smaller than *Crotalus pricei* (e.g., *Tantilla*) may find this method useful. However, the method may prove to be less effective with arboreal species. Arboreal snakes can regulate the blood flow to their tails very effectively while in a vertical orientation, which may mean that less blood would be produced by a lateral vessel puncture (Lillywhite 1987).

**Acknowledgments.**—I thank Mike Schroff for his assistance and encouragement in developing this technique. Matt Kaplan for helping me determine whether my efforts were in vain, Julio Lemos Espin for facilitating work conducted in Mexico, Bruce Weise for some of the accompanying photos, and Kevin Bonine, Raúl Díaz, Matthew Murdoch, and Alexandra Sumarlin for helpful comments on this manuscript. All snake captures, handling, and blood draws were conducted under Arizona Game and Fish Department Scientific Collecting Permits or SEMARNAT permit SGPA/DGVS/07302/14 (in Mexico). This work was done independently so there was no IACUC review, but all procedures followed standard guidelines and consisted of actions routinely approved by IACUC boards.

**Literature Cited**


that the cutaneous antimicrobial peptides and microbiota associated with amphibians may play a key role in maintaining amphibian health and mediating the effects of emerging infectious diseases, which have been implicated as a major cause of amphibian declines worldwide. For example, species-specific differences in both antimicrobial peptides (Rollins-Smith et al. 2002; Rollins-Smith and Conlon 2005) and microbiota (Harris et al. 2009) are associated with susceptibility to B. dendrobatidis, the fungal pathogen responsible for amphibian population crashes in Australia, western North America, and the American tropics. Specifically, cutaneous microbiota and secreted antimicrobial peptides of amphibians can kill or inhibit the growth of B. dendrobatidis (Rollins-Smith and Conlon 2005; Harris et al. 2006; Brucker et al. 2008a; Brucker et al. 2008b; Becker et al. 2009; Harris et al. 2009). Therefore, a better understanding of amphibian microbial communities may increase our understanding of this devastating wildlife disease.

To date, studies on the skin microbiota of amphibians have been variable, with protocols differing substantially among studies. For example, the two most cited papers (Culp et al. 2007; Lauer et al. 2007) describing the methodology for removing environmental microbes prior to skin sampling and characterization of the resident microbial communities differ in methodology (e.g., the number of times each animal was washed and the volume of water used for each wash). However, these two protocols are often cited in a single study, and citing studies do not precisely follow the same protocols (McKenzie et al. 2012; Fitzpatrick and Allison 2014; Kueneman et al. 2014). A standardized washing protocol is necessary to allow for comparison of independent studies on the skin microbiota of amphibians.

Any conclusions regarding resident symbionts depend on the ability of the researcher to separate environmental microbiota from strict host/symbiont associations. To contribute to the study of amphibian skin microbiota, we set out to develop a standardized sampling protocol for biologists studying this topic. We conducted identical protocols using plethodontid salamanders in both the laboratory and the field, to ensure that this protocol can be used in both settings. Moreover, we used an experimental approach to determine if our washing protocol worked. We used a plate-based assay to count colony-forming units to show a sequential reduction of cultureable bacteria in our four-step protocol and molecular confirmation using PCR to show resident microbial community presence after the decontamination protocol. Here, we present evidence for our ability to retrieve microbial communities from amphibian skin after washing off transient environmental microbes.

**Materials and Methods**

**Study species.**—Red-backed Salamanders (*Plethodon cinereus*; *N* = 23) were collected at Jolliffe Nursery in Wetzel Co., West Virginia, USA (39.657°N, 80.555°W), on 20 December 2013. Red-backed Salamanders were kept individually in microcosms (40 × 25 × 18 cm) on natural soil substrate (depth = 10 cm), misted daily with water, and maintained at 21.5°C on a 12 h light cycle in the laboratory (as a component of another research project). After 12 weeks salamanders were subjected to the washing protocol described below. The washing protocol was also field tested on Northern Gray-cheeked Salamanders (*Plethodon montanus*; *N* = 6) in Nantahala National Forest (35.700°N, 082.399°W), North Carolina, USA. Northern Gray-cheeked Salamanders were collected by hand from beneath logs on 12 May 2014, stored with moist substrate in plastic ziplock bags, and subjected to the washing protocol within 8 h of collection.

**Washing protocol.**—This procedure required two researchers beginning the protocol with clean nitrile or latex gloves and is outlined in Table 1. Researcher #1 held the amphibian while

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**Table 1. Standardized washing protocol developed and tested in this study.**

<table>
<thead>
<tr>
<th>Protocol Step</th>
<th>Researcher #1 Description</th>
<th>Researcher #2 Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squirt bottle</td>
<td>Begin the protocol with clean nitrile or latex gloves. Hold the salamander while researcher #2 washes it.</td>
<td>Wash the salamander for 15 seconds (25 mL sterile ddH2O) using a squirt bottle.</td>
</tr>
<tr>
<td>Wash bath #1</td>
<td>Change into fresh gloves at this time to prepare for transfer of the salamander into the second wash bath after the first wash is complete.</td>
<td>Place the salamander into a sterile 90 mm petri dish containing 25 mL sterile ddH2O and swirl the plate in a circular motion for 30 seconds.</td>
</tr>
<tr>
<td>Wash bath #2</td>
<td>Wearing fresh gloves, place the salamander into a sterile 90 mm petri dish containing 25 mL sterile ddH2O and swirl the plate in a circular motion for 30 seconds.</td>
<td>Change into fresh gloves at this time to prepare for transfer of the salamander into the third wash bath after the second wash is complete.</td>
</tr>
<tr>
<td>Wash bath #3</td>
<td>Change into fresh gloves at this time to prepare for swabbing of the salamander.</td>
<td>Wearing fresh gloves, place the salamander into a sterile 90 mm petri dish containing 25 mL sterile ddH2O and swirl the plate in a circular motion for 30 seconds.</td>
</tr>
<tr>
<td>Swabbing</td>
<td>Hold the salamander while researcher #2 performs the swabbing technique.</td>
<td>Swab the salamander on the left, right, dorsal, and ventral sides for a total of 15 strokes from the head to the tip of the tail.</td>
</tr>
<tr>
<td>Storage</td>
<td>Store swabs at -20°C within one hour of being collected for lab specimens or in 99.9% acetone for field collections.</td>
<td></td>
</tr>
</tbody>
</table>
To confirm the presence of resident bacterial communities a subset of the salamanders (N = 10, *P. cinereus; N = 6, *P. montanus*) subjected to the washing protocol were swabbed with a sterile cotton swab autoclaved for two hours (Gefrides et al. 2010) and DNA extracted with the Qiagen DNA Micro Kit according to the manufacturers protocol for isolating genomic DNA from small volumes of blood. The following alterations were made to the DNA extraction protocol: extraction was performed directly from the swab after an incubation period of 7 h (with proteinase K - step 5 of protocol). PCR conditions were as follows for a 25 µl reaction: 12.5 µl New England Biolabs Taq 2X Master Mix, 1 µl (10 µM) forward primer 515F, 1 µl (10 µM) reverse primer 806R (Caporaso et al. 2011), 5 µl DNA, 5.5 µl sterile ddH2O. Electrophoresis was performed in a 1% agarose gel at 140 volts for 40 min. Five µl of each PCR reaction was added to each well. PCR amplicon fluorescence was interpreted as the presence of resident microbial communities on the skin of these salamanders (Figs. 2–3). For *P. cinereus*, 9 of 10 skin swabs showed PCR amplicons (Fig. 2), whereas for *P. montanus* 4 of 6 skin swabs had dull PCR amplicons (Fig. 3).

**Discussion**

This study successfully demonstrates and tests a new standardized protocol for removing environmental microbes for studies on resident microbes composing the amphibian microbiome. We show that successive washes with sterile ddH2O significantly reduce the amount of CFUs and also that PCR amplifiable microbial DNA is present upon concluding the protocol from salamanders in both the field and the laboratory. Field and laboratory experiments revealed similar results, confirming that our methods can be made portable for further applications of this protocol. This technique is similar to previous studies that showed similar results (Lauer et al. 2007). This protocol will help standardize wash time, water volume, and researcher technique, all of which are critical in the removal of environmental microbes from amphibian skin. Adopting a standardized protocol amongst labs will be imperative for direct comparison of future studies on the amphibian microbiome.
We acknowledge that a plate-based assay is a limitation of this study since it only quantifies microbes capable of growing on synthetic medium at 32°C. In addition, gel electrophoresis of PCR amplicons from salamander skin swabs was used to confirm the presence of skin symbionts. It could be argued that these microbes are environmental in origin. However, based on past research by Culp et al. (2007), Lauer et al. (2007), and the plate-based assay results in this study, we conclude that environmental microbes were successfully removed and the PCR amplicons are representative of skin symbionts of *P. cinereus* and *P. montanus*.

We also acknowledge the limitations placed on this washing protocol by the size of the amphibian; studies on larger amphibians will be restricted by Petri dish size. This protocol was designed for small to medium amphibians (< 150 mm length × < 15 mm height) and should prove useful for studies of many similar-sized amphibian species worldwide.

Previous studies have reduced cutaneous microbiota using broad-spectrum antibiotics and/or hydrogen peroxide in salamanders and fish (Lumsden et al. 1998; Russo et al. 2007; Becker and Harris 2010). Our study suggested that these extreme measures might not be necessary to remove environmental microbes. We hypothesize that a volume of 50 ml of sterile water is sufficient to remove the vast majority of environmental microbes, similar to that used in previous studies (60 ml ddH$_2$O in Culp et al. 2007; 50 ml ddH$_2$O in Lauer et al. 2007). If this is the case, then results based on next-generation sequencing studies using variations on these washing protocols are likely accurate in the characterization of the amphibian skin microbiome (McKenzie et al. 2012; Fitzpatrick and Allison 2014; Kueneman et al. 2014; Walke et al. 2014).

The need for standardization of such protocols has arisen due to the precarious health of world amphibian populations. This is due in part to emerging infectious pathogens such as *B. dendrobatidis* and the newly discovered *B. salamandrivorans*, which has caused population crashes of salamanders in Europe and has the potential to cause worldwide declines in species diversity (Martel et al. 2013). Since the associated microbiota of amphibians can be considered an important component of the amphibian immune response, studies focusing on intra- and interspecific differences in the skin of amphibians are needed to better understand interactions between the skin of amphibians and pathogens.

In the future such studies are capable of becoming exceedingly sophisticated with the advent of next-generation sequencing and bioinformatics approaches. However, a necessary first step is a clean sample. We recommend standardization of protocols as studies proceed. This protocol provides researchers with a viable and experimentally tested washing protocol to remove environmental bacteria from the skin of small to medium sized amphibians.

### Table 2. Comparison of washing protocols.

<table>
<thead>
<tr>
<th>Study species</th>
<th>Number of washes</th>
<th>Total wash time (sec)</th>
<th>Total wash water volume (ml)</th>
<th>Type of wash</th>
<th>Swabbing protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>Plethodon cinereus Plethodon montanus Notophthalmus viridescens Lithobates catesbeianus</td>
<td>4</td>
<td>105</td>
<td>100</td>
<td>sterile ddH$_2$O</td>
</tr>
<tr>
<td>Culp et al. 2007</td>
<td>Plethodon cinereus</td>
<td>3</td>
<td>90</td>
<td>60</td>
<td>sterile ddH$_2$O</td>
</tr>
<tr>
<td>Lauer et al. 2007</td>
<td>Plethodon cinereus</td>
<td>2</td>
<td>Not reported</td>
<td>50</td>
<td>sterile dechlorinated H$_2$O</td>
</tr>
<tr>
<td>McKenzie et al. 2012</td>
<td>Lithobates pипiens Pseudacris triseriata Ambystoma tigrinum</td>
<td>3</td>
<td>Not reported</td>
<td>300</td>
<td>sterile H$_2$O</td>
</tr>
<tr>
<td>Fitzpatrick &amp; Allison 2014</td>
<td>Plethodon jordani</td>
<td>1</td>
<td>Not reported</td>
<td>50</td>
<td>sterile dechlorinated H$_2$O</td>
</tr>
<tr>
<td>Kueneman et al. 2014</td>
<td>Anaxyrus boreas Pseudacris regilla Taricha torosa Lithobates catesbeianus Rana cassidae</td>
<td>2</td>
<td>Not reported</td>
<td>100</td>
<td>sterile H$_2$O</td>
</tr>
<tr>
<td>Walke et al. 2014</td>
<td>Lithobates catesbeianus Notophthalmus viridescens</td>
<td>2</td>
<td>Not reported</td>
<td>Not reported</td>
<td>sterile dechlorinated H$_2$O</td>
</tr>
</tbody>
</table>


