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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

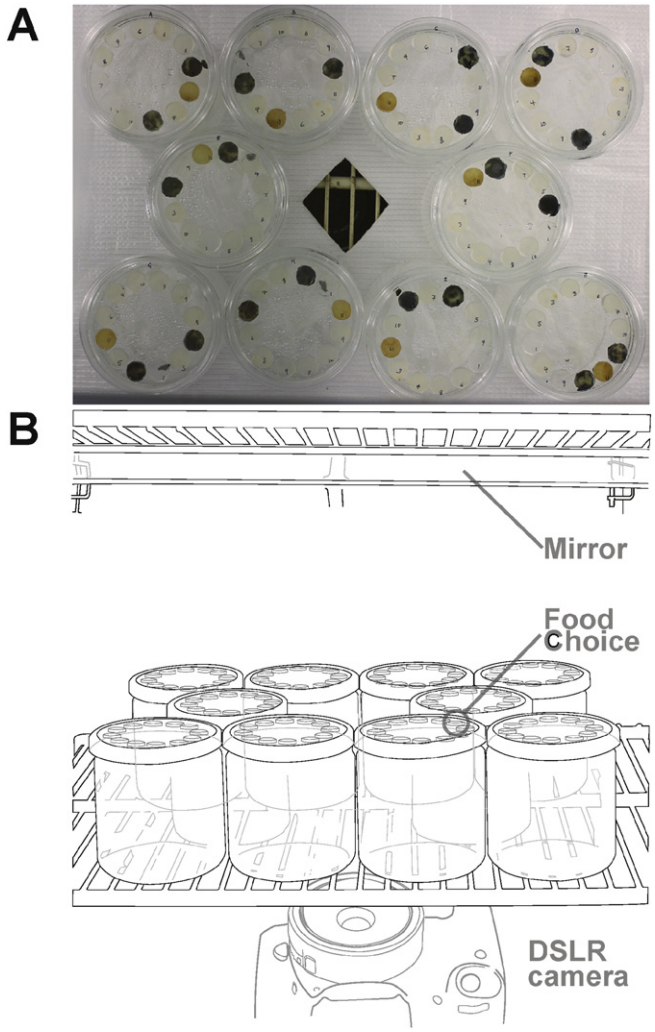

Fig. 1. Experimental setup used to determine if snails do have feeding preferences. Snails were fasted for 12 h and then an individual snail was placed into one of each of ten jars. Twelve different food choices were placed around the perimeter of the underside of the lid of each jar. A digital single lense reflex (DSLR) camera was used to photograph the tops of the jars through a mirror, in order to record how much time each snail spent with each food option. Photographs were taken once every 10 s over 24 h (12 h dark 12 h light) and then assembled into video clips for analyses. Movies are available in supplementary files (S1–S3).

Wilks'-lambda, which provides a value that indicates the proportion of variance that is not explained by differences among groups. Subsequently a ranking matrix is built by the compana command, which formally clusters food choices by time spent in contact with them and then ranks these choices against available food options (Aebischer et al., 1993). Analyses are available as an .r file in Supplementary materials.

3. Results

3.1. Diversity of the ex situ diet

A total of 619,996 high quality DNA sequence reads were obtained from the 1/10th Illumina Miseq run of ITS1-barcoding genes amplified from feces from the HTSCL (NCBI SRA accession PRJNA260291). The diversity of food items in the *ex situ* facility was 0.700 ± 0.042 (S.E.M) and is significantly lower than that observed in snail feces sampled from the wild 0.914 ± 0.010 (S.E.M) Mann–Whitney $W = 747$ and $p = 9.1 \times 10^{-9}$. Differences were driven by a single OTU: "OTU_1" which dominated the dataset and accounted for 38.6% of the reads (Fig. 2A). In comparison OTU_1 accounted for only 1.33% DNA sequence reads of wild snails (Fig. 2B). DNA of OTU_1 was 100% identical to the *Cladosporium* species that is used to supplement the diet of snails in culture.

3.2. Feeding trials

Twenty six *A. diaphana* snails were trialed, but the food in two mazes became dislodged, which meant that these two trials were disregarded and the total number of snails was twenty-four ($N = 24$). Individual snails spent a disproportionate amount of time on a single food choice (Fig. 3). Although there was no single food type that all snails preferred, there was a distinct set of preferred or avoided food choices with a low Wilks'-lambda value of 0.03 ($p = 0.002$) which demonstrates that there were large and significant differences in how much time a snail spent with each particular food choice. Compositional analysis, which was used to cluster and rank food choices based on how frequently snails visited them, found that there were three equally preferred food items: the PDA control, and the fungi *Botryosphaeria* and *Cladosporium* (Fig. 3). Snails spent the greatest time on the PDA control on average (Fig. 3). The bacteria from *Microbacterium* and *Micrococcus* occurred in the next cluster and had a forage ratio of ~1, which is indicative of no preference. All the other fungi and bacteria had a forage ratio < 1, which is consistent with avoidance. Both *Bacillus* strains were clustered together in the most avoided grouping. The snails all spent less than 20 min with the *Bacillus* strains over the 24 h trial, except for one snail which was associated with *Bacillus* strain 2 for 4.48 h. While the PDA-only control was a preferred food type, the two bacterial strains of *Bacillus* sp. also acted as a control to test that the snail responses to similar food were consistent. Video files in which *A. diaphana* are trialed on different foods can be viewed in supplementary materials or through this link wwfoofw.com/trial. Snails were also placed on PDA medium and closely observed to confirm that they did feed on the medium (video file available through the supplementary materials or through this link http://wwfoodw.com/rasping) and visual inspection of PDA controls for radula marks also confirmed that feeding had taken place.

4. Discussion

4.1. Ex situ diet

The Shannon diversity index of the diet of wild snails is significantly greater than that of cultured snails, which is due to the dominance of the *Cladosporium* "supplement" in the cultured diet. Therefore, the *Cladosporium* is less of a supplement and instead a major component of the diet of snails. We were concerned that after twenty-five years of cultivation, this isolate was no longer similar to wild strains due to contamination. However, we determined that this *Cladosporium* species is the sixth most common species of the snail diet in the wild (Fig. 2).

4.2. Feeding trials

Despite the superficial appearance that snails are indiscriminant feeders, we found that snails have significant food preferences. This result is similar to the discovery that aquatic snails are selective feeders despite the apparent evidence that they indiscriminately grazed periphyton (Brönmark, 1989). Oahu tree snails were long believed to eat fungus. The basis for this determination, however, relied on microscopic analysis of fecal pellets (Pilsbry et al., 1912) in which fungi are more easily observed than smaller microbes. We found that classifying the snails as mycophagous is justified, because snails tended to avoid most bacteria tested. The bacteria, *Micrococcus* and *Microbacterium* were occasionally consumed and can be considered as "not repellent" if not attractive to snails (Fig. 3). Both of these isolates are pigmented and belong to clades that do occur in the phyllosphere where pigments act as photo-protectants (Vorholt, 2012), so it is plausible that snails do

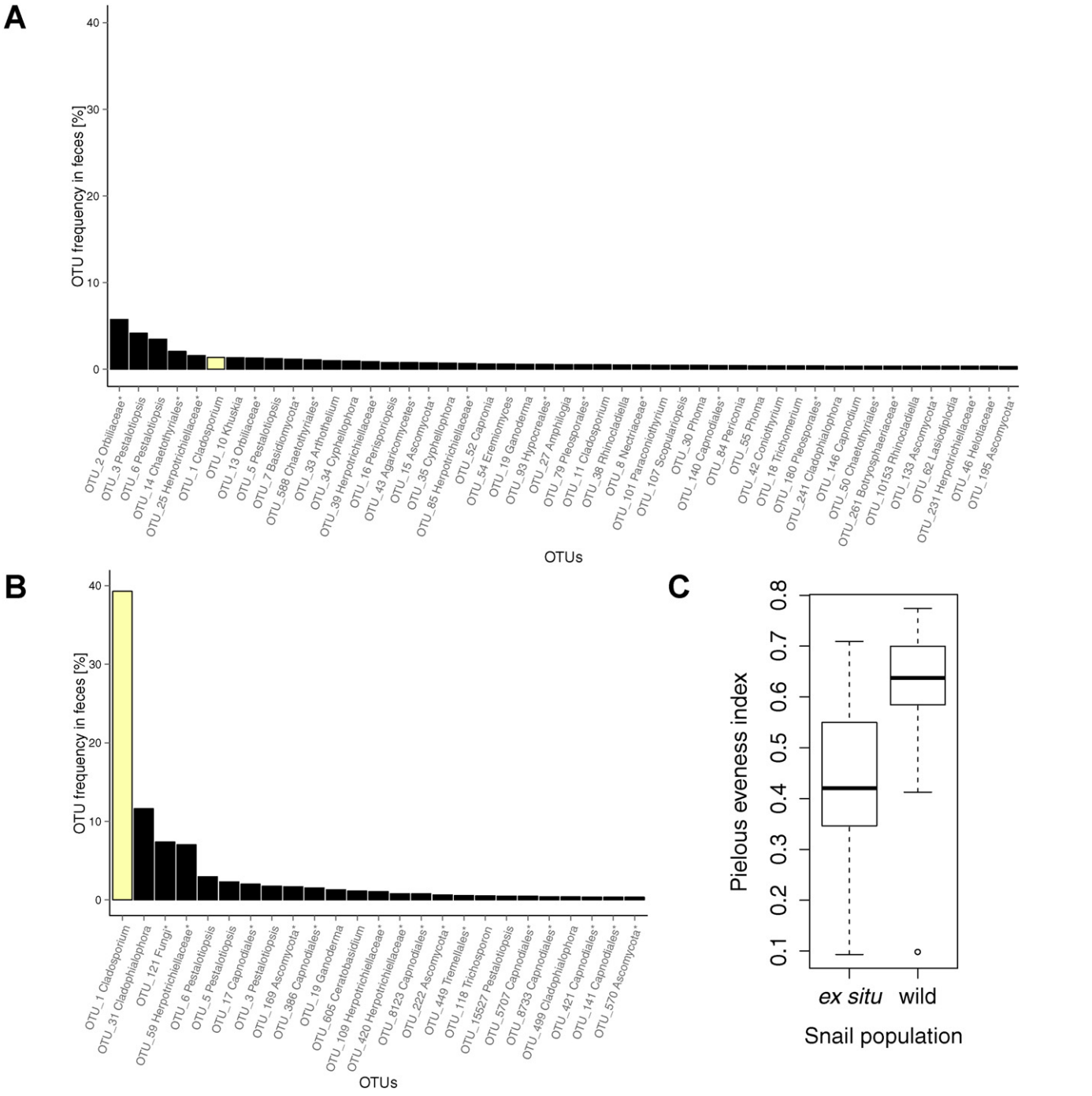

Fig. 2. Ranked abundance of fungal OTUs from DNA sequences obtained from feces of a) wild and b) *ex situ* cultured snails and the c) evenness of food composition in diet (note the difference in scale). (2 A) Wild populations of *Achatinella mustelina* have a diverse diet with no diet items dominating their gut content, (2B) the snails in the *ex situ* facility have a diet that is dominated by a single *Cladosporium* OTU (highlighted yellow rather than black), which took up 38.6% of the sequenced reads from the feces of cultured snails. This OTU also occurred in the wild (highlighted in yellow rather than black), but its overall abundance was 1.33%. (2C) The evenness of the diet composition of wild populations is less dispersed than for *ex situ* cultured snails.

graze on these taxa in the wild. Of the fungi offered to snails, they preferred the dark pigmented *Cladosporium* and Botryosphaeria, which are common colonists of leaves (Baker et al., 1979; Denman et al., 2003; van Niekerk et al., 2004), over either the *Beauveria* or *Annulohypoxylon*. These less preferred fungi are both typical members of a wild fungal assemblage but are not direct colonists of leaf surfaces, as *Beauveria* are typically invertebrate pathogens (and are renamed as *Cordyceps*) and *Annulohypoxylon* are pathogens of fungus. These data therefore suggest that tree snails do have a preference for particular microbes. It would be of interest to determine if tree snails have preferences between the species of fungus that they more frequently in the wild, (such as OTUs 2, 3 and 6 for *Achatinella* snails as seen in Fig. 2a), but we are so far not able to isolate many of these species of fungus in order to conduct this experiment. There is no literature on how tree snails acquire preferences for foods, however studies of other pulmonate molluscs indicate that they can be conditioned to prefer food (Sahley et al., 1992; Desbuquois and Daguzan, 2004). It is plausible that these particular snails preferred *Cladosporium* and *Botryosphaeria*

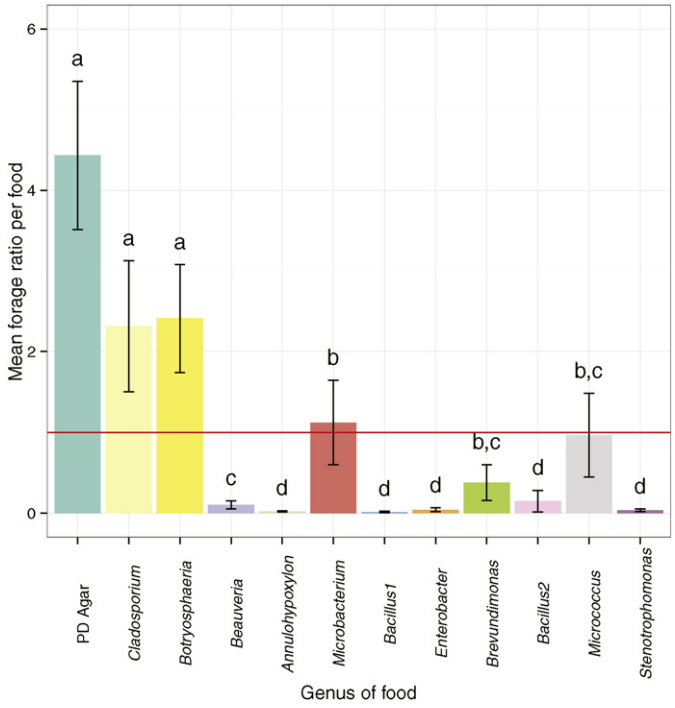

Fig. 3. Feeding preferences of snails. A forage ratio above one (the red line) indicates a favored resource and less than one is an avoided item. The "food" offered to snails was an agar only control, then four fungi and the seven samples to the right of the graph are bacteria, Labels above bars are the results of compositional analysis of preference (Aebischer et al., 1993) and food ranked with an "a" are co-preferred, those with "b" are the next preferred group and those with a "c" and "d" are the next preferred groups respectively.

because they had encountered them before. However, further experimentation is required to determine if these preferences are acquired rather than innate.

That snails show some preference for particular food groups resolves an important long-standing ecological question about these lineages. In previous work it was found that the composition of the snail diet was similar to what was available to them (O'Rorke et al., 2014), but we were unable to resolve whether snails were truly indiscriminant feeders. Tree snails tend to be associated with particular host tree species (Meyer et al., 2014), which is also true of *Achatinella* snails (Price et al., in press). This host preference could be due to differences in the community composition of microbes that occur on those trees, even if those differences are subtle (O'Rorke et al., 2014).

When an endangered animal is in degraded habitat, or threatened by predation, it is common to translocate them to better or safer habitat. However, it has been observed that *Achatinella* tree snails migrate after translocation (USFWS, 1993). Consequently, the recovery plan for *Achatinella* recommends that field workers should remain in the field with translocated snails for at least one week to monitor whether snails leave their new habitat, and return any that do (USFWS, 1993). The present study indicates that snails have preferences for particular microbial foods which suggests that further experimentation needs to be undertaken to determine if microbial community composition modifies the tendency of snails to migrate away from translocation sites. If this were the case, then novel translocation sites can be manipulated so that the phyllosphere compositions resemble preferred mixtures to improve host fidelity after translocation. This is a topic requiring further research, because microbial manipulation could potentially reduce the labor effort associated with translocations.

4.2.1. Consuming carbohydrate rich media

A surprising result from the feeding trials was that snails preferred the control "PDA medium only" treatment over any treatment containing a microbial isolate on the PDA (Fig. 2). PDA is the medium used to grow the *Cladosporium* food that is used for *ex situ* culture and is a very simple and high calorie medium that contains only potato extract and glucose (*i.e.*, a western "junk food" diet). This suggests that the current method of supplementing the *ex situ* diet with fungus on PDA should be re-evaluated, especially because the cultured fungus comprises such a high percentage of the snail diet (Fig. 2).

Achatinella mustelina growth rates are more than two times faster when their diet of microbes grazed from wild sourced leaves is augmented by cultured fungus compared to when they feed on leaf microbes only (Kobayashi and Hadfield, 1996). However, we don't know if increased growth rate is correlated with reproductive fitness of long-term survival of captive snails. The natural phyllosphere is a highly oligotrophic environment, and the snails have not evolved in an environment that provides calorie-rich simple carbohydrates for a sustained period as occurs in the *ex situ* enclosures. Very little research has been conducted on the effect of calorie intake on gastropods, and none on tree snails. However, it has been found that the egg laying activity of the snail *Biomphalaria glabrata* is reduced by 66% when fed on a carbohydrate rich diet compared to a control diet (Stanislawski and Becker, 1979). It is also a common observation in model-animal systems that higher calorie intake has a detrimental effect on longevity, despite proximate gains in growth rate (Guarente and Kenyon, 2000; Bishop and Guarente, 2007).

Tahitian land snails of the genus *Partula* are mostly extinct *in situ*, but have been maintained in captivity in several international institutions where they are fed a "paste" made from native chalk, grass powder, trout pellets, oats, Vitamin E and dog food 'Stress' (Tonge and Bloxam, 1991, Wells, 1995; Gouveia, 2011). The wild diet of *Partula* is not specifically known, although they are mostly believed to be detritivores (Gouveia et al., 2011), and microscopic analysis of dietary remnants in feces has shown that there is considerable variation between these species (Gerlach, 2014 http://islandbiodiversity.com/diet.pdf). The trophic ecology of *Achatinella* or *Auriculella* is therefore not analogous to *Partula*, and the energy available to wild snails might vary considerably, but it is informative that an artificial diet is a possible means of keeping viable *ex situ* populations.

Dietary supplementation is frequently used as a tool to manage the decline of wild animal populations, but recent criticism of this approach points to the need of frequent re-evaluation of whether supplementary feeding is having the intended ecosystem level results (Ewen et al., 2014; Martínez-Abraín and Oro, 2013). The results of the present study indicate important next steps, such as developing a model tree snail system and to use this to determine if there is a similar reduction in fitness for endangered tree snails when fed a carbohydrate rich diet in captivity. It would also be beneficial to determine the energy requirements of these animals through respirometry to better match their energy needs to the energy content of the food with which they are provisioned. This would also be useful for evaluating the carrying capacity of habitats into which the snails are re-introduced.

5. Conclusion

Hawaiian tree snails are under threat and translocating them to protected habitats and *ex situ* facilities is presently the best means to avoid extinction. *Cladosporium* is a disproportionately high component of their *ex situ* diet, and they preferentially feed on the PDA fungal growth media. Therefore there is a need to reevaluate how captive snails are fed, and, having found that the diet of captive snails is significantly different to their wild counterparts, we need to determine whether deviating from their wild diet affects the snails' long-term fitness. We suspect that increasing the diversity of snails' diets is a good initial conservation action, which needs to be evaluated. Understanding that snails have dietary preferences explains key behavioral and ecological traits of these animals, such as their patchy distributions in the wild and provides us with a valuable tool for managing these animals in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.biocon.2016.03.022.

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