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Short communication

Dietary preferences of Hawaiian tree snails to inform culture for conservation

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ABSTRACT

One strategy to safeguard endangered species against extinction is raising subpopulations in *ex situ* facilities. Feeding animals *ex situ* is difficult when their diet is cryptic. We present a combined molecular and behavioral approach to assess the diet of *Achatinella*, a critically endangered genus of tree snail, to determine how diet of captive snails differs from wild snails. Cultured snails are currently fed biofilms growing on leaf surfaces, as well as a *Cladosporium* fungus isolated from this same habitat. Amplicon sequencing of DNA extracted from feces of wild and cultured snails confirms that this *Cladosporium* is abundant in the wild (~1.5% of sequences), but it dominates the *ex situ* snails' diet (~38%) and the diet of captive snails is still significantly less diverse than wild snails. To test the hypothesis that snails have diet preferences, we conducted feeding trials. These used a surrogate snail species, *Auriculella diaphana*, which is a confamilial Oahu endemic, though non-federally listed. Contrary to our expectations we found that snails do have feeding preferences. Furthermore, our feeding preference trials show that over all other feeding options snails most preferred the "no-microbe" control, which consisted only of potato dextrose agar (PDA). PDA is rich in simple carbohydrates, in contrast to the oligotrophic environment of wild tree-snails. These results suggest further research should focus on calorie budgets of snails, devising new approaches to supplementing their *ex situ* diet and determining whether a wild diet is an optimum diet.

1. Introduction

All of the species of the endemic O'ahu tree snail genus *Achatinella* (family Achatinellidae) have been listed under the U.S. Endangered Species Act since 1981 (USFWS, 1981), and all remaining genera and species from throughout the Hawaiian Archipelago are considered either species of concern or critically threatened. Extinctions caused by habitat loss, shell collectors and especially, invasive predators have reduced approximately 41 species of *Achatinella* to just ten species (Holland and Cowie, 2009) with only a single individual remaining in the species *Achatinella apexfulva* and less than ten known individuals of *A. fulgens* in the wild. To minimize the risk of extinction of surviving species, an *ex situ* breeding facility, the Hawaiian Tree Snail Conservation Laboratory (HTSCL), has maintained subpopulations of the snails since the late nineteen-eighties. However, these *ex situ* populations are prone to episodes of high mortality and have not flourished despite the absence of predators. Because wild stocks of these unique animals are quickly declining, managers are anxious to improve lab conservation strategies. The present study examines the use of non-invasive methods and surrogate species to explore how the *ex situ* diet of a critically endangered species differs from their wild diet. This will enable further experimentation to determine if changing the diet to closer approximate the wild diet can improve animal fitness.

The *ex situ* culture facility is modeled on the snails' natural ecosystem (Hadfield et al., 2004), but while temperature and humidity can be monitored *in situ* and simulated in incubators, the diet of wild snails has not been artificially replicated because the composition of their wild diet was not characterized until recently (O'Rorke et al., 2014; Price et al., in press). *Achatinella* graze microbes from leaf surfaces, and so, every two weeks their cages in the *ex situ* facility are provisioned with a supply of leaves collected from the wild. This wild "sourced" diet is supplemented by a cultured *Cladosporium* fungus that was isolated around 1989 from a native *Ohia* tree (*Metrosideros polymorpha*), which is a common host plant for the snails (Kobayashi and Hadfield, 1996). Observations of *ex situ* snails suggest that they will consume almost any microbe that they encounter, but the hypothesis that snails do not have a preference for food items has not been tested in a controlled experiment. Wild populations of tree snails have a very diverse microbial diet (O'Rorke et al., 2014; Price et al., in press), but it is not clear if this is because they indiscriminately consume food from any surface they happen to be on, or if they are targeting particular microbes but accidentally consume non-target diet items as well. Determining snail preferences provides a potential conservation opportunity, because it will indicate whether captive snails should be provisioned with particular foods.

To determine whether the *Cladosporium* isolate that is used to supplement the *ex situ* snail diet is a large component of their diet we

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sequenced fungal DNA from their feces. This also enabled us to determine the degree to which *ex situ* diet overlaps that of the wild populations. We also determined whether snails prefer particular diet items by conducting feeding trials in which isolated fungus and bacteria strains were offered to the tree snail *Auriculella diaphana*. This snail was used as a model for *Achatinella* because although it is of conservation concern, it is more fecund and is not listed as endangered. *Auriculella* are an excellent surrogate for *Achatinella* because they are often sympatric and cohabit the same leaves (Pilsbry et al., 1912) and the dietary remnants in the fecal contents of sympatric *Auriculella* and *Achatinella* are similar, even when sampled almost a year apart (O'Rorke et al., 2014). In addition, both species are members of endemic Hawaiian subfamilies of achatinellid tree snails, the Auriculellinae and the Achaintellinae, which are phylogenetically closely related sister groups (Holland and Hadfield, 2004).

2. Methods

2.1. Snails and microbial isolates

Achatinella snails are housed at the snail culture facility at the HTSCL at the University of Hawaii in Manoa (Table 1). *Auriculella diaphana* used for the feeding trial were collected from the Kalawahine Trail on Mt. Tantalus (Table 1: GPS coordinates available through the US Fish and Wildlife service by request), under Department of Land and Natural Resources permit (FHM13-T&E-11). Microbial cultures were isolated from leaves or snail fecal samples obtained from locations on Oahu (Table 2). The microbial isolates are housed in the University of Hawai'i fungal culture collection and DNA sequence "barcode" regions were obtained using the ITS1F/ITS4B primers for fungi (Gardes and Bruns, 1993) and the 515f/806r 16s v4 primers for bacteria (Caporaso et al., 2012) and these are available from NCBI (Table 2 for accession numbers). Microbial isolates were grown on potato dextrose agar (PDA) for the feeding trial and were offered to the snails on plugs of PDA that were identical to control PDA-only plugs.

2.2. Determining the diet of ex situ snails with DNA sequencing

35 snail fecal samples were obtained from the HTSCL between late February and early March of 2013 (Table 1). The diet of the snails was determined by sequencing DNA extracted from these feces following the methods outlined in O'Rorke et al. (2014). Briefly, a next-generation sequencing (NGS) approach was used, where DNA was extracted from feces using the Powersoil® DNA isolation kit (MoBio) and then PCR amplified with ITS1 specific primers that contained Illumina primers and sequence index tags (Smith and Peay, 2014). Sequences were cleaned using SequalPrep™ Normalization plates (Invitrogen, New York) and subsequently pooled, cleaned using a SPRI plate (Beckman Coulter, California) and Sera-Mag™ Magnetic SpeedBeads™ (Fisher Scientific, Pittsburgh) in an amplicon:bead ratio of 1.8:1, and quantified on a Qubit® fluorometer (Invitrogen) using the dsDNA HS assay. Bioanalyzer Expert 2100 High Sensitivity chip (Agilent Technologies, California) and qPCR determined cluster density before sequencing. Sequencing was undertaken at the University of Hawaii, Genetics Core Facility using 1/10th of an Illumina MiSeq sequencing reaction with the MiSeq Reagent v3 chemistry (Illumina®).

Table 1
Snail species sampled from *ex situ* facility. Some of the species of endemic Hawaiian tree snails kept at the University of Hawaii Tree Snail Conservation Lab and the numbers of fecal samples collected from each for Illumina amplicon sequencing.

Snail species	Number of feces collected
Achatinella apexfulva	2
A. decipiens	1
A. fulgens	1
A. fuscobasis	5
A. lila	11
A. livida	2
A. mustelina	12
A. sowerbyana	1

Table 2
Microbial isolates used in feeding preference trial. Isolates were obtained from either snail feces or leaf surfaces. The isolates from snail feces are assumed to either be undigested food or part of the gut microbiota. DNA sequences of the ITS1-ITS2 (Fungi) and 16S subregion (Bacteria) are available through NCBI.

Genus	ID	Source	Sampling location	NCBI accession
Cladosporium	RH1–01	Ohia leaf	Mt. Olympus	KU552068
Beauveria	PH_14051_6	Snail feces	Pu'u Hapapa	KU552069
Microbacterium	Kea_007	Snail feces	Palikea	KU552062
Bacillus sp. str 2	Kea_012	Snail feces	Palikea	KU552063
Enterobacter	Kea_044	Snail feces	Palikea	KU552064
Brevundimonas	Kea_041	Snail feces	Palikea	KU552065
Bacillus sp. str 2	Kea_043	Snail feces	Palikea	KU552066
Micrococcus	Kea_013	Snail feces	Palikea	KU552067
Stenotrophomonas	Kea_008	Snail feces	Palikea	KU552061
Annulohypoxylon	RH1–04	Ohia leaf	Mt. Olympus	KU552070
Botryosphaeria	Kea_053	Snail feces	Palikea	KU552071

The bioinformatics pipeline used to process the DNA sequences is included in the supplementary materials, but briefly consists of the following steps. Sequences were merged using PEAR (Zhang et al., 2013), demultiplexed in QIIME (Caporaso et al., 2010) and clustered into operational taxonomic units (OTUs) at 97% similarity using UPARSE (Edgar, 2013). The OTU community matrix was imported into R and rarefied to 3500 sequences per sample. Abundances of OTUs were used to generate ranked abundance curves and Shannon alpha-diversity indices (.r file in Suppl materials). Alpha diversity and Pielous evenness indices were compared between feces from *ex situ* populations and the feces of 128 wild *Achatinella mustelina* sampled from four separate geographic locations (O'Rorke et al., 2014) using the Mann–Whitney (Wilcox) test (.r file in Suppl materials).

2.3. Determining food preferences of tree snails

Twenty-four hour feeding trials were conducted in a Percival Intellus environmental incubator on a 12 h dark/light cycle (0.8 lx/1016.2 lx) shifting between 16 °C and 20 °C, based on ambient day/night temperatures recorded in the snail's natural environment. Snails were acclimated to the incubator for at least 14 days before trial and not fed for 12 h prior to the feeding trial. Each individual snail was placed in a 450 mL glass jar. Twelve plugs of PDA agar (diameter = 1 cm) that carried either one of eleven microbial isolates (Table 2) or a PDA-only control were evenly spaced around the perimeter of the ceiling of the jar in a random order (Fig. 1). High-resolution photographs were taken of the snail feeding trial using a Canon 650D DSLR camera through a Canon 40 mm lens. One photograph was taken every 10 s. Shutter speeds were 1.3 s duration through the dark cycle (which caused some blurring when snails were moving) and 0.008 s during the light cycle.

The still images of the feeding trial were assembled into an animated movie in Adobe Premiere Pro. A snail was scored as being associated with food if its head was on a food item. Preference for a particular food item was visualized using the forage ratio, $F = r/p$, where r is the proportion of total time associated with a particular food item and p is the proportion of that food item among all food choices (Savage, 1931; Manly et al., 2002). A food item with a forage ratio < 1 is considered to be avoided and > 1 is preferred. The significance of food selection was tested using the 'compana' command of package (adehabitatHS) in r (Calenge, 2006). This is a routine used to assess resource preference in animals, such as food preferences (Aebischer et al., 1993; Soininen et al., 2013) in which log ratios of proportions of food visited relative to food availability are tested against other food choices to asses if they are distinct (Aebischer et al., 1993). This multivariate test is performed by