

**Preferences and Rates of Feeding in *Ariolimax columbianus*  
and *Arion rufus***

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**This paper is the result of a student project conducted over a single summer and some of our samples are still in process. We are currently working on a peer-reviewed publication. Please contact the authors to ask for an updated version and do not cite this article.**

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## **Abstract**

*Arion rufus* was recently introduced from Europe to the Pacific Northwest and may compete for resources with the native banana slug *Ariolimax columbianus*. I conducted feeding trials with the plants pathfinder (*Adenocaulon bicolor*), sword fern (*Polystichum munitum*), lichen (*Usnea* sp.), and store-bought white button mushrooms (*Agaricus bisporus*) to observe how similar the feeding preferences were across species, which were more closely analyzed by size class, source population (Douglas-fir forest or grassland), and color morph for *Arion rufus*. The white button mushrooms were preferred to the other three food choices across all the different source populations. Mushrooms are typically scarce in both the Douglas-fir and grassland habitats so I found this data interesting and compared oven-dried mushrooms and reverse-osmosis water soaked lichen. All the populations preferred the soaked lichen, which may indicate a preference towards texture and moisture rather than a food preference.

I observed the effects of temperature on feeding rates among the different source populations. *Arion rufus* consumed spinach leaves at the same rate across the three temperature treatments (4°C, 10°C, and 20°C); however, the large *Ariolimax columbianus* may be consuming the least at 10°C. This surprising result suggests that the slugs did not have their typical nighttime feeding temperature as their optimal temperature, nor did they increase feeding rate continually with increasing temperature.

## **Introduction**

Invasive species are alien organisms that are introduced to an ecosystem as the result of population expansion to new, typically far-distant, environments and is often unintentionally aided by humans via various modes of transport (e.g., ship bottoms, ballast water, and airplane wheels). *Arion rufus* (hereafter referred to as *Arion*), “the

European slug”, is an invasive species that has many color morphs that include pale tan, russet orange, milk chocolate, very dark brown, and jet black and was likely introduced to the Pacific Northwest and British Columbia from Western and Central Europe on nursery plants and building materials (Rodriguez-Cabal et al. 2015). Since its introduction, this pulmonate has become a pest to local flora in the area (Joe and Curtis 2007, as cited in Rodriguez-Cabal et al. 2015) decimating typical home garden plants such as spinach and basil (R. Merz, personal communication) and even strawberries (Cates and Gordon 1975). Its generalist diet, which includes plants, lichen, fungi, animal feces, and carrion (Forsyth 2004, as stated in Rodriguez-Cabal et al. 2015), allows *Arion* to have a wide range of habitats and potentially threaten the flora in the introduced area because it is an external stress the flora did not evolve with, like the Hemlock Woolly Adelgid negatively impacting the Eastern Hemlock tree (Abella 2014).

In addition to stress on the flora, invasive species may negatively impact local fauna since resources in nature are typically limited, and the arrival of another species may cause competition to arise. The introduction of *Arion* is the first representation of a slug approaching the size of the native banana slug, *Ariolimax columbianus* (hereafter referred to as *Ariolimax*) in the Pacific Northwest (Rollo and Wellington 1975). These two species are believed to be in competition due to their significantly overlapping diets as generalist herbivores. In addition to similar diets, they inhabit similar areas, typically forested areas that are pristine or near human disturbance (Rodriguez-Cabal et al. 2015). However, *Ariolimax* only exists in the forested areas, like the Douglas-fir forest and the Cedar forest, but *Arion* exists in both the forests and the nearby grasslands as well. This is interesting because it could imply incipient speciation within the *Arion* population. Given

enough time without genetic communication, the forest *Arion* population and the grassland *Arion* population have potential to diverge into different species, resulting in allopatric speciation. There is also potential for sympatric speciation within the forest population because of all the existing color morphs. The black color morph of *Arion* is typically found at much larger sizes than the brown and orange color morphs, and the black morph is also less abundant. To consider these potential divergences and their impacts on my data, experiments considered grassland, Douglas-fir brown morphs, and Douglas-fir black morph as three subcategories of *Arion* so I could observe any differences in their feeding preferences and feeding rates. *Ariolimax* were separated into groups based on size to observe any ontogenetic differences among the size classes.

In addition to the feeding preference trials I compared feeding rates of *Arion* and *Ariolimax* across three different temperatures: 4°C, 10°C, and 20°C to mimic a springtime night, a summer night, and summer day respectively. Since slugs are ectotherms, their metabolic rate is typically dependent upon temperature. With climates changing on a global scale, I wanted to observe if the two slugs and their subpopulations reacted differently, if at all, to temperature. If their rates were positively correlated with temperature, then competition might be more pronounced in the future due to already limited resources being depleted faster because of increased feeding rates.

One of the hypothesized results for this experiment was for slugs to consume the most food at 10°C because *Arion* and *Ariolimax* are most active during the early summer (May and June) (Richter 1976), and the average night temperature then is 10°C. I predicted 5°C and 10°C were too extreme and the slugs would be temperature stressed and become idle and not consume much.

## **Methods**

### ***Specimen Collection***

These experiments took place at the Friday Harbor Laboratories, San Juan Island, WA (48°32'43.2"N 123°00'51.4"W). The surrounding forested area has a dense population of *Ariolimax* and *Arion*. Slugs of both species were hand-collected the morning of experiments from alongside a trail in the nearby Douglas-fir forest either as they roamed free or from underneath haphazardly placed pine wood boards (30.48 cm x 30.48 cm) that covered soil soaked in reverse osmosis water and sponges soaked in beer (to act as slug attractants). Additionally, slugs were hand-collected from a nearby grassland (48° 30' 6N 123°4' 28°W) where only milk brown and dark brown morphs of *Arion* occurred but no individuals of *Ariolimax*.

Immediately after collection, animals were separated by species (in the case of *Arion* they were also sorted by color morph) and collection site and housed in large plastic containers lined with reverse-osmosis (RO) soaked paper towels. All of these containers were placed in a dark room at approximately 18°C until the animals were used in experiments later that day. No slug was used more than once in any experiment and all were placed in experiments on the same day they were collected.

### ***Feeding Preference***

*Ariolimax* were separated into three different size classes: small, medium, and large because they tended to have a much larger range of sizes in the field while, *Arion* slugs were not separated into size classes, just color morph and location site. Weights were taken of each animal before use in experiments to reconfirm the visual size determination and to size match across trials.

Plant material for this experiment was field collected and stored in a refrigerator until use to maximize freshness. The collected plants were pathfinder (*Adenocaulon bicolor*), sword fern (*Polystichum munitum*), and lichen (*Usnea* sp.). The fourth food option presented to the slugs was store-bought white button mushrooms (*Agaricus bisporus*). Mushrooms were bought because wild mushrooms could not be collected in the quantity needed in the Douglas-fir forest or the grassland due to the extreme drought the summer the research was conducted. Also Rodriguez-Cabal et al. (2015) claimed that *Arion* consumed up to 7 times more mushrooms than *Ariolimax*, and I wanted to reconfirm this hypothesis and observe any possible preference switch. These options were chosen because previous studies (Rodriguez-Cabal et al. (2015), Thompson and Iyengar (2014), and Richter (1976)) have shown that slugs prefer these species, and personal observations reconfirm that the slugs consume these materials.

A single slug and the four food options (*Adenocaulon bicolor*, *Polystichum munitum*, *Usnea* sp., and *Agaricus bisporus*) were weighed and placed in a plastic container (9.4 cm x 30.5 cm x 22.4 cm) with a mesh-screened section on the lid. The bottom of the container was lined with 1 cm of topsoil (G&B Organics Premium Topsoil), which was moistened with 20 ml of reverse osmosis water. Additionally, small sponges (approximately 2 cm x 2 cm x 2 cm) soaked in reverse osmosis water were placed in each corner to ensure moisture throughout the trial. Moisture is critical for terrestrial slugs because they will desiccate without it. Previous work (Richer 1976), has shown that slug activity is dependent on moisture levels. I placed a sponge in each of the four corners to insure equal moisture across all four-box quadrants that contained the food choices.

To control for any compass-related effects, the location of each food type was rotated clockwise by one corner between each replicate. The initial location and orientation for slug placement remained constant across all replicates. The experimental boxes were placed in a 10°C dark incubator, with treatments interspersed to avoid any effects of placement within the room. After approximately 20 hours, each of the four food options in every container was re-weighed to determine the change in mass during the experiment. Because evaporation or absorption of moisture could alter the mass of the food options separately from the impacts of herbivory, and because this impact was likely to differ across the four food options, I included a set of replicates that contained the same experimental set-up except there were no slugs added to the container. Therefore, the gain or loss of mass of the replicates in this “no slug” treatment was used as a standard. I plotted initial weight of given food type against its final weight for each replicate. The average best-fit line equation from that graph for that food type for each replicate in the slug herbivory treatments was then used to correct the final weights by accounting for different absorption/ evaporation levels.

Based on the results of the trials just described, I conducted a second set of experiments with only two food options: white-button mushrooms (*Agaricus bisporus*) that were oven-dried at 70°C for 24 hours and lichen (*Usnea* sp.) that was soaked in reverse-osmosis water for 24 hours. This experiment investigated whether the food preference of the slugs was primarily driven by moisture content and texture or the nutritional content and chemical taste of the food items.

After the experiment, I disposed of all plant material, euthanized all *Arion* individuals via freezing, and released all *Ariolimax* individuals about 0.5 km from their collection area to avoid recollecting any slugs in future experiments.

### ***Feeding Preference in Various Temperatures***

To determine whether the amount *Ariolimax* and *Arion* eat differ when exposed to various temperatures of 4°C, 10°C, and 20°C, each slug was weighed and then placed individually in a small plastic container (17.5 cm x 17.0 cm x 12.4) with a mesh screened section on the lid and 1 cm of topsoil (G&B Organics Premium Topsoil) that was moistened with 20 ml reverse osmosis water lining the bottom. They were size matched across trials and allowed to feed for approximately 18 hours. I ensured that, across temperatures, the slugs had the same time to feed.

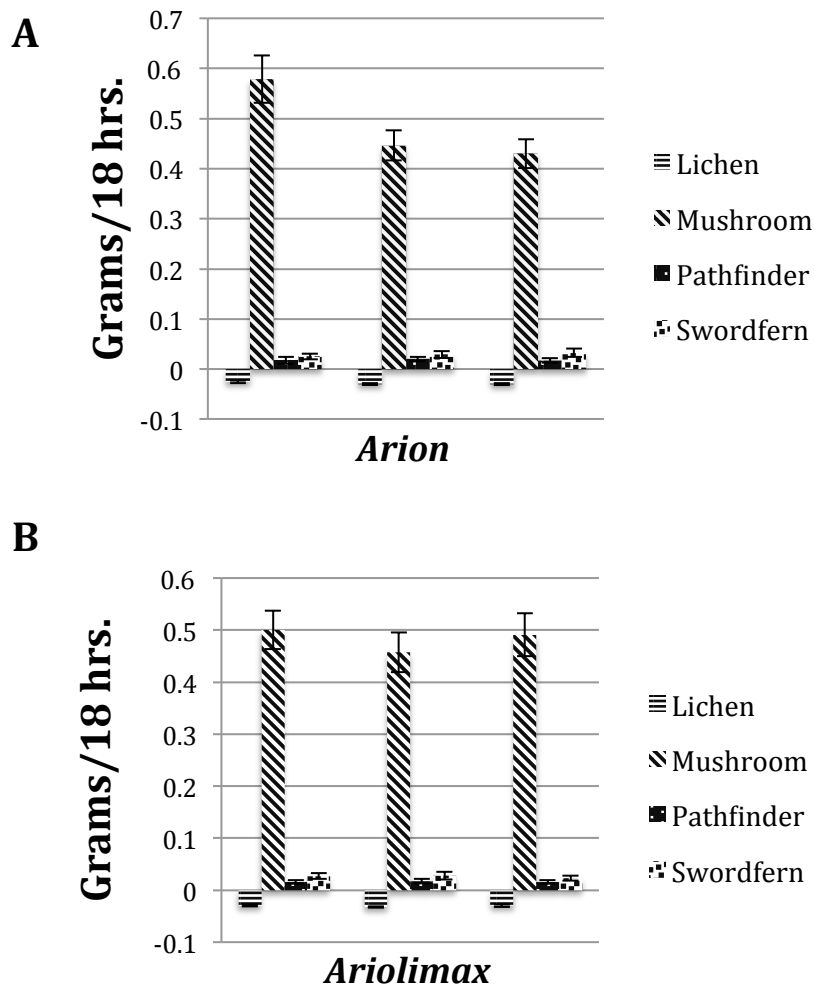
An excess amount (more than the slug could eat during the trial period) of the experimental food (Organic Girl- Baby Spinach or field-collected stinging nettle *Urtica dioica*) was placed directly on the soil. Pictures were taken before and after the experiment and leaf area consumed was analyzed with ImageJ. The experimental design lends itself to a two-way ANOVA; however, due to significant interaction terms I opted to run separate one-way ANOVAs for each slug subpopulation with amount eaten as the dependent variable and temperature as the independent variable. Pairwise comparisons were conducted using Bonferonni correction factors for an overarching family alpha level of  $p=0.05$ .

After the experiment, I disposed of all plant material, euthanized all *Arion* individuals via freezing, and released all *Ariolimax* individuals about 0.5 km from their collection area to avoid recollecting any slugs in future experiments.

## Results

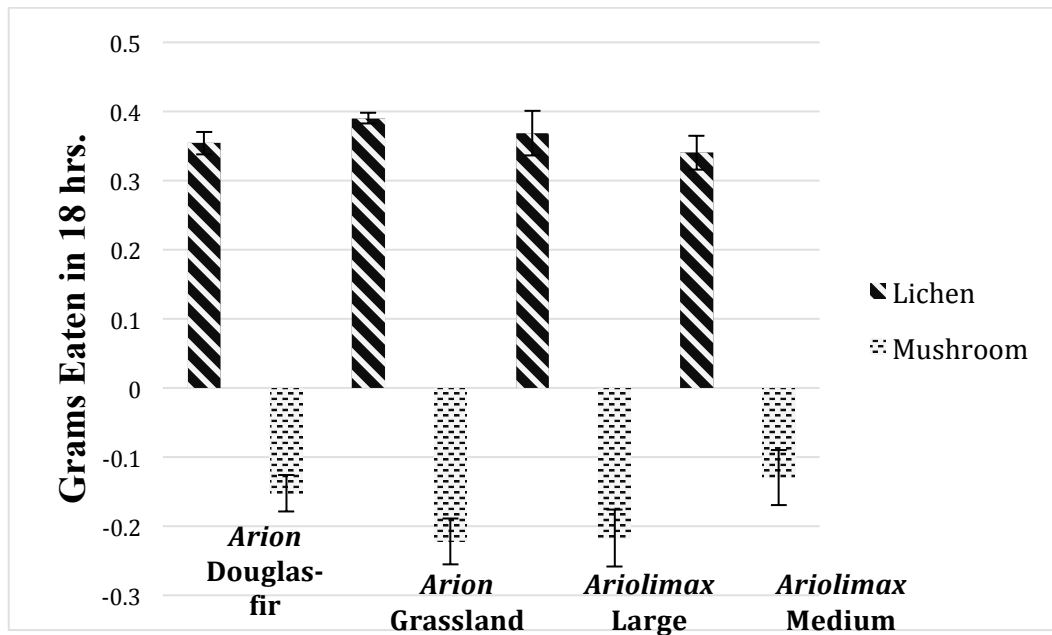
### Feeding Preference

The slugs preferred mushrooms to pathfinder, sword fern, and lichen, regardless of the subpopulation of slug: *Arion* black, *Arion* grassland, *Arion* Douglas-fir ( $p < 0.01$ ) and all three-size classes of *Ariolimax* ( $p < 0.01$ ); (Figure 1). Additionally, all slug subpopulations preferred RO soaked lichen to oven-dried mushrooms (*Arion* Douglas-fir and *Arion* Grassland:  $p < 0.001$ ; *Ariolimax* Large and Medium:  $p < 0.001$ ); (Figure 2).



**Figure 1.** A) Average consumption ( $\pm 1$  SE) of lichen, mushroom, pathfinder, and swordfern by *Arion* black (N=11), *Arion* Douglas-fir (N=27), and *Arion* Grassland

(N=27) slugs. B) Average consumption ( $\pm 1$  SE) of lichen, mushroom, pathfinder, and swordfern of *Ariolimax* large (N=25), medium (N=25), and small (N=22) slugs.



**Figure 2.** Average consumption ( $\pm 1$  SE) of reverse-osmosis soaked lichen and oven-dried mushrooms of *Arion* Douglas-fir (N=20), *Arion* Grassland (N=18), *Ariolimax* large (N=20), and *Ariolimax* medium (N=18) slugs.

### ***Feeding Preference in Various Temperatures***

There was no significant effect of temperature on the feeding rate of the *Arion* from the grasslands (Figure 3A;  $df=2; 41; F=0.73, p>0.1$ ) There was a significant effect of temperature among the *Arion* slugs from the Douglas-fir forest (Figure 3B;  $df=2; 63; F=10.85; p<0.0001$ ), with significantly less eaten by slugs maintained at 4°C than those at 10°C or 20°C ( $p<0.05$  for each pairwise comparison), but no significant difference between the slugs in 10°C and 20°C ( $p>0.1$ ). Within the large *Ariolimax* slugs, temperature did not significantly affect the feeding rate (Figure 3C;  $df=2; 12;$

F=1.89;p>0.1). The feeding rates of small *Ariolimax* respond significantly (Figure 3D; df=2; 32; F=7.39; p<0.01), with significantly more spinach consumed at 20°C than 4°C or 10°C (p<0.05 for each pairwise comparison). There was no significant difference in the amount of spinach consumed by slugs maintained at 4°C and 10°C (p>0.1).

**Figure 3**

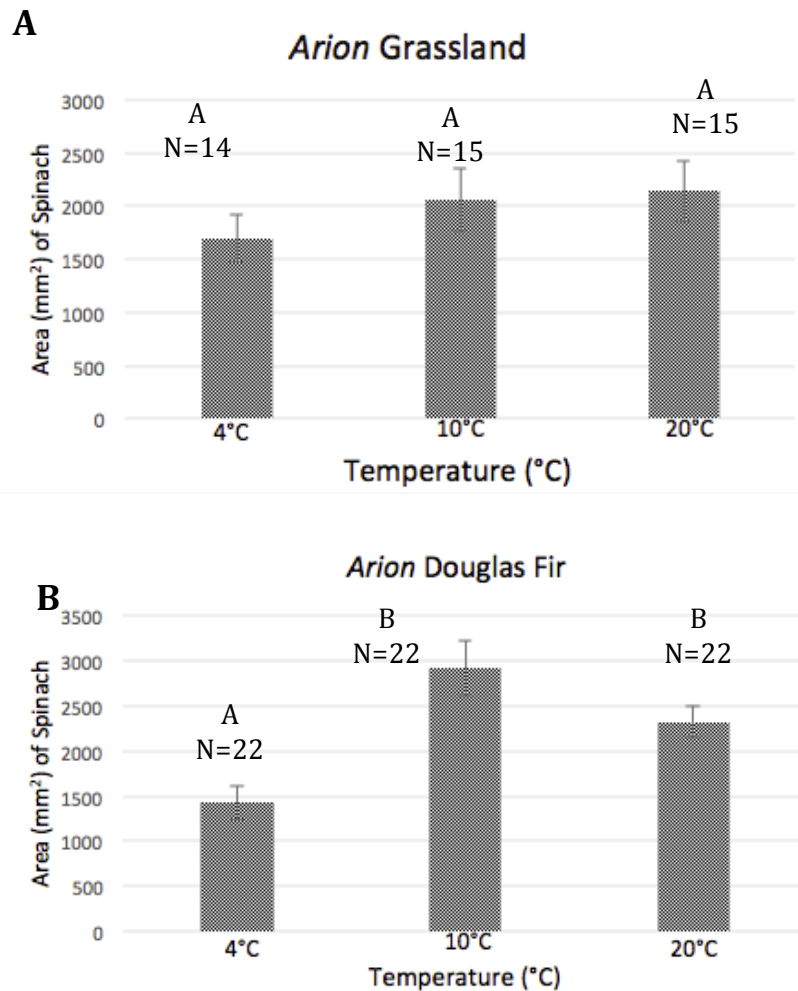
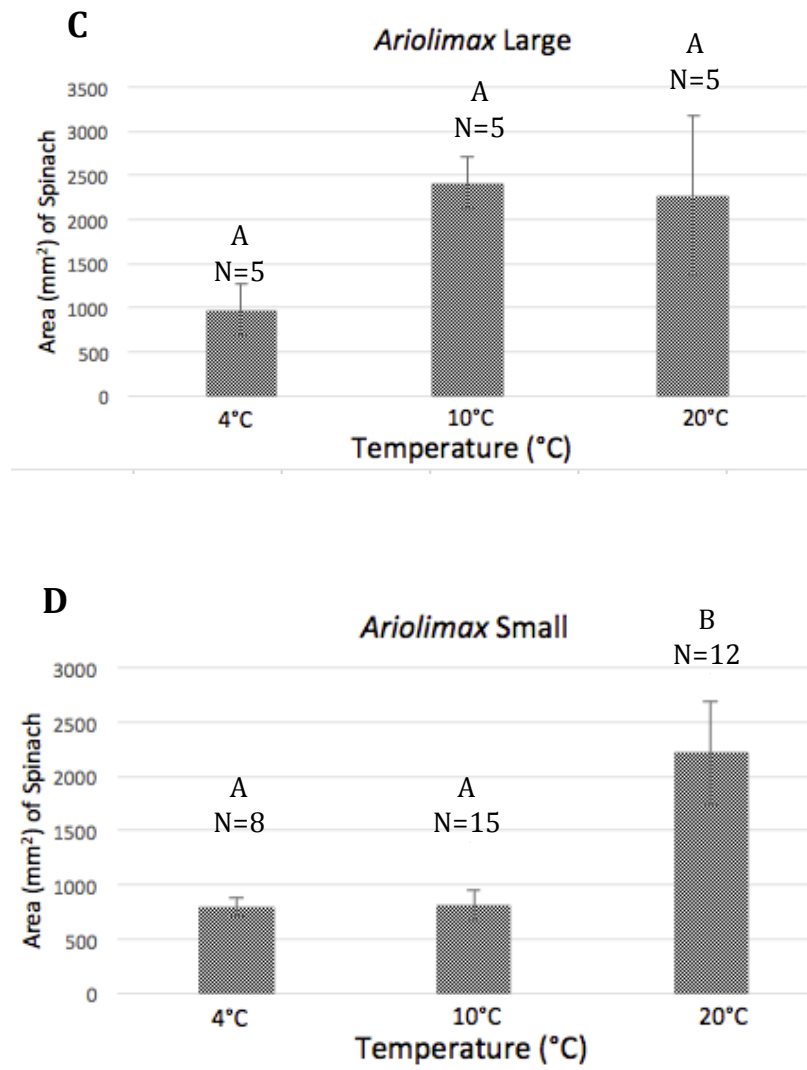


Figure 3 continued



**Figure 3:** Average ( $\pm 1$  SE) spinach leaf consumed by different slug subpopulations: A) *Arion* Grassland, B) *Arion* Douglas-fir, C) *Ariolimax* Large, and D) *Ariolimax* Small. Different letters above the bars indicate statistical significant pairwise differences ( $p < 0.05$ ) within that particular graph. Comparisons were not made across graphs due to interaction factors.

## **Discussion**

These present day interactions between *Ariolimax* and *Arion* may greatly affect the population dynamics and even eventual evolution of the two species because they did not co-evolve, and have only co-occurred for fewer than 100 years. Further, *Arion* slugs can exist in habitats that possess very different microclimates, and the slugs from these subpopulations may respond very differently, reflecting local adaptations. My experiments demonstrated that across all *Arion* and *Ariolimax* populations, store-bought white button mushrooms were consumed much more than the other choices. These results suggest that there may not yet be local food preference adaptations among the *Arion* populations or ontogenetic differences within the *Ariolimax* population. Mushrooms may seem a surprising preferred food because they are ephemeral and more difficult to find compared to fern, lichen, and pathfinder. However, slugs are terrestrial animals that have to combat desiccation and food with high water content, such as mushrooms, may help alleviate the stress.

Oven-dried mushrooms, stripped of both their moisture and malleable texture, were not consumed much. All slug populations preferred RO-soaked lichen to the oven-dried mushrooms suggesting that it is the moisture of the mushrooms, not their nutrients that is important. Lichen was used as a comparison, over pathfinder and fern, because it has fungi components, which make it ideal for absorbing moisture. If future climate change increases local average temperature and decreases moisture, mushrooms will likely become even less common and the slugs may be forced to eat something else. This shift can exacerbate competition between *Arion* and *Ariolimax*, especially in the forested areas where they cohabitate.

To examine the effect of temperature on the slug's feeding and enable prediction as to how these various subpopulations of slugs might react to future climate change, I measured feeding rates in different temperature incubators. I was expecting, based on principles of ectothermy, that the optimal temperature with highest feeding rates to be 10°C because *Arion* and *Ariolimax* are most active during the nights in early summer (Richter 1976). Since these animals are ectotherms and metabolic rate is typically directly correlated with temperature, I suspected temperatures 5°C and 20°C to be too extreme for the slugs and that they would remain idle to conserve energy. Another possible result would be for feeding rate to continuously increase along with increasing temperature along the three temperatures I examined, which would be consistent with the findings of Rodriguez-Cabal et al. (2015). I was surprised that while different slug subpopulations reacted differently to changing temperatures, none of them displayed either of these two patterns- there was no evidence of a single optimal middle temperature or a consistent positive correlation.

This was not completely surprising for the *Arion* Grassland subpopulation, which showed no significant effect of temperature, because they experience many temperature fluctuations throughout the day and should have a wide temperature tolerance for optimum performance. However, slugs from the *Arion* Douglas-fir subpopulation ate significantly less at 4°C than those at 10°C and 20°C, which suggests that higher temperatures do promote elevated metabolic rates in these slugs from more thermally stable habitats, but there appears to be a step function response that occurs between 4°C and 10°C since rates at 10°C and 20°C were not statistically different. Why does *Arion* potentially perform better at higher temperatures? It may be because *Arion* is originally

from Western Europe, where there is a temperate climate, and therefore, is evolutionarily adapted to higher temperatures.

Even more surprising was our finding that there was no significant effect of temperature on feeding rate among the large *Ariolimax* slugs, but a statistically significant difference among the small *Ariolimax* population where slugs consumed most in the 20°C environment. While these results must be viewed conservatively due to low sample size, some interesting conclusions emerge. Higher temperatures do not seem to negatively affect the feeding rate of *Ariolimax*, so grassland avoidance is likely not due to temperature stress on metabolic enzymes but perhaps moisture limitations are critical. The mushroom feeding experiments pointed to moisture as an important driving factor for these slugs. I predict that the impacts of future climate change on these species will depend more on the effect of these alterations on pattern of precipitation rather than thermal change.

Note: I am still measuring area consumed for many of our treatments, so our replicates and subpopulations will increase. Additionally, we examined the effect of temperature on consumption rate of stinging nettle, but those data are not yet compiled. I plan to present the complete data set at the Society for Integrative and Comparative Biology conference in January 2016.

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