrose Agar), the latter specific for fungi isolation. Plates were incubated at room temperature for two days. Single colonies from each bacterial morphotype, discriminated by eye inspection, were streaked on fresh agar plates and subcultures were made until pure cultures were obtained. Each isolate was cryopreserved in nutritive broth with 30% glycerol at -80°C. To identify (if possible) the isolated strains and determine which strains correspond to the same bacterial species, we used matrix-assisted laser

				desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry. Unidentified morphotypes by the MALDI, were submitted to sequencing of the 16S ribosomal gen. We have all the results but we are still performing statistical analysis.
To describe the whole bacterial microbiome, using culture-independent techniques.			X	To determine if the microbial-associated community changes among life stages and between sympatric species that co-occur, we collected skin swabs from 17 tadpoles, 10 juveniles and 17 adults, we also took 10 samples of pond water. All individuals were collected using a dip net or a new plastic bag. Prior to sampling, individuals were rinsed twice with 50 mL of sterile water in order to remove transient microbes and sediments ensuring that samples correspond mostly to skin-associated bacteria. Whole community DNA was extracted from each of 54 samples using the Qiagen DNeasy blood & tissue kit (Valencia, CA, USA) following manufacturer's protocol slightly modified. We obtain 16S rRNA gene amplicons amplifying the V3 and V4 regions of the gene obtaining a single amplicon of approximately 460 bp.

2. Please explain any unforeseen difficulties that arose during the project and how these were tackled (if relevant).

We consider that the main difficulties in this kind of research projects are to standardise the protocols and procedures in the laboratory. First, we spend a couple of months trying to have all the extractions and PCR reactions in the proper conditions for the analysis in the system Illumina miSeq. Also, we experimented unforeseen delays to run the challenge assays. One of our goals was to determine which of the cultivable bacteria exhibited antifungal activity. Despite we have ample experience growing the chytrid fungus (*Bd*), for a couple of months we were unable to grow *Bd* to the required zoospores concentration to perform the tests. Two months ago, we finally managed to grow the pathogen and we started the test. In addition, it took several attempts until we standardised the protocol to measure inhibition. All these difficulties experienced during the project were almost always very worrying because we did not know if we would be able to find a solution. However, the situation confers ourselves with capacities to find various strategies in order to solve each problem we faced.

3. Briefly describe the three most important outcomes of your project.

a) Our goal was to determine if there are differences in prevalence of *Bd* infection depending on the life-stage (larvae-juvenile-adult) and host species (*Dendropsophus labialis* and *Rheobates palmatus*). We did not find differences in prevalence between species (31.7% in *D. labialis* and 31.2% in *R. palmatus*), but we detected a higher prevalence of *Bd* in the juveniles (88% of juveniles were *Bd* positive). Infection intensity ranges from 2.5 to 27676 ZE (Zoospore Equivalents), and individuals from *D. labialis* have higher zoospores loads.

b) Based on the whole-skin microbiome data, we found that juveniles and adults cannot be separated in either host species based on their microbes. On the contrary, we confirmed that tadpoles harbour a very different bacterial assemblage compared to adults and juveniles.

c) In order to determine the potential antimicrobial activity against *Bd*, we combined a culturing strategy to increase the novelty in isolation of the skin microbiota, by: i) using three different isolation media, and ii) extending the screening itself by filtering the bacterial collection through MALDI-TOF MS. We isolated 615 morphotypes, and 88 bacterial species were identified.

4. Briefly describe the involvement of local communities and how they have benefitted from the project (if relevant).

Our project did not involve the participation of local communities, but we involved an undergraduate student who helped us with some procedures. She will be co-author in any publication that result from this research project.

5. Are there any plans to continue this work?

Yes, we are very interested in continue our research on the ecology of amphibian diseases. We are also interested in determining how changes in temperature affect the performance of symbiotic bacteria when tested against a fungal pathogen. To assess this question we can use species widely distributed altitudinally, including *Dendropsophus labialis* and *Rheobates palmatus*. This information will allow conservation biologists to make specific and more accurate predictions about changes in amphibian populations under expected future climatic scenarios and improve conservation strategies locally.

Aside from the symbionts microbes on the skin of amphibians, antimicrobial peptides (AMPs) also play an important role in protection against invasive pathogens including the chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*). We would like to determine if AMPs composition vary among life stages, between sympatric species and *Bd* condition (infected or *Bd*-free). In addition, we hope to correlate the information obtained on the amphibian skin peptides with the data on microbial community composition in order to have a more accurate interpretation on how these two defence mechanisms interact and modulate the host response to the fungal pathogen.

6. How do you plan to share the results of your work with others?

Preliminary results were presented at the Rufford Small Grant Conference held in Chile in May 2015. We also participated at the BoMM -2015 (Bogotá Microbial Meeting) in August where we obtained the Best Talk Award. Currently we are preparing a scientific manuscript, and we hope to submit it to the ISME Journal. For the next year we are planning to attend the 8th World Congress of Herpetology in China and present the final results of our research.

7. Timescale: Over what period was The Rufford Foundation grant used? How does this compare to the anticipated or actual length of the project?

The funds from RSG were expected to be use during one year (from October 2014 to October 2015). However due to the delays caused by the problems when we tried to grow the fungus we asked for one-month extension until November 2015.

8. Budget: Please provide a breakdown of budgeted versus actual expenditure and the reasons for any differences. All figures should be in £ sterling, indicating the local exchange rate used.

Item	Budgeted Amount	Actual Amount	Difference	Comments
Transportation expenses	300	104.23	+195.77	This amount indicated what we spent in gas and the toll tickets. Since we only visit the site twice we did not spend all the money we asked for. Two field trips were enough to collect all the data we needed for the project.
Food expenses	200	22.41	+177.59	We provided food to each person who helped us during the fieldwork. However, since we only travel twice to collect data we saved a lot of money.
Field supplies	350	98.13	+251.87	We spent £91.42 in supplies for the field. Since we have some material available in the lab we did not use all the money.
Bacteria isolation	350	449	-99	Although we asked for £350, and we had some material available, later we found out that we required more supplies to isolated the bacteria As we used three different media this

				increased the amount of p.e petri dishes, agar, tubes, etc.
Bacteria identification	2300	3450	-1150	To identify the isolated morphotypes we had to use more resources than we expected when we submitted the proposal. Also we performed challenge assays to determine which bacteria exhibit antifungal activity. Supplies were also expensive, and since we had problems and delays growing the pathogen to run the tests we expended more money than we ask for at the beginning.
Reagents and supplies for qPCR	2000	1271.82	+728.18	We bought extraction kits, reagents and lab supplies to perform the quantitative PCR and to run the Illumina miSeq system.
Total	5500	5395.59	104.41	

We assumed 1 GBP = 3195 COP according to the exchange rate for the date when we received the money. (17.572.938 COP, October 3th, 2014)

9. Looking ahead, what do you feel are the important next steps?

We consider that one of the most important step is to try a treatment using some of the bacteria with antifungal activity as a probiotic, in order to evaluate the capacity of these isolates to help another endangered tropical species that have been affected by the fungal pathogen.

10. Did you use The Rufford Foundation logo in any materials produced in relation to this project? Did the RSGF receive any publicity during the course of your work?

The RSGF logo was displayed in the presentations during the Rufford Small Grant Conference and the Bogotá Microbial Meeting (BoMM-2015). The logo will also be displayed in every opportunity we have to present our results. Additionally, the RSGF will be mentioned in the acknowledgments section of our manuscript entitled "Skin microbiota differs across life stages in two co-occurring Andean frog species" that we expected to submit to the ISME Journal next year.

11. Any other comments?

With financial support from Rufford Foundation we could take all the samples we needed to determine how microbial communities vary among species and stages. We also were able to establish new collaborations with other partners in the country. We would like to thank RSGF for the financial support to accomplish our goals.