Escaping the captive diet: enhancing captive breeding of endangered species by determining dietary preferences

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Abstract
Endangered species can be safeguarded against extinction by raising subpopulations in ex situ facilities that mimic their wild habitats. This is difficult when the endangered animal’s diet is cryptic. We present a combined molecular and behavioral approach to assess the ex situ diet of Achatinella, a critically endangered genus of tree snail, to determine how diet of captive snails differs from wild snails. Ex situ snails are currently fed biofilms growing on the surface of leaves, as well as a cultured fungus isolated from this same habitat. Amplicon sequencing of DNA extracted from feces of cultured snails confirms that this cultured fungus is abundant in the wild, but that it dominates the diet of the ex situ snail diet (comprising ~38% of sequences). The diet of captive snails is significantly less diverse compared to 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 congeneric 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 and on devising new approaches to supplementing their ex situ diet.
**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 & Cowie 2009) with only a single individual remaining in the species *A. apexfulva* and less than ten known individuals of *A. fulgens* in the wild. To safeguard the genetic stocks 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 can be improved in order to improve their 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. n.d.). *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 & 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. n.d.), 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 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 Auriculementinae and the Achaintellinae, which are phylogenetically closely related sister groups (Holland & Hadfield 2004).

**Methods**

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 & 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.

Determining the diet of *ex situ* snails with DNA sequencing

34 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 & 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®).

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 wild (O’Rorke et al. 2014; Price et al. n.d.) and *ex situ* populations using the Mann-Whitney (Wilcox) test (.r file in Suppl materials).
Determining food preferences of tree snails
Twenty-four hour feeding trials were conducted in an Percival Intellus environmental incubator on a 12 hour 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 hours prior to the feeding trial. Each individual snail was placed in a 450 mL glass jar. Twelve plugs of 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 (Figure 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, \( F = \frac{r}{p} \), where \( r \) is the proportion of time associated with a particular food item and \( p \) is the proportion of that food item amongst 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 assess if they are distinct (Aebischer et al. 1993). This multivariate test is performed by 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.

Results
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 XXXXXXXX). 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) W=747 and p=9.1 × 10^{-9}. Differences were driven by a single OTU: "OTU_1" which dominated the dataset and accounted for 38.6% of the reads (Figure 2A). In comparison OTU_1 accounted for only 1.33% DNA sequence reads of wild snails (Figure 2B). DNA of OTU_1 was 100% identical to the Cladosporium species that is used to supplement the diet of snails in culture.
Individual snails spent a disproportionate amount of time on a single food choice (Figure 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* (Figure 3). Snails spent the greatest time on the PDA control on average (Figure 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 minutes with the *Bacillus* strains over the 24 hour trial, except for one snail which was associated with *Bacillus* strain 2 for 4.48 hours. 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 snails responses to similar food was consistent. Movie files in which *A. diaphana* are trialed on different foods can be viewed in supplementary materials. Snails were also placed on PDA medium and closely observed to confirm that they did feed on the medium (Movie file also in supplementary materials) and visual inspection of PDA controls for radula marks also confirmed that feeding had taken place.

**Discussion**

**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 (Figure 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 repellant” if not attractive to snails (Figure 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 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 *Cordyceps* or *Annulohypoxylon*. These less preferred fungi are both typical members of a wild fungal assemblage but are not direct colonists of leaf surfaces, as *Cordyceps* are typically invertebrate pathogens and *Annulohypoxylon* are pathogens of fungus. These data therefore suggest that tree snails do have a preference for particular microbes. 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 but also physiologically respond to particular components of food (Sahley et al. 1992; Desbuquois & Daguzan 2004). It is plausible that these particular snails preferred *Cladosporium* and *Botryosphaeria* because they had encountered them before.

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. n.d.). 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, when translocation is used as a conservation measure it is frequently the case that animals attempt to return to an environment resembling that to which they are habituated (i.e. natal habitat preference induction, Stamps & Swaisgood 2007). This problematic phenomenon has been observed in *Achatinella* tree snails, which migrate when translocated (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). That the present study indicates that snails have preferences for particular microbial foods suggests that translocating snails to environments to which they are habituated might reduce the chances of snails migrating away from translocation sites. Furthermore, if novel translocation sites can be manipulated so that the phyllosphere compositions resemble those of natal host trees, transplant fidelity may be improved. This is a topic requiring further research, because microbial manipulation could potentially reduce the labor effort associated with translocations.

**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 (Figure 2). [Here might be a place to mention Partula culture, since they are provided with a dietary supplement that is high in carbohydrate.]

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 & 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 & 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 & Kenyon 2000; Bishop & Guarente 2007).

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.

**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. However, the practice of provisioning captive bred snails with Cladosporium grown on PDA is clearly falling short of the objectives of making the ex situ habitat mimic that of the wild. 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 to understand how deviating from their wild diet composition affects snail’s long-term fitness. We suspect that increasing the diversity of snails’ diets is a good initial conservation action. 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.

Acknowledgements
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References


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.

<table>
<thead>
<tr>
<th>Snail species</th>
<th>Number of feces collected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Achatinella apexfulva</em></td>
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</tr>
<tr>
<td>A. decipiens</td>
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</tr>
<tr>
<td>A. fulgens</td>
<td>1</td>
</tr>
<tr>
<td>A. fuscobasis</td>
<td>5</td>
</tr>
<tr>
<td>A. lila</td>
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</tr>
<tr>
<td>A. livida</td>
<td>2</td>
</tr>
<tr>
<td>A. mustelina</td>
<td>12</td>
</tr>
<tr>
<td>A. sowerbyana</td>
<td>1</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Genus</th>
<th>ID</th>
<th>Source</th>
<th>Sampling location</th>
<th>NCBI Accession</th>
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<td>Mt Olympus</td>
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<td>Snail Feces</td>
<td>Pu‘u Hapapa</td>
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Figure 1. Experimental setup used to determine if snails do have feeding preferences. Snails were fasted for 12 hours 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 hr (12 hr dark 12 hr light) and then assembled into video clips for analyses. Movies are available in supplementary files (S1-S3).
Figure 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). (2A) 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), 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), 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.
Figure 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.