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Soil Habitat Characteristics and Laboratory Care of Invasive Terrestrial Flatworms

Blake Hudson
Environmental Studies: Ecology and Environmental Biology
University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisors: Jake Hagedorn and Dr. Camila Filgueiras

Abstract

For decades, there have been numerous instances of exotic terrestrial flatworms (commonly known as planarians) invading soil ecosystems in the United States. This has been impactful because many of these invasive planarians are carnivores and their preferred prey are native earthworm populations. Earthworms play an important role in the health of the overall soil ecosystem as well as the flora and fauna it supports aboveground. Due to their status as apex predators, invasive flatworms are able to reduce earthworm populations unhindered, leading to potential absence of these earthworms and subsequent degradation of the soil. The research on the ecology of these invasive flatworms is largely a new realm of study, and there have been few effective methods of management to combat their spread. In order to set the stage for future targeted study, this project aimed to assess the relative abundance of flatworm populations at UNC Asheville, their ecological preferences in the field, and optimal conditions for rearing in a laboratory setting. Two carnivorous planarians were identified in the gardens on UNC Asheville's campus, *Bipalium adventitium* and *Diversibipalium multilineatum*. Individuals of these species were collected and reared in a lab with the goal of encouraging their reproduction to grow a laboratory population. A general protocol was developed for the care of collected specimens. Limited reproduction was recorded from *B. adventitium*, which refutes past studies of a hypothesized breeding season for this species. Soil samples were also collected from the field site and assessed for moisture and organic matter to evaluate flatworm soil condition preferences. This study provides a better understanding of the feeding and reproductive ecology of three exotic planarians that have invaded western North Carolina and provides a basis for future study.

1. Introduction

The invasion of soil ecosystems by exotic flatworms (Family Geoplanidae) has been detrimental in the US and in many other parts of the world due to their predation of native mollusks, arthropods, and especially annelids.[1] Initially native to Asia and some Pacific islands, many planarians have been unintentionally released outside these ranges through exotic plant trade, either as eggs or fully formed individuals.[2] Several species of flatworms have been observed to decimate earthworm populations following introduction to local soil ecosystems.[3] Earthworms are important in their native ranges, being a keystone species of soil ecosystems. They shred and decompose organic matter to a form that can help nourish plants and other organisms within the system, which contributes valuable biomass to the nitrogen cycle of the soil. Beyond this, the burrowing paths of earthworms facilitate soil aeration and water infiltration, further emphasizing their importance to plants and other organisms.[4] When earthworms are removed from their native ranges due to predation from invasive planarians, many of the soil ecosystem services they contribute to are reduced.[3]

Due to their indirect repercussions on plant life via soil degradation, exotic flatworms have become agricultural pests in many parts of the world. However, relatively little is known about their ecology and natural predators in their native ranges. Much of the relevant literature is not very recent, leaving scientists with few large-scale management strategies. Traditionally, integrated pest management (IPM) has been employed by scientists in agricultural systems to manage pests, using methods ranging from chemical application to winter cover crop planting.[5] One of the additional components of IPM has traditionally been the application of biological control in these systems. Ideally, biocontrol as a component of IPM is conducted by facilitating the success of natural predators of pests that are already present in the system, either by habitat manipulation or population augmentation.[6] Due to the lack of literature and knowledge about natural enemies of flatworms in native ecosystems, it has been difficult to identify an appropriate biocontrol agent with a comparable ecological niche. The few studies investigating this in the past have assessed herpetofauna[7], snails[8], or beetles[9] as potential natural enemies due to perceived similarities in habitat, but have either ended in failure or been deemed inconclusive.

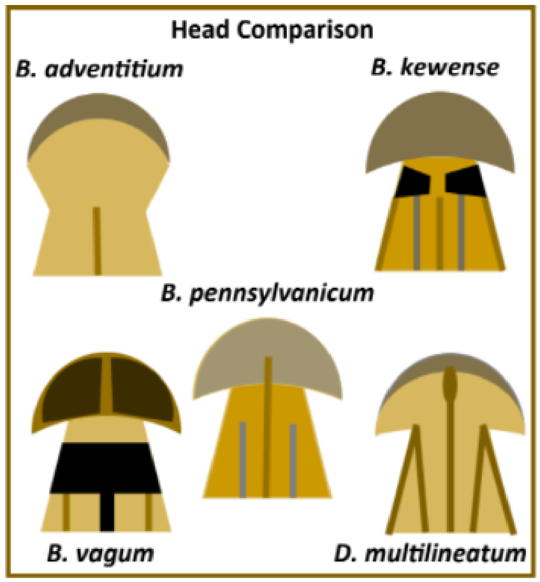

Figure 1. Comparison of *Bipalium adventitium* (upper left) and *Diversibipalium multilineatum* (lower right) with similar species that have colonized North America.[10]

In order to be able to make informed and effective management decisions in the future for this invasive group of invertebrates, it is important to gain a better grasp of their feeding and reproductive habits, habitat preferences, and any co-occurring organisms that could be potential biocontrol agents. Therefore, the large majority of this research can be divided into two main sections, 1) field collection and habitat assessments, and 2) maintenance of a lab colony of the collected planarians. With the ROOTS garden on campus at UNC Asheville as the primary field site, three introduced species of terrestrial planarians, commonly recognized by head shape and pattern (Figure 1), were identified to have colonized this area: *Bipalium adventitium*, *Diversibipalium multilineatum*, and *Microplana terrestris*, with the two former being identified as apex predators in soil ecosystems[3, 11]. Along with the collection of individual flatworms, a number of environmental characteristics were also recorded that could be potentially meaningful for planarian activity. These included soil moisture content and soil organic matter content between the garden beds, as well as additional organisms found such as earthworms, which could serve as prey for the predatory flatworms.

A final variable assessed from soil collection was the natural co-occurrence of the invasive planarians with a common biocontrol agent, entomopathogenic nematodes (EPN). One other past study has been conducted applying nematodes as a biological control for an invasive flatworm species.[12] The principal goal of the study was to determine effectiveness of the nematodes on a slug pest, with unintentional effects on native earthworms and the invasive flatworm present in the system being secondary measures. While this particular species of nematode did not appear to be effective against the flatworm, it holds potentially intriguing implications. Many specimens of invasive planarians, namely *Bipalium kewense* and *Dolichoplana striata*, have been collected in the past and noted to be parasitized by nematodes following dissection.[1] This proves the ecological possibility of parasitic nematodes infecting flatworms in a natural setting. Nematodes have been widely studied as a means of biological control in the past two decades, with promising success when applied in other ecosystems.[13] Within an agricultural setting, nematodes have shown success being employed against the larvae of problematic arthropods, responding to chemical stimuli to target their hosts in much the same way flatworms do.[14, 15] With the growing success and expansion of nematodes as biocontrol, as well as the current lack of successful management methods against invasive flatworms, the coexistence of any flatworms and EPN in the ROOTS garden could pave the way for future trials to determine biocontrol efficacy.

Within the lab, collected planarian specimens were observed in order to better understand their full life cycle. Flatworms were fed their preferred prey, earthworms, and maintained in a controlled environment as similar as possible to the environment they were collected from. Platyhelminthes are hermaphroditic and capable of both sexual and asexual reproduction, so specimens were monitored for the release of reproductive caccoons or any other signs of offspring.[16] To our knowledge, this is the first study to quantify the feeding ecology and growth of either *B. adventitium* or *D. multilineatum*, as well as detail their breeding capabilities and clutch sizes. This makes the study a significant contribution to the existing literature and can give future studies a baseline for lab colonies that can be utilized for further research.

2. Methods

2.1. Soil Collection and Assessment

Soil samples were collected evenly across the five garden beds surveyed for flatworms (Figure 2). Each bed was labeled A-E, with five soil probe sites per bed with the exception of Bed A, which was slightly larger than the rest and had a sixth probe site. Each bed was a roughly 1.5m x 11m rectangle, with Bed A having a lateral extension at one end denoting the sixth probe. A 2.5 cm diameter soil probe was used to collect soil to a depth of 20 centimeters and the samples were separated into two plastic deli containers per probed site, one for soil 0-10 centimeters in depth and one for 10-20 centimeters. The depth separation was done because other Geoplanid flatworms have been noted to primarily inhabit the top 10 centimeters of soil while only burrowing down to around 20 centimeters to reproduce.[2]

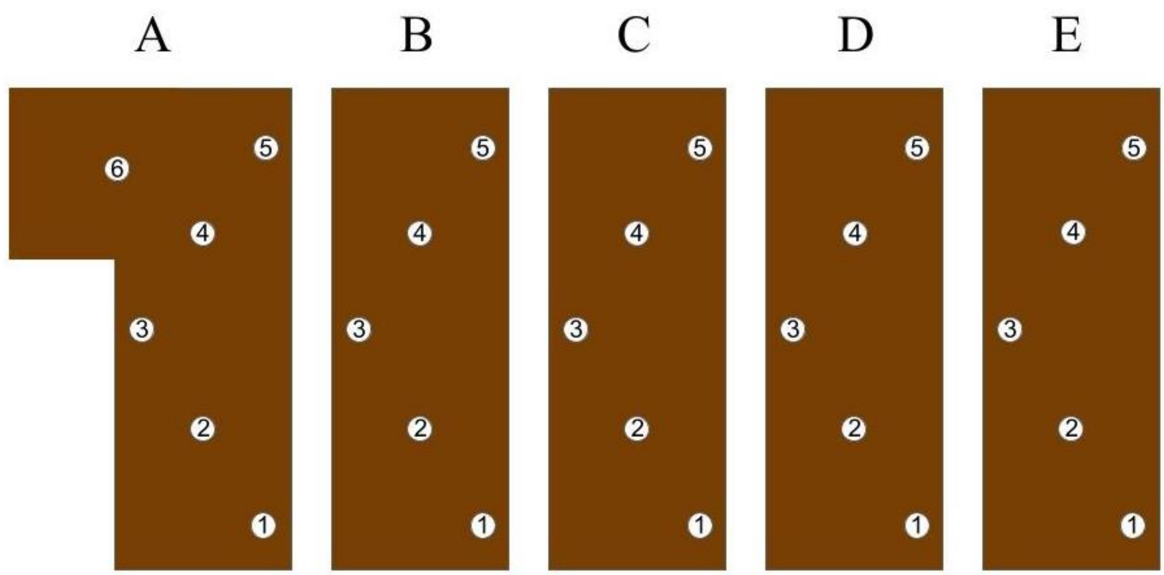

Figure 2. Garden Plots A-E, with soil sampling sights numbered along each bed.

Once the soil samples were transported back to the lab, five waxworm larvae (sp. *Galleria*) were placed on the surface of the collected soil and the entire container was turned upside down, where it remained for one week. These waxworms were utilized to draw any EPN already present into a vulnerable body for further identification. After the week, each *Galleria* was removed from its soil container, the container was resealed, and physiologically intact larvae with color changes were grouped on a petri dish with a damp filter paper underneath. After another week, the remaining larvae and the bottom half of their petri dishes were placed into a larger petri dish with water covering the bottom. This secondary petri dish was used as a reservoir for nematodes to gather following their emergence from infected *Galleria* hosts.

Soil formerly occupied by the *Galleria* was weighed, with the moisture still largely retained due to being constantly sealed, and then each sample was allowed to air dry for two weeks with the lid removed. Samples were then reweighed to calculate the difference in weight due to moisture loss.

The protocol for assessing soil organic matter percentage in each sample was largely adapted from Schulte and Hopkins (1996). A 15-mL crucible was used for each sample and weighed. A fraction of each air-dried sample was processed with mortar and pestle to pass through a size 10 sieve. Afterward, approximately 5 grams of sample soil were weighed into each crucible and placed in an isothermal oven to dry further at 105 degrees C. The samples were weighed again following oven drying and then combusted at 360 degrees C for another two hours. The combusted samples cooled off in the isothermal oven again for an additional hour, then weighed again.[17]

2.1.1. statistics

The variables of gravimetric water content and organic matter were assessed by bed. This was done via an ANOVA test. The distributions of the input variables and model residuals were assessed for normality and heteroskedasticity. If an ANOVA test was significant, a post-hoc Tukey test was performed.

2.2. Laboratory Maintenance of Specimens

2.2.1. environmental management

Upon laboratory arrival, individual flatworms were placed into petri dishes. Due to other terrestrial flatworms showing cannibalistic tendencies in past studies[1, 18], individuals were each initially given their own dish to occupy in isolation. Terrestrial flatworms can be vulnerable to dehydration, so a paper filter was placed on the bottom interior of each dish to maintain moisture. To further mimic their native environment, soil collected from field sites was added to each petri dish. This method evolved into caking fresh soil into a flattened solid layer across the entirety of the floor of each petri dish. Once patted into the dish, soil was initially sprayed with water until it could be seen penetrating to the paper filter underneath. Following this, soil was remoistened regularly, going no longer than three days in between watering. Lids of petri dishes were also removed at these intervals to allow air to be replenished inside the sealed dishes. Individuals were removed to new dishes only when mold or fungus was noted to propagate from the soil. Bycatch soil organisms such as hatchling slugs, microarthropods, and juvenile myriapods were allowed to remain in the petri dishes with flatworms if present.

2.2.2. feeding

Many Geoplanid flatworms are noted to be top generalist predators[3], while some are more specialized to prey on gastropods or annelids.[19] Feeding trials were conducted using earthworms collected from the same field site and earthworms from fishing bait stores. Once feeding trials began, they generally continued once a week and no longer than two weeks passed between two trials. Specimen earthworms used as food were cut into smaller pieces to make them easier to be targeted by their generally smaller flatworm predators. Individual flatworms were weighed before being fed, along with their prey item, which was then placed in the same dish in physical contact with the flatworm to encourage feeding. Individual flatworms were paired with prey items based relatively on mass. In some cases, feeding was evident almost immediately, with the flatworm coiling around the body of its prey and the pharynx visibly penetrating the skin of the earthworm. After twenty-four hours, the dishes were inspected again for additional signs of predation, and if any pieces of earthworm remained they were removed to deter any possible contaminants from growing in the dish. Flatworms were reweighed following this twenty-four-hour period, whether they were noted to have predated or not.

2.2.3. reproduction

While individual flatworms were originally kept separate upon bringing them to the lab to avoid the chance of cannibalism, some couples were placed in shared petri dishes to encourage breeding. As all the species collected are hermaphroditic and capable of both sexual and asexual reproduction, the identification of sex was not necessary when coupling potential partners, and instead, they were paired based on similar mass, as in McDonald and Jones, 2013.[20]

This was only conducted with *B. adventitium* and *M. terrestris*, as individuals of *D. multilineatum* were of drastically different sizes.

Aside from couples paired in the lab, some specimens of *B. adventitium* collected from the field proved to have already been impregnated in the field. When an offspring capsule was released, it was left in the original dish in which it was laid and the parent was moved to a new dish. The dishes containing only capsules were maintained with the same protocol as if they housed adult flatworms, also adapted from McDonald and Jones, 2013. Once capsules hatched, siblings stayed together in the same petri dish.

2.2.4. statistics

The difference between pre-feeding weight of flatworms and their prey was assessed relative to their choice to feed. Significance was determined via a t-test. The variable of post-feeding weight was assessed over time as a linear regression for each individual with five or more weighed feeding events. In order to determine activity level following reproduction, the number of successful feeding events was assessed for individuals who had and had not reproduced. Significance was determined via a t-test. The variables clutch size and average hatchling size were assessed via linear regression. The threshold of significance for all statistical tests was $p < 0.05$.

3. Results

3.1. Soil Collection and Assessment

Three different species of terrestrial planarians were collected from surveys in the ROOTS garden (35.614, -82.556) at UNC Asheville: *Microplana terrestris*, *Bipalium adventitium*, and *Diversibipalium multilineatum* (Table 1). Flatworms were primarily found along the edges of garden beds, inhabiting microhabitats under rocks and logs that had been placed along the outline of each bed.

Table 1. Soil characteristics and flatworm populations by garden bed.

Garden Bed	A	B	C	D	E
M. terrestris	1	2	0	2	0
B. adventitium	4	5	3	3	0
D. multilineatum	0	1	1	2	0
Total Flatworms	5	8	4	7	0
Min. OM %	3.5	2.2	1.6	1.6	1.6
Max. OM %	10.7	8.5	5.8	5.3	6.2
Mean OM %	5.9	5.8	3.6	3.9	3.6
Min. Water Content	12.4	15.4	9.7	14.6	13.2
Max. Water Content	26.8	31.6	22.7	26.1	25.5
Mean Water Content	19.6	24.4	16.2	19.6	18.9

For soil organic matter, bed A was borderline significantly different than beds C (p=0.0577) and E (p=0.0559), while all other beds were statistically the same (Figure 3). Beds A and B had similarly high mean organic matter values, while C, D, and E were lower. Regarding gravimetric water content, bed B had the highest average measurement and was significantly different compared to bed C, the lowest (p=0.001), while differences between all other beds were insignificant (Figure 4).

Of the 24 collected planarians, 13 had at least one earthworm adjacent to them under the same rock at the time of collection, and 5 of these were near more than one earthworm. Additional flatworms were incidentally found and brought back to the lab colony, but they were not connected to any particular garden plot. In total, we found 28 *B.*

adventitium, 10 *M. terrestris*, and 6 *D. multilineatum* in the ROOTS garden at UNC Asheville. EPN were present in five different soil samples across three of the five garden beds, namely A, D, and E (Figure 5). These nematodes consisted of at least two different species, one from the genus *Steinernema* and one from the genus *Heterorhabditis*.

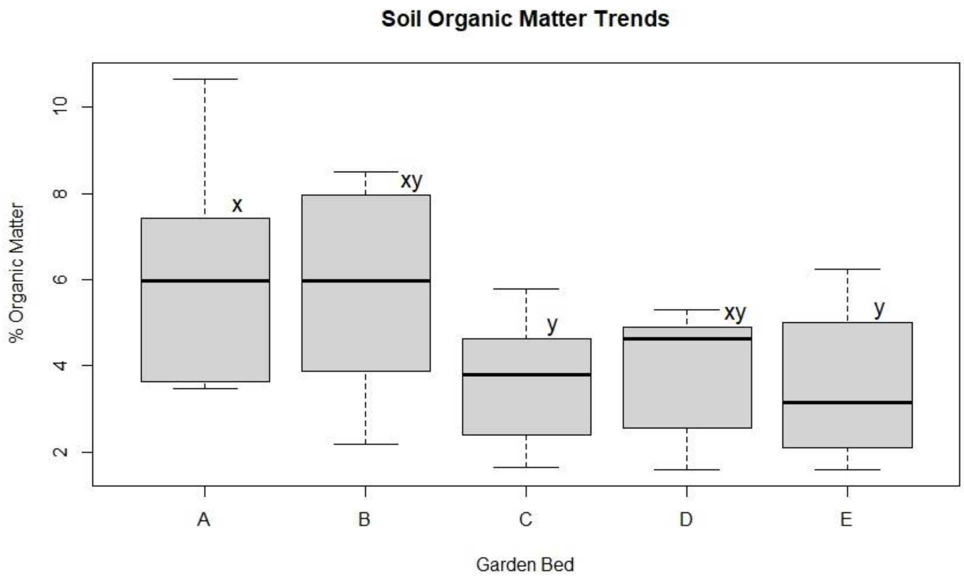

Figure 3. Organic Matter Trends by Garden Bed. Full range of data by bed is indicated by dashed lines, while the interquartile range is indicated by gray boxes. Median organic matter is represented by black lines. Significant differences are denoted by x and y. (p=0.007)

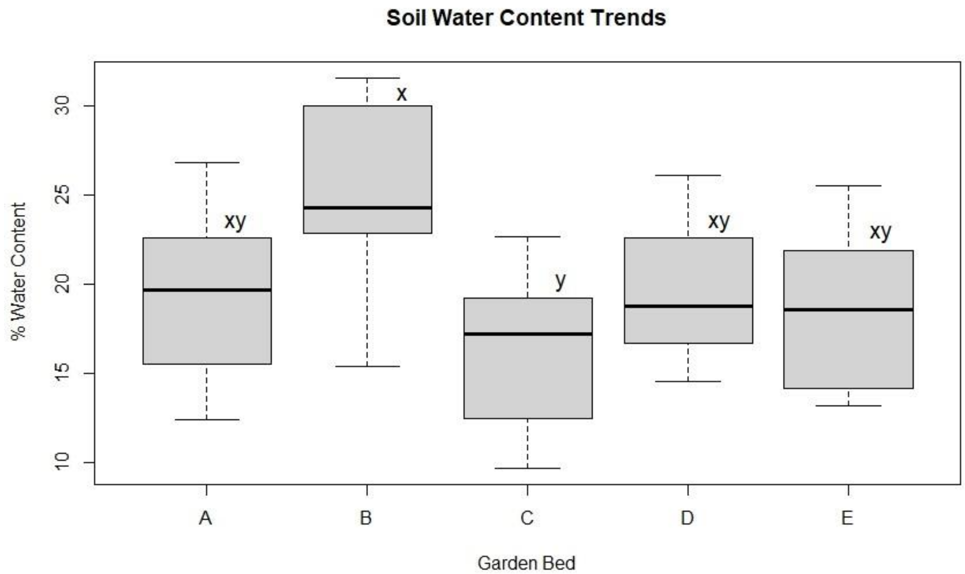

Figure 4. Gravimetric Water Content Trends by Garden Bed. Full range of data by bed is indicated by dashed lines, while the interquartile range is indicated by gray boxes. Median gravimetric water is represented by black lines. Significant differences are denoted by x and y. (p=0.004)

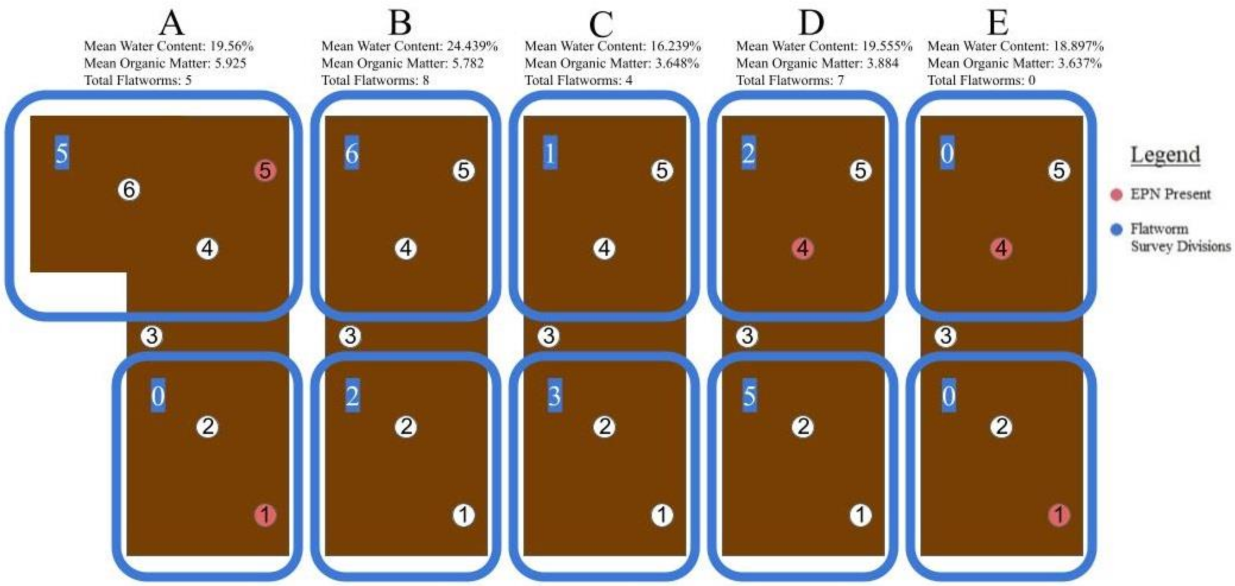

Figure 5. Map of Surveyed Garden Plots. Circles indicate soil sampling sites. Blue numbers indicate the number of flatworms collected per sampling division.

3.2. Laboratory Maintenance of Specimens

3.2.1. environmental management

While individuals of each species were initially stored in petri dishes with only a paper filter on the bottom to maintain moisture, this environment proved to be too variable for the flatworms to survive. This method proved to be highly prone to both dehydration and oversaturation, both resulting in death to the specimens (although it should be noted that dehydrated specimens could sometimes be revived with the addition of moisture). Individuals that died from too much exposure to water appeared to liquefy, sometimes melting completely to the point only a stain remained. It should be noted that liquefication did not affect each species equally, however. Of the six *M. terrestris* present in the lab at the time, four died after being exposed to excess moisture. Of the twelve *B. adventitium*, one died following oversaturation. All three *D. multilineatum* present at that time survived.

Following this high level of mortality, soil was compacted into each petri dish over a moistened paper filter to regulate the environment. Following this adjustment, only one individual, a *B. adventitium*, dissolved in a similar fashion for the remainder of the study, and none died due to dehydration.

3.2.2. feeding

When conducting feeding trials, *B. adventitium* chose to feed on average 56.6% of the time, and *D. multilineatum* chose to feed on average 57.5% of the time when given freshly chopped earthworms, making an average feeding rate of 56.7% overall. No *M. terrestris* specimens ate throughout the entirety of the study, so they are excluded from this calculation. An individual flatworm eating was indicated by three factors: witnessing a predation event, in which the flatworm's pharynx is visibly extended into the prey (Figure 6a); weight gain noted from reweighting the following day; and at times color change. This was only noted in some *B. adventitium* specimens, which would gain a pinkish hue while or immediately following gorging on their earthworm prey (Figure 6b).

Figure 6. (a) An adult *B. adventitium* feeding on an earthworm, with the crater on the lower part of its body indicating its pharynx penetrating its prey. (b) A *B. adventitium* specimen taking on a pinkish hue while feeding on a segment of earthworm.

Individuals were given the opportunity to feed on intact, live earthworms on a few occasions, but none were successful. There was also no observed predation of any other bycatch soil organisms left in the planarians' containers. Planarians attempted to feed on pieces of earthworms ranging from 8.2% of their body mass in size to one instance of an individual feeding on an earthworm 3600% of its body mass. Some individuals also increased to over double their initial body mass after a single feeding event. There was no significant relationship between an individual's body mass relative to its prey and its decision to feed ($p > 0.05$). Over the course of the study, change in weight was recorded for each individual flatworm. Eighteen individuals had at least five or more feeding events recorded in the lab (Figure 7), but even when given prey substantially larger than themselves, only four increased in weight overall after being brought to the laboratory, while 14 decreased in weight.

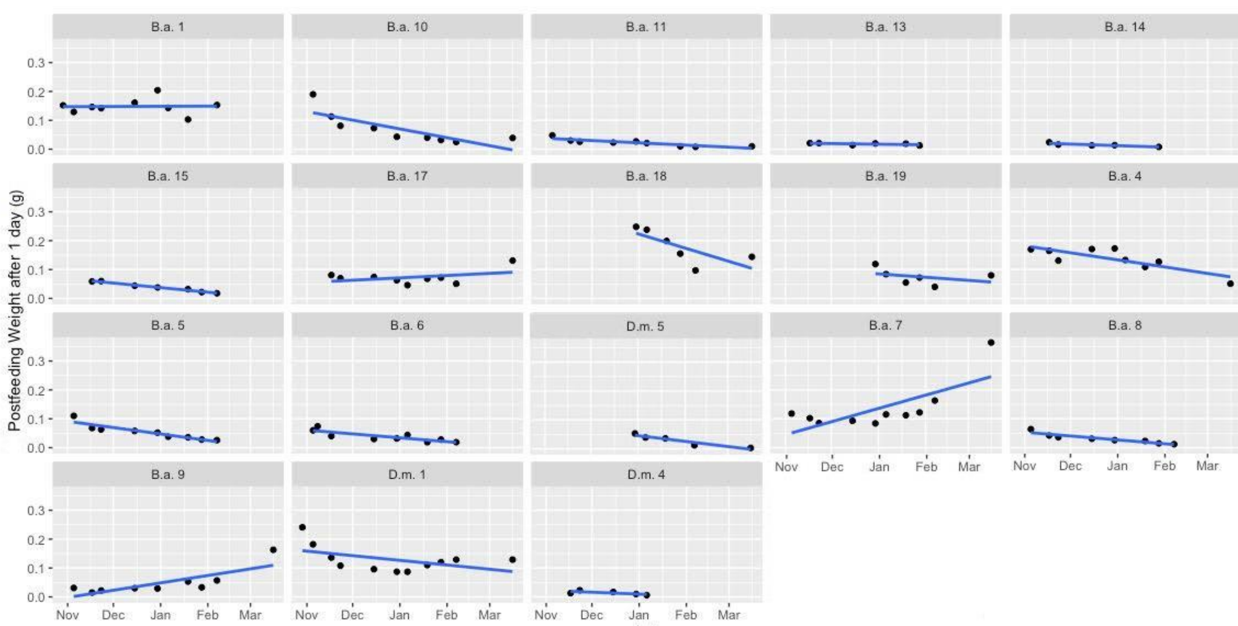

Figure 7. Linear regressions modeling change in weight over time of all individuals with at least five weighted observations.

Also noted was what seemed to be decreased activity by individuals following reproduction, characterized by relative sluggishness and a decreased likelihood of feeding. The average feeding rate of *B. adventitium* following reproduction was 45.9%, a decline from 59.6% successful feeding rate of individuals who had not reproduced recently (P=0.05528).

3.2.3. reproduction

While no couples paired after being brought to the lab-produced any offspring within this study, a total of eight capsules were laid by individuals who had copulated in the field prior to being collected. Of these eight capsules, four hatched and produced juvenile individuals. Time between laying and hatching ranged from 14-21 days, and clutch sizes ranged from 1-7 individuals hatching from a single capsule. The largest individual upon time of hatching weighed 0.016 g and was the sole juvenile produced from its capsule. The smallest individual produced weighed 0.004 g and was one of seven juveniles to hatch from its capsule. This appeared to indicate a negative correlation, but data was limited. Of the individuals that reproduced, one released four separate capsules over the course of four weeks, while the individual it was found copulating with in the field released a single capsule roughly four weeks after being separated from its mate. Capsules from all reproductive individuals were laid between January 11th 2022 and February 21st 2022.

4. Discussion

The purpose of this study was to gain a better understanding of terrestrial planarian ecological preferences and lab rearing for the purpose of providing a baseline for future study. Within the ROOTS garden on campus at UNC Asheville, the three exotic terrestrial flatworms *D. multilineatum*, *B. adventitium*, and *M. terrestris* all occurred adjacent to one another in similar environmental conditions, with *B. adventitium* being the most abundant. There was some considerable soil condition variation between certain garden beds used as the surveying site. Beds A and B were the two beds highest in soil organic matter and had similar means, while C, D, and E were noticeably lower. There were no major trends between either species richness nor number of individuals collected compared to the organic matter contents of their beds. Variation in moisture between the beds was significant overall, with the wettest, Bed B, being significantly higher in hydrometric moisture content than Bed C, the driest bed. There appears to be some correlation between these metrics, as all three species of flatworms were present in Bed B and Bed B also produced the largest number of individuals, while Bed C lacked any *M. terrestris* specimens during surveys. It should be noted, however, that Bed E had a greater moisture content compared to Bed C based on our measurements, yet Bed E did not produce any planarians of any species during surveys. This is likely due to Bed E not having adequate habitat for the flatworms, as there was not a substantial amount of rocks or logs for them to hide under like there were in neighboring beds.

EPN presence also did not show any trend compared to where flatworms were. Flatworms and nematodes co-occurred in two beds (A and D), and nematodes showed a slight tendency to emerge from soil samples richer in moisture and organic matter, but these relationships were not significant based on our data. Of the nematodes found, one species was found in both Beds A and E, which would imply that dispersal to the beds in between these two is possible, and the lack of detection could simply be from *Galleria* being killed by fungi or other pathogens first before being infected by the nematodes. Overall, future studies could be improved by taking soil samples from similar locations over multiple days to get a better understanding of soil chemical, structural, and ecological trends over time.

Although not formally quantified in this study, the most important indicator of terrestrial planarians in a garden may be adequate shelter for them, as we did not find any flatworms other than ones that were hiding under rocks and stumps placed in and around the garden beds. Additionally, another bias could be the time of day the surveys were carried out, as they all took place during the hottest and brightest hours of the day from late morning to mid-afternoon, when flatworms generally gravitate to the humid underside of rocks for refuge.[21] It is possible that planarian activity and distribution would be different if surveys taken during evening or nighttime hours contributed to the dataset, giving the flatworms a cooler, damper environment in which they could disperse freely outside their daytime shelter.[22]

We constructed a general lab protocol for the care of the flatworms found in a laboratory setting, taking note of successful practices and failures resulting in mortality throughout the process. Although the protocol was based largely on McDonald and Jones (2013), a study with *M. terrestris* as the primary study organism, *M. terrestris* appeared to be the least successful under laboratory conditions in our project, resulting in 100% mortality in all collected individuals of this species. There was no evidence of feeding or reproduction when any individuals of this species were given the opportunity.

Although terrestrial planarians can be highly susceptible to dehydration and thus were routinely monitored with moisture in their dishes, they also appear to have a certain degree of sensitivity to high moisture levels. When exposed to an excess amount of moisture on only a paper filter in their dish, several individuals died via dissolution.[20] Adding soil into each petri dish as an environmental buffer and means of regulating moisture proved to mitigate these fatal results drastically, with only one death for the remainder of the study due to dissolution. This mortality did not affect all three species equally, however, and *M. terrestris* appeared to be the most sensitive to excess water, while *D. multilineatum* had no individuals die in this nature throughout the study. The number of *D. multilineatum* present in the lab during the initial string of deaths due to dissolution was relatively low and thus hard to draw firm conclusions from.

When conducting feeding trials for the two species that ate successfully in the lab, specimens successfully ate the food given to them 56.7% of the time at one-week intervals, with near-identical rates of predation between *D. multilineatum* and *B. adventitum*. Of the 18 specimens with at least five different feeding events to analyze, only four grew in size overall throughout the study, meaning 77.8% of these individuals consistently lost weight over their time in captivity, even when given a surplus of food. Taking these two findings into account, it is possible this decreased tendency to feed could be a result of stress from being in the lab.[23] Furthermore, given that they managed to survive while only feeding roughly half the chances offered to them, it could be possible in future studies that lab colonies of either species could sustainably survive feeding only half as frequently, at two-week intervals instead. This decreased rate of disturbance may also lower their overall stress and make them more likely to feed at healthier rates.

Aside from visible absence of the prey following a feeding event, we also observed a few other indicators of predation: the dorsal indentation of a flatworm extending its pharynx into its prey, mass gained if the individual is weighed before and after feeding, and on limited occasions, color change. Color change was subtle and did not occur regularly across individuals, however. Although in the wild predatory flatworms are known to eat earthworms and other free-living invertebrates alive[3, 24], individuals did not seem readily eager to feed on live prey in the lab. The only successful means of predation we observed was on pre-cut segments of earthworms, and although some predatory flatworms have been described as generalists,[19] we did not observe any predation on any other bycatch soil organisms present in their environments. We did not find any correlation between the body size of an individual relative to its prey, and in some cases *B. adventitium* would opt to feed on prey outweighing it by an order of magnitude.

Although we attempted to facilitate some mating pairs in the lab with both *B. adventitium* and *M. terrestris*, neither yielded any offspring in this study. However, some individuals of *B. adventitium* collected in the field proved to have already been fertilized, with one pair found in the middle of copulation. Both mates in this pair producing reproductive capsules could be further evidence of mutual fertilization noted in studies of other Triclad flatworms[25], but one of these partners produced four capsules over a span of four weeks. Without genetic analysis, it is impossible to know if any or all of these resulted from the same copulation as the single capsule produced by its partner, which released its sole capsule three days after its first partner's final one. Overall, eight capsules were released by five individuals, of which four hatched. There seemed to be a negative correlation between clutch size and mass of juveniles produced, but data for this were insufficient to draw a significant conclusion. We observed that all capsules laid in the lab were released in mid-winter between January 11th and February 21st, 2022, refuting past studies that *B. adventitium* reproduction is limited to a single season[26], although this study (published in 1998 in New York State) may also differ based on temporal, climatic and population differences. Following reproduction, parents appeared to be more sluggish and less active compared to before. When comparing feeding events prior to and following a capsule being released, parents were borderline significantly less likely to feed right after reproduction compared to their counterparts who had not reproduced.

Overall, within this study, *B. adventitium* appeared to be the most abundant species of terrestrial flatworm in the ROOTS garden at UNC Asheville. While there was some significant variability between some of the garden beds in terms of gravimetric moisture and organic matter, neither of these factors were significantly varied enough to explain flatworm occurrence. Flatworms were not found residing outside refuge structures such as rocks and logs during surveys, which may be important ecological refuges for the taxon. These species of flatworms were observed co-occurring in the same habitat as two entomopathogenic nematodes from the genera *Steinernema* and *Heterorhabditis*, but this was also not significant in explaining flatworm occurrence or absence. Individuals of *B. adventitium* were observed to be capable of successfully reproducing during mid-winter, when they had previously only been recorded reproducing in early summer. Reproductive capsules were capable of producing as little as one and at least as many as seven juveniles. No predation was observed on live prey, and no preference for prey size was observed. Reproduction was observed to notably reduce activity of the parent in the period of time immediately after birth. Lab colonies may be capable of being sustained with feeding events only once every two weeks, a practice that may reduce stress and benefit collected flatworms.

4.1. Supplemental Discussion

This study was constructed with sparse foundational literature. Many recent projects related to our study organisms have been reports of flatworm geographic dispersal, and sources looking at planarian life cycles are largely at least a decade old. That being said, there were many phenomena we noticed throughout this project that we would not have expected due to lack of reporting on the subject. Although not one of our original hypotheses, and thus we did not include procedures to try to quantify these in our experimental design, we believe they may be worth studying in future projects to contribute to the literature of this taxonomic group.

As we reported above, over-exposure to moisture appeared to affect the different species from the ROOTS garden in different degrees of severity, with *D. multilineatum* being largely unaffected overall. It is possible that this species may have evolved to prefer more saturated environments compared to the other planarians in this study, which may also explain why *D. multilineatum* was the least abundant in our surveys. Furthermore, when individuals in the lab needed to be weighed and were stuck to the surface of the petri dish's lid, it was common for them to latch onto the smooth surface to avoid being picked up. Typically, a light application of moisture was enough to allow *B. adventitium* and *M. terrestris* to lose their grip and slip off, but *D. multilineatum* appeared to be able to maintain its hold on the surface without slipping from the water. This may also point to this species being more specialized to generally wetter environments than our other study organisms.

Another phenomenon that we had no reason to suspect was the apparent disturbance of soil caused by the flatworms in their enclosures. This was observed several times with a number of individuals, where after being placed in a petri dish with manually compressed soil covering the bottom, the soil would have trails of disturbed substrate following the addition of the flatworm (Figure 8). In one instance, we noticed this occurred only after a reproductive capsule hatched in the dish and juveniles were released. The apparent irregularity of these trails over several regulated environments is odd, but if it is indeed due to the planarians' presence, it would be interesting to quantify their effects on soil infiltration and bulk density. This could hold implications that some species of planarians may have ecosystem engineering capabilities.

Figure 8. Example of compressed soil being disturbed in specific areas after the addition of a flatworm (in this case, *B. adventitium*).

As we noted above, we did not encounter any planarians in the garden other than under rocks and other natural shelters, although earthworms were present both in these environments as well as free-living in the center of the garden beds. Microhabitats are notably important for many other taxa as well, such as amphibians and arthropods.[27, 28] It may be valuable to assess the inherent characteristics of these microhabitats where planarians reside to see how they differ from their surroundings. Studies have been conducted in the past with other Geoplanid species[21], with little indication of preferences, but this was conducted in a largely undisturbed forest ecosystem rather than an agricultural one. Understanding any trends in the pH and soil moisture content, as well as how long it might take these microhabitats to develop with the addition of a new structure, could give important insight into the ecology of the flatworms that are commonly found there.

We observed one instance of a mated pair of *B. adventitium* mid-copulation, which were brought back to the lab to reproduce. However, one partner produced four separate reproductive capsules over a span of nearly a month, while the second partner only produced one following its mate's final one. While mutual fertilization has been noted in planarians in the past[25], it is unknown if all four of the caccoons produced by the first mate were a result of this observed copulation with the second, or if they were perhaps from early fertilization events or even examples of asexual reproduction. Further genetic analysis of reproductive habits in this species and others like it could contribute to the growing literature on its overall life cycle.

We observed our three study organisms all living in similar environments and co-occurring adjacent to one another in the wild. While they were separated from each other once brought back to the lab, how they may interact naturally with one another was not assessed. Planarians have been known to feed on members of their own species, so it is possible that some predator-prey relationships may occur when given the opportunity. *B. adventitium* was the most abundant by far in our study, but its dominance as a predator of its fellow flatworms has yet to be assessed. That being said, *B. adventitium* was discovered in the past to naturally produce a neurotoxin[29], so this may or may not protect it from predation by other planarians it shares a habitat with.

Aside from few feeding attempts with chopped earthworms in the enclosures of siblings produced from the same capsule, we did not attempt to assess group hunting with any of our study species. However, it has been common among other terrestrial flatworms for more than one individual to gang up on a larger prey item.[24] Furthermore, we did not observe any predation of live prey in the lab, although it has been noted to happen in the wild. It would be interesting to observe if groups of flatworms sharing an enclosure may be more inclined to feed on a prey item together, and if they may be more successful predating on a live specimen when feeding as a group. Understanding if there is any preference in hunting techniques would give important insight into how these invasive predators decimate populations of their prey.

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