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## Recovery of Domestic Behaviors by a Parasitic Ant (*Formica Subintegra*) in the Absence of Its Host (*Formica Subsericea*)

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**RECOVERY OF DOMESTIC BEHAVIORS BY A PARASITIC ANT (*FORMICA*  
*SUBINTEGRA*) IN THE ABSENCE OF ITS HOST (*FORMICA SUBSERICEA*)**

A Master's Thesis

Presented to

The Graduate College of  
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Amber Nichole Hunter

May 2019

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# **RECOVERY OF DOMESTIC BEHAVIORS BY A PARASITIC ANT (*FORMICA SUBINTEGRA*) IN THE ABSENCE OF ITS HOST (*FORMICA SUBSERICEA*)**

Biology

Missouri State University, May 2019

Master of Science

Amber Nichole Hunter

## **ABSTRACT**

Dulosis is a type of social parasitism in which a parasitic ant has become dependent on captured workers (hosts) of a closely related species. Dulosis can be either facultative or obligate depending on the degree of dependence on host workers. As parasites become more specialized over evolutionary time, their domestic abilities degenerate until they become unable to survive without the host. However, the ‘lost’ behaviors may display some degree of recovery when host workers are unavailable to do the tasks. The *Formica sanguinea* group consists of 12 species of parasitic ants, which are all traditionally considered to be facultative parasites. However, recent studies suggest that *F. subintegra* has characteristics that are more consistent with obligate parasitism. To explore the degree to which *F. subintegra* has become obligate, this study examined the degree of recovery of foraging and nest excavation behaviors of parasites when hosts are removed. When parasites were isolated from hosts, they spent more time foraging, fed regurgitated food to nestmates (oral trophallaxis) more often, and tended to begin feeding earlier than when hosts were present. Isolated parasites were able to excavate suitable tunnels and engaged in nest building behavior significantly more often than parasites in groups with hosts. These results indicate that *F. subintegra* demonstrates behavioral recovery in its foraging and nest excavation abilities, and can be considered a less specialized (primitive) obligate parasite. *Formica subintegra* and its facultative relatives can be used as models for studying the progression of dulotic evolution within a closely related group of social parasites, and may shed light on which factors contribute to the transition from facultative to obligate parasitism.

**KEYWORDS:** dulosis, facultative parasite, obligate parasite, social parasitism, *Formica subintegra*, *Formica sanguinea* group, ant behavior

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A Master's Thesis  
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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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## INTRODUCTION

Dulosis is a type of social parasitism in which a parasitic ant has become dependent on captured workers of a closely related host species (Holldobler & Wilson, 1990; Wheeler, 1910; Wilson, 1975). Parasite workers raid host colonies, capture host larvae and pupae and carry the brood back to their nest. After eclosing, the host adults are fully functioning members of the parasite-host mixed colony. The host workers engage in normal domestic duties, such as foraging, nest maintenance, and care of the parasitic brood and queen. In contrast, parasite workers generally do not engage in typical domestic duties, but have the primary function of scouting out neighboring host colonies to raid to acquire more host workers for the colony (Holldobler & Wilson, 1990; Wheeler, 1910). Dulosis is a rare life strategy, occurring in approximately 50 species belonging to two subfamilies (Formicinae and Myrmicinae) (Holldobler & Wilson, 1990) out of ~12,000 described species of ants (Bolton, 2019; Topoff, 1990).

Dulosis may be either facultative or obligate based on the degree of dependence on the hosts and specializations for raiding (Mori & Le Moli, 1988; Savolainen & Deslippe, 1996; Wheeler, 1910). Facultative parasites are able to live in productive colonies without host workers and are more often found without hosts (Savolainen & Deslippe, 1996; Wheeler, 1910). Facultative parasites have a high retention of domestic abilities as they can resume normal domestic duties if hosts are scarce (Mori & Le Moli, 1988; Wheeler, 1910). When present, hosts account for a small proportion of workers in the nest of facultative parasites (Savolainen & Deslippe, 1996; Wheeler, 1910). Because facultative parasites have not evolved efficient behavioral or morphological specializations for host raiding, their raids tend to last several hours

and have intensive fighting with a large number of parasite casualties (Savolainen & Deslippe, 1996; Wheeler, 1910). Facultative parasites are typically only able to exploit host species whose workers are smaller than the parasite workers (Savolainen & Deslippe, 1996).

Obligate parasites are not found in nests without hosts, suggesting that they lack the retention of behaviors needed to resume domestic duties essential to colony survival if hosts are absent (Wheeler, 1910). Obligate parasites typically are not active outside of the nest until the raiding season (Savolainen & Deslippe, 2001), and their nests have a large proportion of host workers since they are dependent on hosts for maintenance of the colony (Savolainen & Deslippe, 1996). They also tend to be rarer than facultative parasites since their distribution is dependent on host availability (Holldobler & Wilson, 1990; Savolainen & Deslippe, 1996).

Obligate parasites exhibit morphological adaptations for raiding host colonies. A hypertrophied Dufour's gland is considered a derived state that is presumably an adaptation for specialized parasitism. This gland produces large amounts of acetates that are sprayed offensively at host workers during raids, presumably as an alarm substance that confuses and scatters the host workers (Wilson & Regnier, 1971). Sharp, sickle-shaped mandibles, another derived trait in obligate parasites, are used offensively for piercing hosts during raids (Holldobler & Wilson, 1990; Wheeler, 1910; Wilson & Regnier, 1971). While modified mandibles are beneficial for fighting, they are not very useful for domestic tasks (Wheeler, 1910; Wilson & Regnier, 1971). Raids by obligate parasites tend to be more organized and shorter than raids by facultative parasites, with fewer parasite and host casualties (Talbot & Kennedy, 1940; Wheeler, 1910).

Although dulotic ants may be classified as facultative or obligate based on whether colonies are able to survive without host workers, each dulotic species may demonstrate varying degrees of specialized behavioral and morphological traits (Stewart & Alloway, 1985; Wilson,

1975). It is generally thought that domestic abilities of parasites degenerate as they evolve to become more specialized at raiding behavior (Stuart & Alloway, 1985; Wheeler, 1910; Wilson, 1975). Consequently, facultative parasites are more primitive and obligate parasites are more derived in terms of dulotic specialization (Mori & Le Moli, 1988). Therefore, it has been proposed that obligate parasites may have evolved from facultative parasites, at least in the Subfamily Formicinae (D'Ettorre & Heinze, 2001; Wilson, 1975).

While parasite workers exhibit domestic degeneration when host workers are present, they may still possess some flexibility in these behaviors which could be expressed when hosts are absent (Mori & Le Moli, 1988; Wesson, 1940; Wilson, 1975). By comparing parasite behavior in groups with and without host workers across species showing differing degrees of dulotic specialization, we will be better able to understand how and why certain species cross the threshold from a facultative to obligate dulotic lifestyle.

The *Formica sanguinea* complex consists of 12 dulotic species. They have all traditionally been considered facultative parasites (Wheeler, 1910). However, more recent studies have categorized one species, *F. subintegra*, as an obligate parasite (Savolainen & Deslippe, 1996, 2001). This species has not been found in nests without host workers (Savolainen & Deslippe, 1996), has a large proportion of host workers in their nests (~80%), and workers are not active outside the nest until raiding occurs in July and August (Savolainen & Deslippe, 2001). Workers of this species do a negligible amount of foraging, and although they move soil on the nest mound, they appear to be inept at effective nest maintenance (Savolainen & Deslippe, 2001). In addition, *F. subintegra* have an extremely hypertrophied Dufour's gland (Wilson & Regnier, 1971).

However, *F. subintegra* lack some traits of specialized obligate parasites and appear more similar to their facultative relatives in these traits. For instance, their mandible shape is more characteristic of free-living *Formica* species than the derived sickle-shaped mandibles characteristic of specialized obligate parasites (Wilson & Regnier, 1971). Behaviorally, *F. subintegra* seem to have retained at least some domestic abilities, even in the presence of host workers. For instance, *F. subintegra* have been seen carrying insects to the nest mound (Savolainen & Deslippe, 2001), foraging both on sugar water and mealworms, and engaging in some level of brood care and nest maintenance alongside their host workers (personal observation).

Because obligate parasitism involves a loss of behaviors from the free-living condition (Wilson, 1975), behavioral studies are an appropriate starting point for exploring evolutionary transitions between facultative and obligate dulosis. Host-removal behavioral experiments have been conducted with some species of dulotic ants, and have suggested an apparent continuum between wholly facultative and wholly obligate. At the wholly facultative end of the continuum, the facultative parasite *Formica sanguinea* completely recovered its foraging and brood care behaviors when host workers were absent (Mori & Le Moli, 1988). Some primitive obligate parasites can partially care for themselves in the absence of hosts, but lack one or more domestic behaviors essential for independent living. For example, foraging behaviors are recovered by some obligate parasites when host workers are removed (Kutter, 1969, as cited by Wilson, 1975; Stuart and Alloway, 1985; Wesson, 1940; Wilson, 1975). Some obligate parasites show an increase in brood care behaviors when hosts are removed (Wesson, 1940; Wilson, 1975), while others have lost the ability to care for brood (Kutter, 1969, as cited by Wilson, 1975). Obligate parasite workers of *Strongylognathus huberi* do not capture insect prey without host workers

(Kutter, 1969, as cited by Wilson, 1975). *Harpagoxenus canadensis* workers are able to successfully relocate their nest after disturbance without the aid of host workers, but *H. sublaevis* and *H. americanus* workers are not consistently successful at this task without host workers (Stuart and Alloway, 1985). At the far end of the obligate spectrum, workers of the highly specialized obligate parasite *Polyergus rufescens* display no brood care behaviors and cannot even sustain themselves nutritionally without host workers (Mori & Le Moli, 1988; Wheeler, 1910).

Where does *F. subintegra* fit on the facultative/ obligate spectrum? Previous behavioral studies for this species have reported that host workers were always present in field observations of *F. subintegra* colonies (Savolainen & Deslippe, 2001), suggesting that they may be near the wholly obligate end of the spectrum. However, as *F. subintegra* possesses some specialized characteristics but lacks others, I hypothesize that this species may possess latent domestic behaviors that can be evoked by the removal of their host workers. To my knowledge, there have been no behavioral observations of this species in the absence of host workers to determine to what extent they are capable of recovering their domestic behaviors upon host removal. This study aims to fill this gap by comparing *F. subintegra*'s foraging and nest excavation behaviors when host workers are present versus absent.

Dulotic ants typically do few domestic duties and instead allow the host workers to fill those roles (Holldobler & Wilson, 1990; Wheeler, 1910). If the parasites possess latent domestic abilities and are deprived of the host workers, they should 'fill the gap' left by the absent hosts and begin to do more of the domestic duties themselves. Therefore, if *F. subintegra* possesses latent domestic abilities in relation to foraging behaviors, I predict that: (1) Parasite workers in isolated groups will forage more often, and for a greater amount of time, than parasites in mixed

groups with host workers. (2) Parasite workers in isolated groups will begin foraging sooner than parasites in mixed groups with host workers. (3) Parasite workers in isolated groups will regurgitate liquid food (oral trophallaxis) to nestmates more often than parasites in mixed groups with host workers. With regard to nest excavation behaviors, I predict that: (4) Parasites in isolated groups will engage in nest excavation behaviors more often than parasites in mixed groups with host workers. (5) Parasites in isolated groups will be able to construct a tunnel system typical of those constructed by mixed groups with host workers.

## METHODS

### Experiment 1: Foraging Experiment

**Collection and maintenance.** Worker ants of the host (*F. subsericea*) and parasite (*F. subintegra*) species were collected from two mixed *F. subintegra*/*F. subsericea* colonies in Two Rivers Mountain Bike Park (Christian Co., MO) on 10 June 2017, and from two colonies in Meramec State Park (Franklin Co., MO) on 11 June 2017. Ants were collected by digging into the upper layers of the nest and scooping the nest material into an Insect-A-Slip™ -lined plastic collection container. Effort was made to collect ants mostly from inside the nest so that the sample was not biased toward foragers who may be older in age. The collection containers were taken to the lab where the ants were removed from the nest material. Ants from each colony were placed in an Insect-A-Slip™ -lined plastic foraging container (34 cm×21 cm×12 cm) containing a test tube of dechlorinated water with a cotton plug, and a 100 mm×15 mm round Petri dish to serve as a nest chamber (Figure 1). The bottom of the nest chamber and foraging box were lined with hydrostone, a gypsum cement that holds moisture and resists mold.

The nest chamber was moistened as needed. Food was made available for the ants *ad libitum* and was changed three times per week. The carbohydrate food source was alternated between the Bhatkar diet (Bhatkar & Whitcomb, 1970), brown sugar water, and honey water. The protein source was alternated between fresh mealworms, fruit flies, and small crickets. The feeding dish consisted of a white PVC knock-out plug with a 4.5 mm lip around the edge on which the ants could sit while feeding. Containers were randomly assorted on shelves with a 14-h light/ 26-30°C: 10-h dark/ 20-22°C cycle. Ants were held in the lab for 3 months prior to beginning the foraging experiment.



**Figure 1.** Photograph of foraging container (34 cm×21 cm×12 cm). The small circular feeding dish is shown at the top of the picture, and the large circular nest chamber is shown at the bottom of the picture.

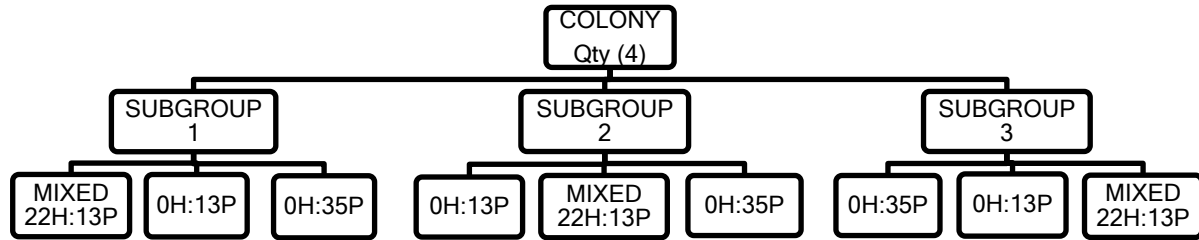
**Experiment protocol.** To test for recovery of foraging behavior, each of the four colonies was divided into three colony subgroups, each containing approximately 40 parasite workers and 70 host workers. Subgroups were subjected to three treatments in all possible orders as shown in Appendix A (Figure 2). Order was varied so that the number of starvation events the groups experienced, which corresponds to treatment order, was not confounded with treatment group. The mixed treatment group (22H:13P) consisted of 35 ants in total with 22 host workers and 13 parasite workers, in keeping with a natural worker species ratio. The parasite-only treatment groups consisted of 13 and 35 parasite workers, respectively. Parasite-only treatments 0H:13P controlled for the number of parasites in the mixed treatment group, and 0H:35P controlled for the total number of ants in the mixed treatment group. The sample size for each of these three treatments was  $N = 12$  replicates.



To form the treatment groups, individual parasite and host workers were taken at random from the colony subgroups and placed in testing containers identical to the subgroup containers. Treatment groups were fed *ad libitum* for 4 d so that they could become accustomed to the treatment container. Ants were then deprived of food for 72 h before testing. If there were any ant mortalities in the treatment groups prior to the day of testing, ants were replaced with ants from the subgroup container, which were also deprived of food during the same days.

At the start of testing, a dish of brown-sugar water at a concentration of 3g sugar per 20 mL water was placed in the container and then the ants were observed for 45 min. The first 30 min of observations were video recorded using a DSLR camera. The number of visits to the dish and number of oral trophallaxis events for each species present were recorded for the full 45 min, while duration of feeding was recorded for 30 min using the videos. Latency to begin feeding was recorded when the first ant in each group began feeding from the dish. A visit to the dish was counted when an ant mounted the lip of the food dish and began feeding on the sugar water. A visit was concluded when the ant stopped feeding and did not resume feeding again before dismounting. Regurgitated food donations via oral trophallaxis were counted when the following stereotypic behavior was observed (Holldobler & Wilson, 1990): Two ants faced each other and the donor ant opened its mandibles. The receiving ant placed its mandibles between those of the donor and both ants rose up on their rear pairs of legs, while the receiving ant rapidly drummed on the donor ant with its antennae and forelegs. For the mixed treatment groups containing both parasites and hosts, a record was made of which species donated liquid food and which received the food. Following testing, ants were placed back in their colony subgroups for 1 wk and were fed *ad libitum* before being placed into the next treatment group, until each subgroup had

experienced all treatments. If at least one ant did not feed from the dish during the first 10 m of the trial, the trial was ended and the group was re-tested on the following day.



**Figure 2.** Diagram showing an example of a colony divided into 3 colony subgroups that were subjected to three treatments in different testing orders. Treatment ‘Mixed’ consisted of 22 hosts and 13 parasites, treatment ‘0H:13P’ consisted of 13 parasites only, and treatment ‘0H:35P’ consisted of 35 parasites only.

**Statistical analyses.** Statistical analyses were performed using Minitab v. 16.1.0 (Minitab Inc., 2010). Initial normality tests revealed that the data differed significantly from normal for all response variables except number of food donations by oral trophallaxis, so the non-normal data were transformed using the aligned rank transformation procedure (ARTool 1.6.2; Wobbrock et al., 2011).

To test the hypothesis that parasites behave differently in the different density treatments, the GLM procedure ( $\alpha = 0.05$ ) was used with treatment and treatment order as factors in the analysis. The behavioral response variables were number of visits to the dish, latency to begin feeding, duration of feeding, and number of food donations (via oral trophallaxis). To control for density of ants, the data for each response variable were converted to ‘behavior per parasite’ values by dividing the behavioral data by the number of parasites in each treatment group (e.g., number of visits/parasite).

To test the hypothesis that parasites and hosts within the Mixed groups behaved differently from each other, again the GLM procedure ( $\alpha = 0.05$ ) was used with treatment and treatment order as factors. The response variables were converted to ‘per parasite’ or ‘per host’ values as described above, and the same response variables were used as above.

For all tests, post-hoc multiple comparisons were made for factors with significant results using Tukey’s multiple comparison tests.

## **Experiment 2: Nest Excavation Behavior**

**Collection and maintenance.** Host (*F. subsericea*) and parasite (*F. subintegra*) worker ants were collected from 3 mixed *F. subintegra*/*F. subsericea* colonies located in Two Rivers Mountain Bike Park on 19 May and 9 June 2018. Collection was conducted in the same way and ants were housed in identical setups as in Exp. 1. Ants were kept in the lab for 2-3 days before beginning the nest excavation experiment. The ants were exposed to natural light through windows with a 14.5-h light/ 9.5-h dark cycle for the duration of the experiment. Room temperature varied between 22 °C and 27 °C.

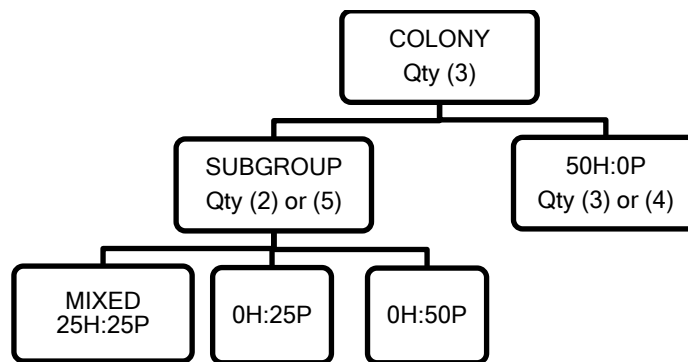
**Experiment protocol.** Because unequal numbers of worker ants were collected from the three colonies, workers from two of the colonies were divided into five colony subgroups and workers from the third colony were divided into two colony subgroups. Each colony subgroup contained 60 parasite workers and 60 host workers. Subgroups were subjected to three treatments in all possible orders as shown in Appendix A (Figure 3). Order was varied so that experience in the ant farms was not confounded with treatment group. The mixed treatment group (25H:25P) consisted of 25 parasite workers and 25 host workers, for a total of 50 ants. The parasite-only groups (0H:25P and 0H:50P) consisted of 25 and 50 parasite workers, respectively. Treatment

0H:25P controlled for the number of parasites in the Mixed group, and treatment 0H:50P controlled for the total number of ants in the Mixed group. The sample size for each of these three treatments was N = 12 replicates. A host-only treatment group consisting of 50 host workers and no parasites (50H:0P) was used as a control. The host workers used in these groups were ‘extra’ ants collected from the same three mixed *F. subintegra*/*F. subsericea* colonies, and were not part of the replicate groups mentioned above. The sample size for the host-only treatment group was N = 10 replicates.

The testing apparatus (Figure 4) consisted of an ‘ant farm’ formicarium constructed of a wooden frame and two sheets of 15 cm×37 cm clear acrylic. The acrylic sheets were separated by a gap of 5 mm. A permanent horizontal ‘fill line’ was drawn on the acrylic at a height of 33 cm to standardize the depth of substrate and serve as the reference line for tunnel excavation measurements. Timberline™ sterilized organic top soil was sifted and then added to the farms until the top was level with the fill line. A feeding tube filled with brown-sugar water was inserted into a hole drilled in the acrylic at dirt level and the ants were fed *ad libitum* during the experiment.

To begin the trials, individual parasite and host workers were taken at random from the colony subgroups, or from the extra host ant containers for the host-only (50H:0P) groups, were placed on top of the substrate in an ant farm, and the lid was secured. Each individual parasite worker took part in the 0H:50P treatment, and either the 0H:25P treatment or the Mixed (25H:25P) treatment since the 0H:25P and Mixed treatments took place simultaneously. The treatment groups were visually observed by scan sampling (Martin & Bateson, 2007) three times per day at random intervals. Number of parasites engaging in digging behavior was recorded, and the room temperature was noted. Every 24 h, the progression of tunnels was outlined on the

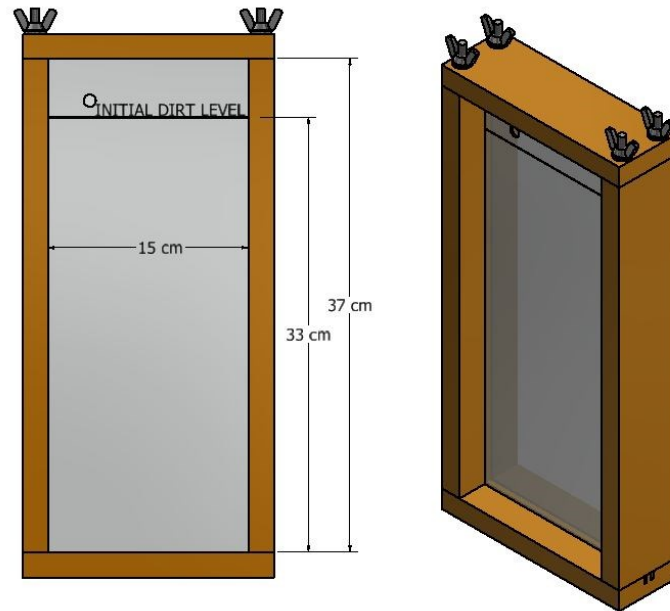
outside of the acrylic with a wet-erase marker. After 3 d, the trials were ended and the ants were removed from the ant farm formicaria and placed back in their subgroup containers for 2 d before beginning the next treatment, until each subgroup had experienced all treatments. The fill lines and tunnel outlines were then permanently transferred to transparent sheets and scanned (see Appendix B). ImageJ™ v1.52a image processing and analysis software was used to measure the maximum depth, tunnel area and perimeter below the initial dirt level, and area and perimeter of dirt moved above the initial dirt level for each day.



**Figure 3.** Diagram showing an example of a colony divided into colony subgroups that were subjected to three treatments in different testing orders. Treatment ‘Mixed’ consisted of 25 hosts and 25 parasites, treatment ‘0H:25P’ consisted of 25 parasites only, and treatment ‘0H:50P’ consisted of 50 parasites only. Host-Only groups (50H:0P) were used as a control.

**Statistical analyses.** Statistical analyses were performed using Minitab v. 16.1.0 (Minitab Inc., 2010). Initial normality tests revealed that the data differed significantly from normal, so the data were transformed using the aligned rank transformation procedure (ARTool 1.6.2; Wobbrock et al., 2011). Correlation analyses revealed that area and perimeter of dirt moved above the reference line were tightly correlated with area and perimeter of tunnels below

the reference line ( $p < 0.0005$ ), so only measurements below the reference line were used in the analyses.



**Figure 4.** Diagram of experimental ‘ant farm’ formicarium used in the nest excavation experiment.

As in exp. 1, the data were converted to ‘per parasite’ values by dividing by the number of parasites in each treatment group, and were thus denoted as digging bouts per parasite. To test the hypothesis that the number of digging bouts by parasite workers was influenced by density treatments, the GLM procedure ( $\alpha = 0.05$ ) was used with treatment and treatment order as fixed effects factors, subgroup as a random effects factor, and room temperature as a covariate. Post-hoc multiple comparisons were made for factors with significant results using Tukey’s multiple comparison tests.

To test the hypothesis that the quality of tunnels differed among the density treatments, the maximum tunnel depth and area of excavated tunnels were used as response variables and treatment was used as the single factor. Treatment order was deleted from the model since it did not have a significant effect on digging behavior. Congruence of tunnels between days 1-2 and days 2-3 was quantified by measuring the area of tunnel that was dug on one day and subsequently filled in on the next day. The area of tunnels filled in on days 2 and 3 were summed to get the total area of tunnel filled in. These data were converted to percentage of tunnel area filled in by dividing the total area of tunnels filled in by the total area of tunnels dug over days 1 and 2 of testing, and multiplying by 100 (% Area Filled In). Tunnel analyses were conducted using the GLM procedure ( $\alpha = 0.05$ ). Post-hoc multiple comparisons were made for factors with significant results using Tukey's multiple comparison tests.

## RESULTS

### Experiment 1: Foraging Behavior

**Treatment (density) effects.** Treatment significantly affected all response variables. For oral trophallaxis, the number of times parasites donated regurgitated food, parasites in the Mixed and 0H:35P Parasite-Only groups both donated food least often, and parasites in the 0H:13P Parasite-Only group donated food the most ( $F_{2,27} = 16.21$ ,  $p < 0.0005$ ; Figure 5). There was no significant main effect of treatment order ( $F_{2,27} = 1.72$ ,  $p = 0.198$ ) and no interaction between treatment and treatment order ( $F_{4,27} = 0.49$ ,  $p = 0.766$ ) for oral trophallaxis.

For number of visits to the food dish per parasite, parasites in the 0H:13P and 0H:35P Parasite-Only groups visited the food dish significantly more times than did parasites in the Mixed group. ( $F_{2,27} = 9.89$ ,  $p = 0.001$ ; Figure 6). There was no significant main effect of treatment order ( $F_{2,27} = 2.52$ ,  $p = 0.099$ ) and no interaction between treatment and treatment order ( $F_{4,27} = 1.38$ ,  $p = 0.268$ ) for number of visits to the food dish.

For duration of feeding per parasite, parasites in the 0H:13P and 0H:35P Parasite-Only groups fed from the food dish for a significantly longer duration than did parasites in the Mixed group ( $F_{2,27} = 9.52$ ,  $p = 0.001$ ; Figure 7). There also was a significant main effect of treatment order on duration of feeding per parasite ( $F_{2,27} = 6.13$ ,  $p = 0.006$ , Figure 8). Treatment orders 1 and 2 had higher mean durations of feeding from the dish than did treatment order 3. For treatment group and treatment order, the two-way interaction effect on duration of feeding was nearly significant ( $F_{4,27} = 2.66$ ,  $p = 0.054$ , Figure 8).

For latency of parasites to begin feeding, the 0H:35P Parasite-Only group began feeding significantly sooner than did the Mixed group, but there was no significant difference between



the 0H:13P Parasite-Only group and the Mixed group or the two Parasite-Only groups ( $F_{2,27} = 3.92$ ,  $p = 0.032$ ; Figure 9). There was no significant main effect of treatment order ( $F_{2,27} = 1.16$ ,  $p = 0.330$ ) and no interaction between treatment and treatment order ( $F_{4,27} = 0.82$ ,  $p = 0.521$ ) for latency of parasites to begin feeding.

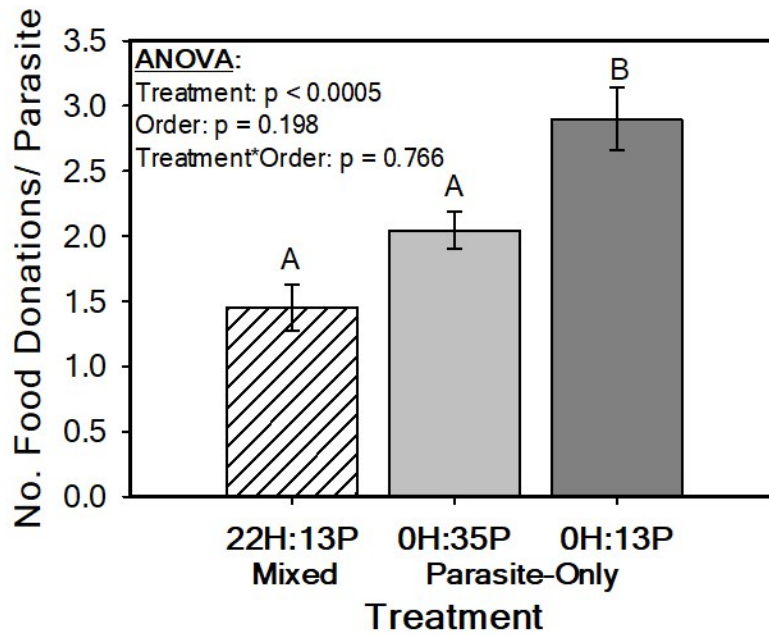
All trial failures, where at least one ant did not feed from the dish within 10 min of the start of a trial, were in three of the Mixed treatment groups. One Mixed group had to be re-tested three times, one group had to be re-tested twice, and one group had to be re-tested once.

**Behavior within the mixed groups.** Parasites were as likely as hosts to be food donors ( $F_{1,44} = 0.19$ ,  $p = 0.667$ ). There was also no difference in which species was the receiver of the regurgitated food ( $F_{1,44} = 0.52$ ,  $p = 0.473$ ). In other words, a parasite was as likely to donate to a host as to another parasite, and a host was as likely to donate to a parasite as to another host.

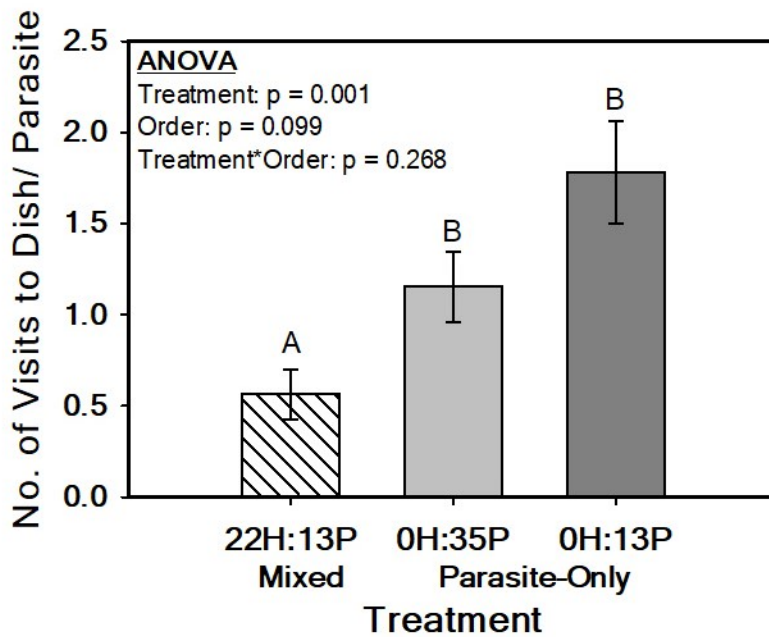
For duration of feeding per parasite/host, the interaction between treatment group and treatment order was significant ( $F_{2,18} = 9.10$ ,  $p = 0.002$ ; Figure 10). Parasites fed for shorter durations when first tested, and hosts fed for longer durations when first tested.

## **Experiment 2: Nest Excavation Behavior**

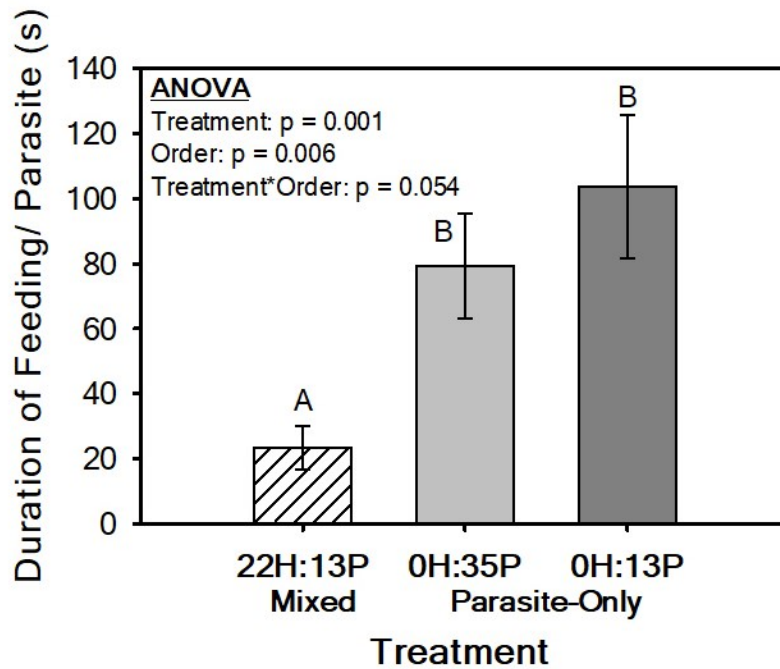
**Parasite digging behavior.** Treatment significantly affected all aspects of digging behavior. For number of digging bouts per parasite, the Parasite-Only groups had significantly more digging bouts per parasite than did the Mixed group ( $F_{2,317} = 8.92$ ,  $p < 0.0005$ ; Figure 11). There was no significant main effect of treatment order ( $F_{1,317} = 0.00$ ,  $p = 0.957$ ) and no interaction between treatment and treatment order ( $F_{2,317} = 0.96$ ,  $p = 0.383$ ) for number of digging bouts per parasite.



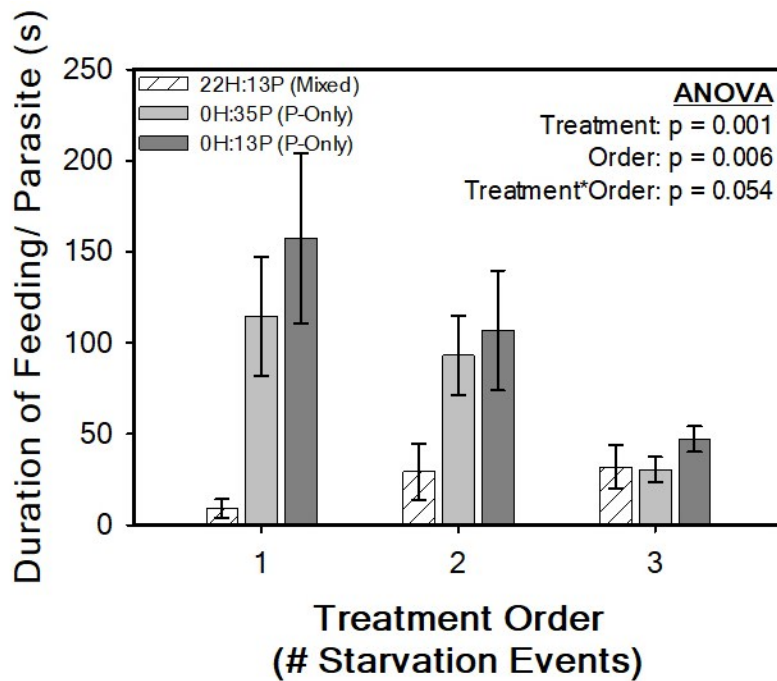
**Figure 5.** Mean ( $\pm$ SE) number of food donations per parasite (P) when parasites were in mixed groups with host workers (22H:13P), or Parasite-Only groups containing 35 or 13 parasites. Means that do not share a letter are significantly different.



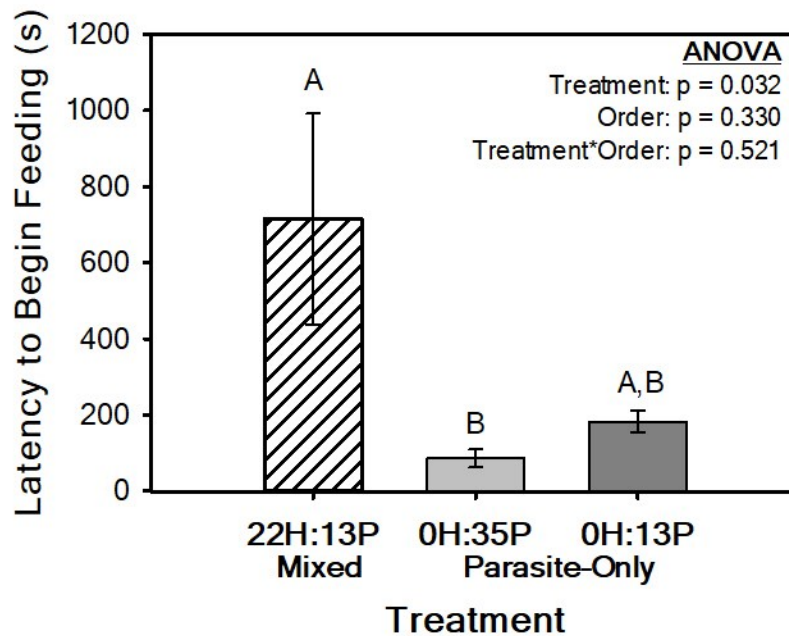
**Figure 6.** Mean ( $\pm$ SE) number of visits to the food dish per parasite (P) when parasites were in mixed groups with host workers (22H:13P), or parasite-only groups containing 35 or 13 parasites. Means that do not share a letter are significantly different.



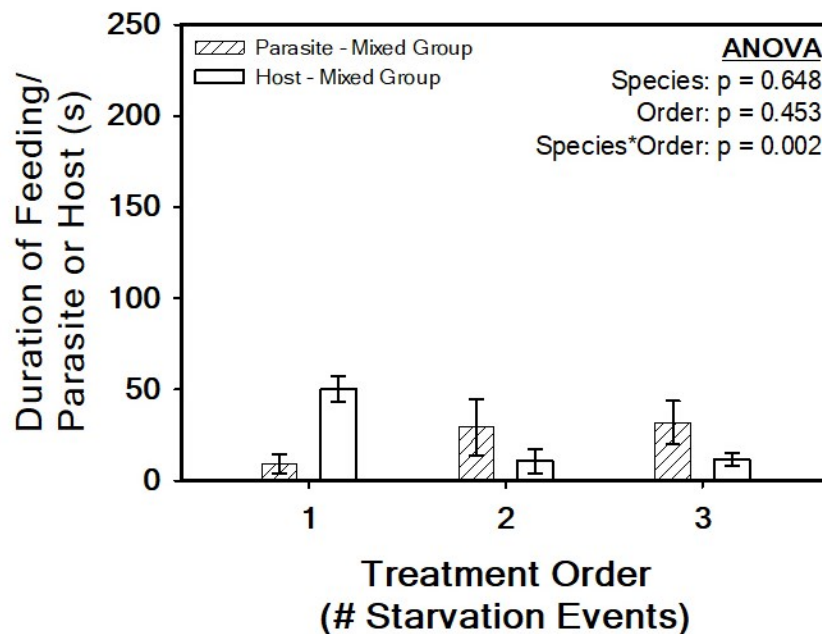
**Figure 7.** Mean ( $\pm$ SE) duration of feeding per parasite (P) when parasites were in mixed groups with host workers (22H:13P), or parasite-only groups containing 35 or 13 parasites. Means that do not share a letter are significantly different.



**Figure 8.** Mean ( $\pm$ SE) duration of feeding per parasite (P) for the three treatment groups vs. treatment order. Treatment order corresponds to number of starvation events experienced.

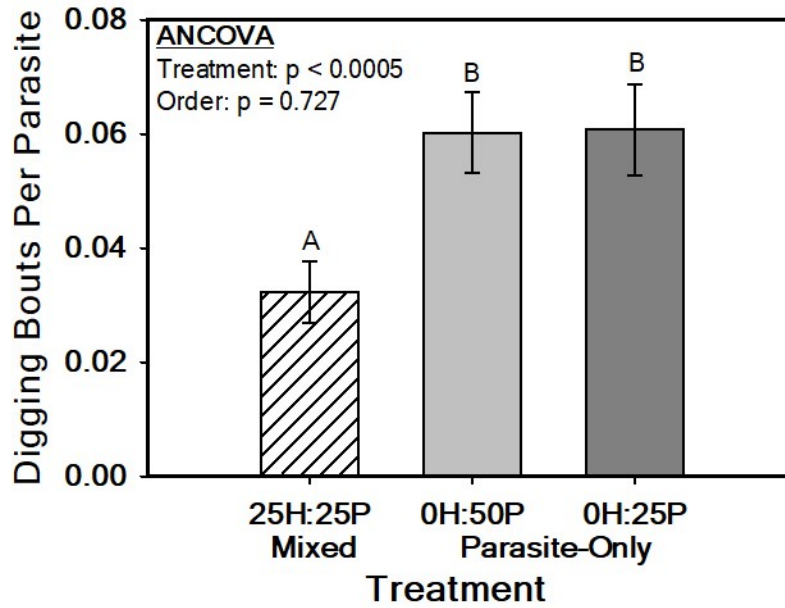


**Figure 9.** Mean ( $\pm$ SE) latency for parasites (P) to begin feeding from the dish when in mixed groups with host workers (22H:13P), or parasite-only groups containing either 35 or 13 parasites. Means that do not share a letter are significantly different.

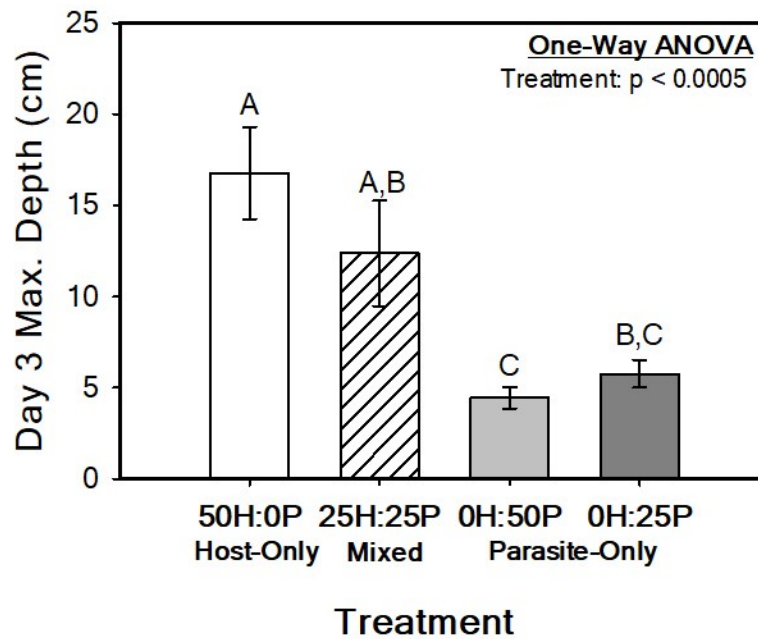


**Figure 10.** Mean ( $\pm$ SE) duration of feeding per parasite and per host in the mixed treatment group (22H:13P) vs. treatment order. Treatment order corresponds to number of starvation events experienced.

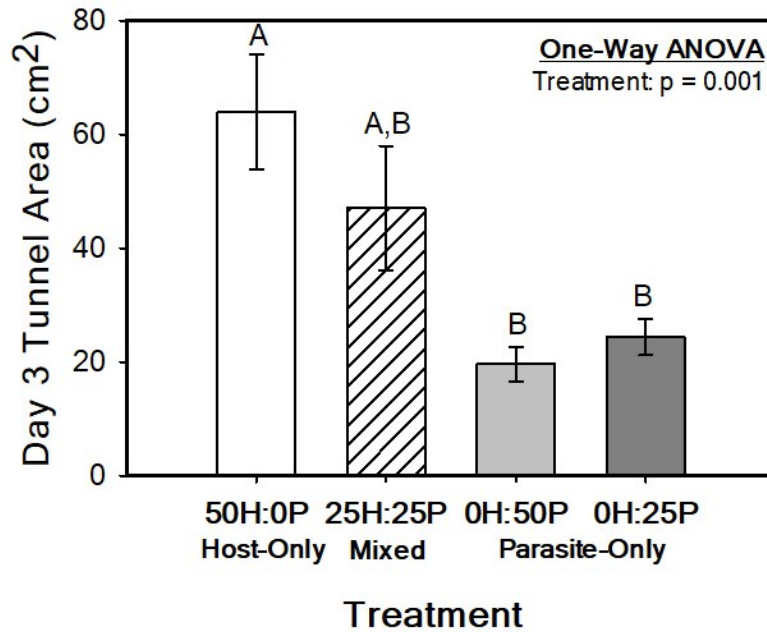
**Tunnel metrics.** Treatment significantly affected all aspects of digging behavior as it pertained to tunnel metrics. For maximum depth of tunnels, the Host-Only group (50H:0P) and the Mixed group constructed deeper tunnels than did the 0H:50P Parasite-Only group, but the Mixed group did not differ significantly from the 0H:25P Parasite-Only group ( $F_{3,42} = 8.67$ ,  $p < 0.0005$ ; Figure 12). After day 3, the tunnel area of the Host-Only group (50H:0P) was significantly greater than the tunnel area of both Parasite-Only groups, but the Mixed group did not differ significantly from any other treatment group ( $F_{3,42} = 6.50$ ,  $p = 0.001$ ; Figure 13). For percentage of tunnel area filled in on subsequent days, the 0H:50P and 0H:25P Parasite-Only groups filled in significantly more tunnel area than did the Host-Only group (50H:0P), and the 0H:50P Parasite-Only group filled in significantly more tunnel area than did the Mixed group ( $F_{3,42} = 6.47$ ,  $p = 0.001$ ; Figure 14). The 0H:50P Parasite-Only group was not significantly different than the 0H:25P Parasite-Only group, and the 0H:25P Parasite-Only group was not significantly different than the Mixed group.



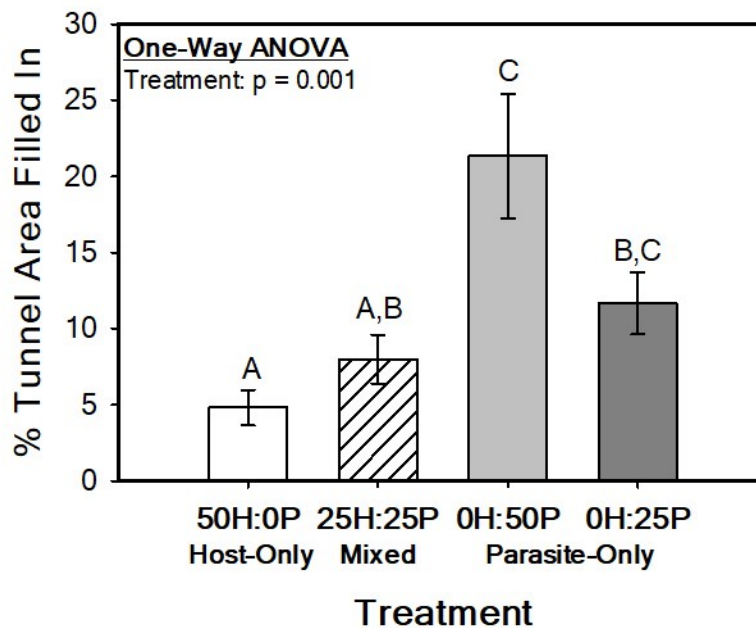
**Figure 11.** Mean ( $\pm$ SE) number of digging bouts per parasite (P) when parasites were in Mixed groups with host workers (25H:25P), or Parasite-Only groups containing 50 or 25 parasites. Means that do not share a letter are significantly different.



**Figure 12.** Maximum depth of tunnels (mean  $\pm$ SE) at the conclusion of trials for Host-Only groups (50H:0P), Mixed groups (25H:25P), and Parasite-Only groups containing 50 parasite workers (0H:50P) and 25 parasite workers (0H:25P). Means that do not share a letter are significantly different.



**Figure 13.** Area of tunnels (mean  $\pm$ SE) dug at the conclusion of trials for Host-Only groups (50H:0P), Mixed groups (25H:25P), and Parasite-Only groups containing 50 parasite workers (0H:50P) and 25 parasite workers (0H:25P). Means that do not share a letter are significantly different.



**Figure 14.** Percentage of tunnel area filled in (mean  $\pm$ SE) at the conclusion of trials for Host-Only groups (50H:0P), Mixed groups (25H:25P), and Parasite-Only groups containing 50 parasite workers (0H:50P) and 25 parasite workers (0H:25P). Means that do not share a letter are significantly different.

## DISCUSSION

### Experiment 1: Foraging Behavior

Previous field studies have suggested that *F. subintegra* do not forage when in natural mixed nests (Savolainen & Deslippe, 1996, 2001). However, the results of experiment 1 of this study indicate that *F. subintegra* can recover its foraging behaviors when deprived of its host workers. In comparison with parasites in mixed groups containing host workers, isolated parasite groups generally began foraging sooner, foraged longer, visited the food dish more often, and donated liquid food to nestmates through oral trophallaxis more often.

Similar behavioral experiments examining foraging behavior of parasites in the absence of hosts have been conducted with some other species of dulotic ants. The facultative parasite *Formica sanguinea* completely recovered its foraging behavior after 32 days of isolation from its host workers (Mori & Le Moli, 1988). Upon removal of its host workers, the obligate parasite *Leptothorax duloticus* (subfamily: Myrmicinae) began to feed itself on honey water but never retrieved insect prey (Wilson, 1975). Three obligate parasitic species of the genus *Harpagoxenus* (subfamily: Myrmicinae) all increased foraging behavior to different degrees when isolated from host workers (Stuart & Alloway, 1985). Although they are all obligate parasites, *L. duloticus* and the three species of the *Harpagoxenus* genus are considered to represent earlier stages in the evolution of dulosis than species in the *Polyergus* genus (Stuart & Alloway, 1985; Wilson, 1975). The highly specialized obligate parasite *Polyergus rufescens* was not able to sustain itself even with an abundance of food available and consequently had a very high mortality rate (Mori & Le Moli, 1988).



The results for the present study indicate that *F. subintegra* show foraging behavior in the absence of hosts that is similar to the more primitive obligate parasites (*L. duloticus* and *Harpagoxenus* spp.). Workers of *F. subintegra* are able to forage on liquid food without assistance from hosts, but more studies are needed to determine if they could actually sustain themselves in the long term. Their ability to independently gather and feed on insect prey, an essential part of their diet, would also need to be evaluated.

It is interesting to note that expansion of foraging behavior was generally greater for the smaller parasite-only groups than for the larger parasite-only groups. While it is unclear why this is the case, one possible explanation could be related to task allocation. In a small group of only 13 parasites, it seems reasonable that all individuals would need to explore to locate food sources. However, in a larger group, all individuals may not need to forage and ‘extra’ ants could fill other roles. While observing the groups, I noticed that several individuals in the larger groups remained inactive during the trials, whereas most of the individuals in the smaller groups were active. Future studies could individually mark ants to determine if specific individuals were filling certain roles within the groups.

Parasite workers donated liquid food to nestmates in both the mixed and parasite-only groups, but parasites tended to donate to nestmates less often when hosts were present. A similar pattern was seen with the obligate parasite *L. duloticus*, which is considered at an early stage in the evolution of dulosis (Wilson, 1975). Workers of *L. duloticus* donated regurgitated food even in natural mixed groups, but did so more frequently when hosts were removed than when they were present (Wilson, 1975).

Within the mixed groups, parasites were just as likely as hosts to donate food to nestmates. There was also no bias in which species served as receivers of food donations. This

lack of bias was somewhat surprising. Although the obligate parasite *L. duloticus* was also observed donating food to its host workers and to other parasites, it donated food to nestmates with much less frequency than host workers donated food to nestmates (Wilson, 1975). I hypothesize that the lack of discrimination among nestmates is indicative of *F. subintegra* being more primitive in this regard than *L. duloticus*.

Although all treatment groups were held without food for 72 h before testing, I observed trophallaxis in some of the mixed groups, but not the parasite-only groups, before the food dish was located by the first ant in the trial, indicating that food was still stored in the crops of the ants even after 72 hours of not feeding. Therefore, a difference in hunger level between the mixed group and the parasite-only groups could partially explain some of the differences between the mixed and parasite-only groups. A study examining foraging dynamics at varying nutritional states of a colony found that with increased starvation, ants accepted liquid food more readily, fed for longer and more continuously, and filled their crops with more food (Josens & Roces, 2000). Several aspects of the isolated parasites' behavior suggest that the hunger level in those groups was greater than that in the mixed groups. The parasite-only groups located and fed from the food dish sooner, suggesting a hunger-induced increase in exploratory behavior. They also fed for longer durations. There were no failed trials in the parasite-only groups due to no ants feeding in the first 10 min of the trial, whereas the mixed treatment group had six failed trials. The evidence for an increased state of hunger in the parasite-only groups could indicate that during the evolution of dulosis, a change in function and/or behavior occurs as it pertains to long-term internal food storage and the role the parasite plays in the 'social stomach' of the colony.

The treatment order effects seen in the duration of feeding by hosts and parasites may indicate that parasites can adjust their food storage behavior when hosts are removed. The behavior of host workers can be considered to be the ‘normal’ state. Host workers fed for the longest duration during the first treatment to which they were subjected, then decreased the duration of feeding during the second and third treatments. An interpretation could be that the hosts were able to anticipate another food shortage and compensated by storing extra food in their crops when food was available between treatments. Therefore, during subsequent treatments, hosts may not have been as hungry and did not need to forage as much during those trials. The parasites in the mixed group with hosts did not display this trend. While the host workers decreased their duration of feeding, the parasites in the mixed group slightly increased their duration of feeding. However, parasites in the groups without hosts displayed the same trend as the host workers, although they did still feed for longer durations overall than hosts. Parasites-only groups fed for the longest duration during the first treatment, then decreased the duration of feeding during subsequent treatments. These trends could indicate that when parasites are isolated, they become somewhat better at preparing for future conditions and storing food accordingly. Even so, if the parasite-only groups had higher hunger levels as stated above, they may not be as effective at long-term food storage as host workers. To my knowledge, a change in behavior related to crop food storage in obligate parasites has not yet been explored. Future studies could investigate this idea further by comparing crop morphology/ capacity and storage behaviors of free-living species with parasites of differing dulotic specialization.

## Experiment 2: Nest Excavation Behavior

Previous field observations have suggested that *F. subintegra* workers are not competent at nest maintenance activities when in their natural mixed nests, but these results were not quantified (Savolainen & Deslippe, 1996, 2001). This current study quantified nest excavation behavior when parasites were in groups with and without host workers to determine if *F. subintegra* workers have retained the ability to independently perform nest maintenance tasks during the course of their dulotic evolution.

In this study, *F. subintegra* workers displayed qualitatively similar digging behavior as their host species, *F. subsericea*. They were observed moving to the bottom of a tunnel, picking up dirt with the mandibles, and returning to the surface to deposit the dirt near the tunnel entrance. Occasionally, *F. subintegra* workers were seen rapidly digging at the dirt with their front legs in a canine-like fashion. This method did not appear to be very productive, and host workers were never observed using this digging method. Sakagami & Hayashida (1962) also observed this leg-only digging behavior in the facultative parasite *Formica sanguinea*, and pointed out that while it is rare in ants, this type of digging is a common behavior for digger wasps. It is unclear why these parasitic ants at least occasionally engage in this type of digging behavior.

Parasites in isolated groups exhibited more digging behavior per parasite than parasites in mixed groups with host workers, indicating that *F. subintegra* expand their nest building behavior if hosts are absent. These results agree with my prediction that, although they may not regularly participate in nest maintenance when host workers are available to do the task, the ants of this species have retained the ability to fill this role to some degree if needed. Even though parasites could not be observed continually over the course of the trials, the construction and

progression of tunnels in groups without host workers is further evidence of the parasites' ability to excavate.

This study has shown for the first time that *F. subintegra* are capable of digging functional tunnels without the aid of host workers. The maximum tunnel depth reached after 3 days of excavation was not significantly different between the mixed and 0H:25P parasite-only group. There was no significant difference in the area of the tunnels after day 3 for the mixed group and both parasite-only groups. However, the trends in the data indicate that the parasite-only groups are probably not as proficient at nest excavation as the groups containing all or some host workers. The retention of general digging behavior may have adaptive value when considering the defense strategies of *F. subsericea* host colonies. Field observations on Gibraltar Island, Ohio, by Talbot & Kennedy (1940) confirmed that *F. subsericea* colonies that are regularly raided by *F. subintegra* parasites conceal their nest entrances by plugging them with soil, rocks, sticks, and other debris. During raids, the *F. subintegra* workers must locate these entrances and unplug them by removing bits of the plugging material with the mandibles and discarding them to the side, a task analogous to excavating a short tunnel. If the drive to dig was completely lost, the parasites would not be successful at raiding host nests and replenishing the host work force, thus eliminating the obligate parasite colony's chance of survival.

Over the course of the 3 day trials, some tunnels that were dug on a previous day were subsequently filled in with soil. This equates to work inefficiency since it is essentially undoing work that was previously done. Work efficiency in nest-building has been studied for groups of dulotic ants and their hosts. Sakagami & Hayashida (1962) compared the work output of various group compositions of *Polyergus samurai* (a highly specialized obligate parasite), *F. sanguinea* (a facultative parasite) and their common host species *Formica japonica*. The study found that *P.*

*samurai* did no digging at the nest at all, whether they were in parasite-only or mixed groups with hosts, but they also did not hinder the nest building output in mixed groups. *F. sanguinea* dug tunnels in parasite-only and mixed groups, but their work output was less than host worker output. In this regard, *F. subintegra* is more similar to its facultative relative, *F. sanguinea*, than to the highly specialized *P. samurai*. In the study by Sakagami & Hayashida (1962), work efficiency for the groups was calculated as the mixed group's mean output divided by the expected output of the same number of individual ants of each species. Using this index, the researchers found that work efficiency decreased with increase in group size, especially with the addition of *F. sanguinea* workers (Sakagami & Hayashida, 1962). For the present study, if percent of tunnels filled in is used as a metric for work inefficiency, then similar results are seen for *F. subintegra*. It appears that the addition of *F. subintegra* workers increases the percentage of tunnels that are subsequently filled in, which indicates a decrease in work efficiency. Groups with 50 host workers and no parasites only filled in an average of 4.8% of their tunnels. The addition of 25 parasite workers to a group of 25 host workers increased the average percentage of tunnels filled in to 8.0%. Parasite-only groups with 25 and 50 parasite workers filled in an average of 11.7% and 21.3%, respectively.

The genus *Polyergus* represents a highly specialized state of dulosis in which domestic ability is extremely degenerate or completely absent (Wheeler, 1910). It appears that workers of *Polyergus samurai* have completely lost the drive to participate in nest building and maintenance (Sakagami & Hayashida, 1962). However, like *F. subintegra*, they do move soil in the context of raiding host nests and uncovering their entrances (Mori et al., 1991). The retention of digging behavior in the raiding context, but loss of digging behavior in the nest maintenance context, could represent an end state in the evolution of dulosis. The adaptive value of retaining digging

behavior while raiding is obvious. Perhaps there is also adaptive value in the loss of digging behavior at the nest. For *P. samurai*, loss of interest in digging at the nest could be viewed as positive since they did not hinder the superior nest-building abilities of their hosts. In this study, *F. subintegra* has still retained the drive to dig, but it appears that digging negatively impacted nest building in mixed groups. Tunnels constructed by host-only groups were generally deeper and more organized than groups that contained some or all parasite workers, and tunnels by mixed groups appeared more organized than tunnels of parasite-only groups. The parasite-only group of 50 ants (0H:50P) had the poorest nest-building performance. The 0H:50P group also had the most parasites digging during the observational samples (325 total digging observations vs. 164 and 87 for the 0H:25P and mixed groups, respectively). Parasite ants with subpar nest maintenance ability that still participate in the task could be a case of “too many cooks in the kitchen.” If so, parasites losing the drive to participate in nest building and maintenance tasks could actually benefit the mixed parasitic colony.

## **Conclusion**

This study, along with host-removal behavioral studies conducted by other researchers, confirm that while domestic behaviors degenerate with increasing dulotic specialization, these behaviors can sometimes be recovered following removal of the host species (Kutter, 1969, as cited by Wilson, 1975; Mori & Le Moli, 1988; Stuart and Alloway, 1985; Wesson, 1940; Wilson, 1975). The degree to which recovery occurs seems to be a function of how specialized the parasite has become in the course of evolution from the free-living state to that of complete dependence on host workers (Stuart & Alloway, 1985; Wheeler, 1910; Wilson, 1975).

Dulotic specialization ranges from facultative parasites that possess all behaviors necessary to maintain a colony without host workers, to the highly specialized obligate parasites like those in the genus *Polyergus* that have lost nearly all domestic behaviors and are entirely dependent on the host workforce. Less specialized, or ‘primitive’, obligate parasites fall somewhere in the middle. In this study, the behavior of *F. subintegra* workers when isolated from host workers was comparable to other species that have been identified as primitive obligate parasites, namely *L. duloticus*, *H. canadensis*, *H. sublaevis*, and *H. americanus* (Stuart & Alloway, 1985; Wilson, 1975). Workers of *F. subintegra* generally recovered foraging and nest excavation behaviors when isolated from host workers. It is clear that its domestic abilities have degenerated beyond those of facultative parasites, but not as far as those parasites in the highly specialized *Polyergus* genus.

The nature of dulosis in the sanguinea group leads to the following question: Why have *F. subintegra* evolved into an obligate parasite, while their sanguinea group relatives have not? This is a complicated question that encompasses multiple areas of research, such as phylogenetics, physiology, biogeography, ecology, and behavior. It is interesting to note that *F. rubicunda*, a member of the sanguinea group, shares a habitat and a host species with *F. subintegra* (Talbot, 1985) but has not made the switch from facultative to obligate parasitism. In fact, a *F. rubicunda* nest containing host workers was found only 53 m from a *F. subintegra* nest containing the same host species. Both species would seem to have the same ecological pressures, yet *F. rubicunda* does not have an enlarged Dufour’s gland (Wilson & Regnier, 1971) and is still considered a facultative parasite (Wheeler, 1910). Another member of the sanguinea group, *F. pergandei*, does possess an enlarged Dufour’s gland and has a large proportion of host



workers in its colonies (Apple et al., 2014). Perhaps further studies may classify *F. pergandei* as a second obligate parasite in the sanguinea group.

This behavioral study is a necessary step in examining the only known crossover from facultative to obligate parasitism in the *Formica sanguinea* species complex. The species *F. subintegra* and its relatives can be used as models for studying the progression of dulotic evolution within a closely related group of social parasites. Phylogenetic studies have indicated that the *F. sanguinea* complex represents a monophyletic group, which suggests that dulosis evolved only once in the group (Romiguier et al., 2018). However, I am not aware of any phylogenetic studies that examine the relationships between the 12 species within the sanguinea group. Such a study, along with comparative behavioral and genetic studies between species in the group, could shed light on how and why *F. subintegra*, and other obligate dulotic parasites, may have crossed the threshold from facultative to obligate parasitism.

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## APPENDICES

### Appendix A. Treatment Order Charts

#### Foraging Experiment:

	Subgroup/Treatment	Treatment Order		
		22H:13P	0H:35P	0H:13P
Colony TA (Two Rivers)	TA1	3	1	2
	TA2	1	2	3
	TA3	2	3	1
Colony TB (Two Rivers)	TB1	3	2	1
	TB2	2	1	3
	TB3	1	3	2
Colony MA (Meramec)	MA1	3	2	1
	MA2	2	1	3
	MA3	1	3	2
Colony MB (Meramec)	MB1	1	3	2
	MB2	2	1	3
	MB3	3	2	1

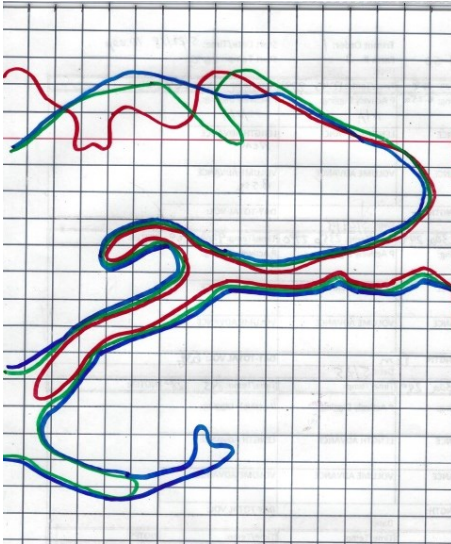
#### Nest Excavation Experiment:

	Subgroup/Treatment	Treatment Order		
		0H:50P	0H:25P	25H:25P
Colony TA (Two Rivers)	TA1	1	2	2
	TA2	2	1	1
Colony TB (Two Rivers)	TB1	1	2	2
	TB2	2	1	1
	TB3	1	2	2
	TB4	2	1	1
	TB5	1	2	2
Colony TC (Two Rivers)	TC1	2	1	1
	TC2	1	2	2
	TC3	2	1	1
	TC4	1	2	2
	TC5	2	1	1

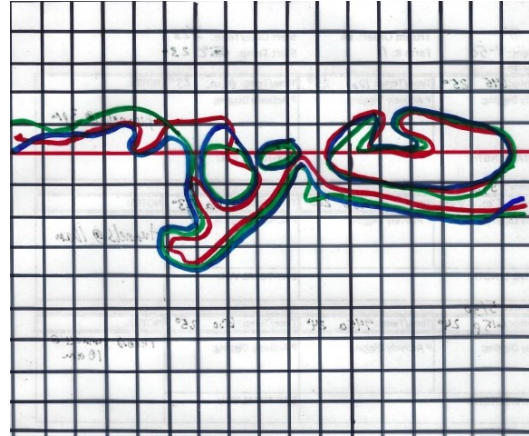
**Appendix B. Nest Excavation Tunnel Outlines (Red = Day 1; Green = Day 2; Blue = Day 3)**

**Host-Only Groups (50H:0P):**

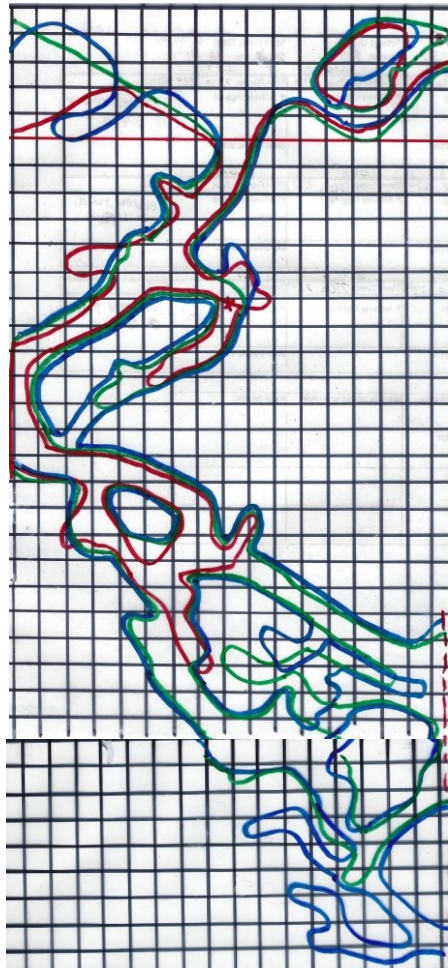
**(1)**



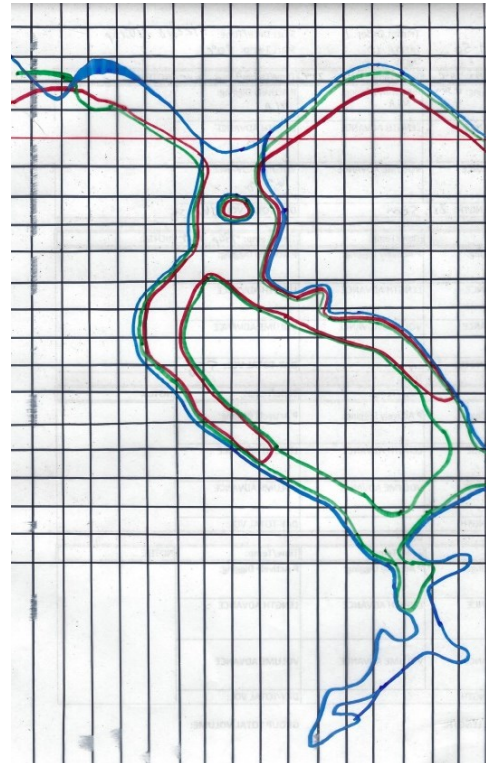
**(2)**



**(3)**

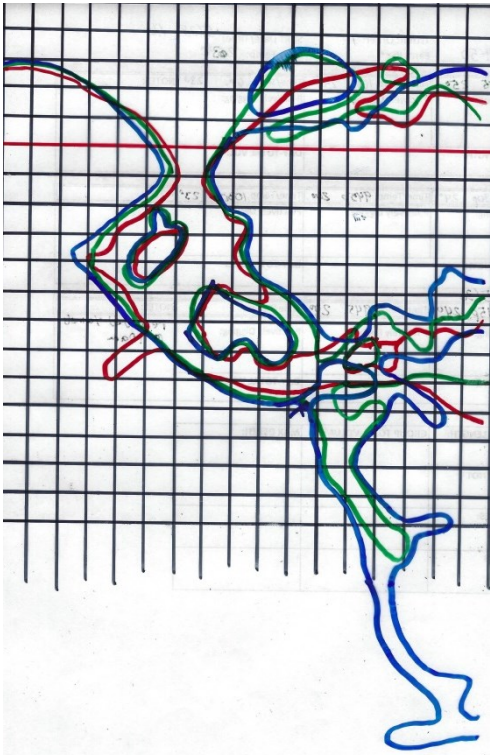


**(4)**

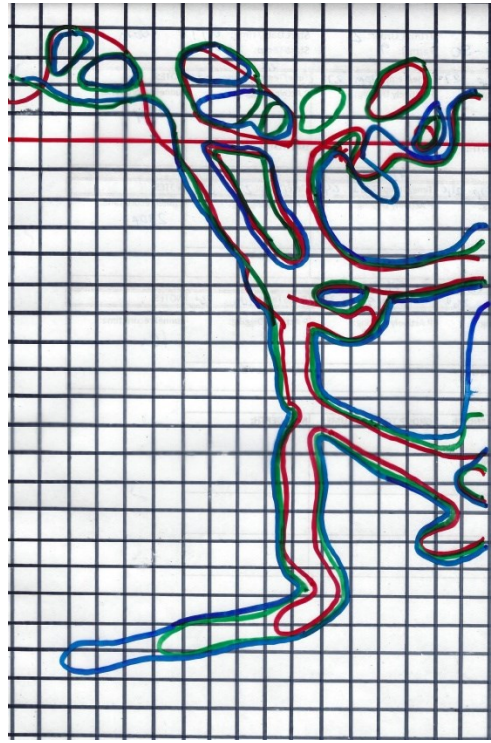




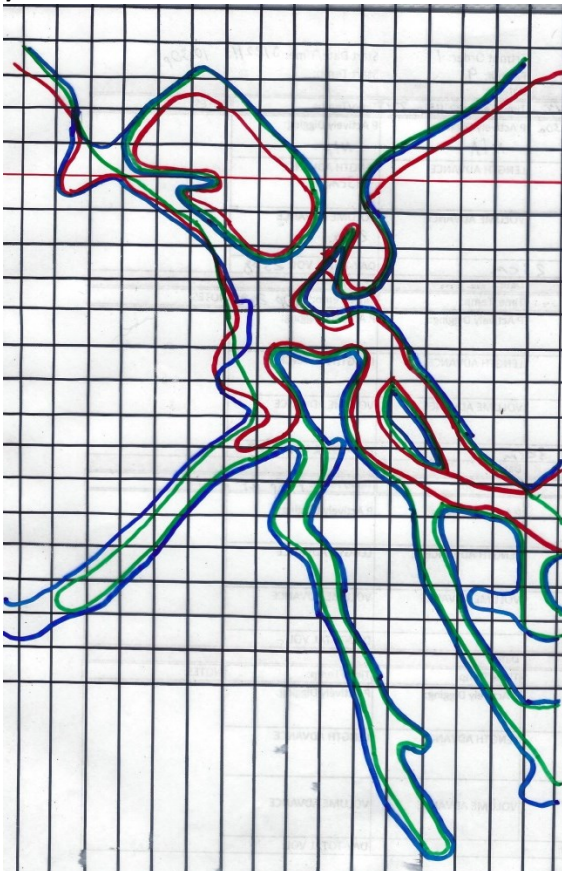
(5)



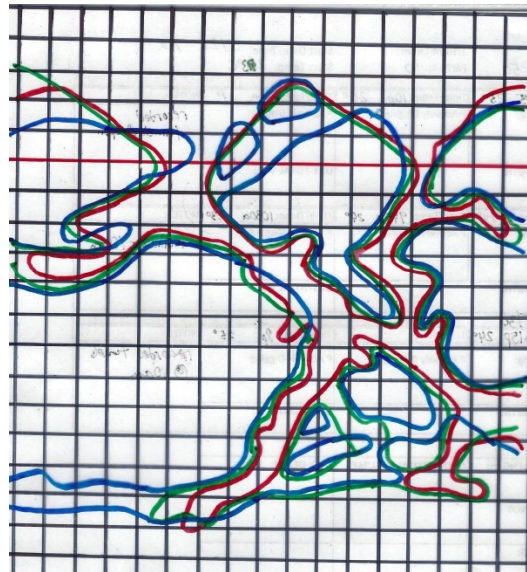
(6)



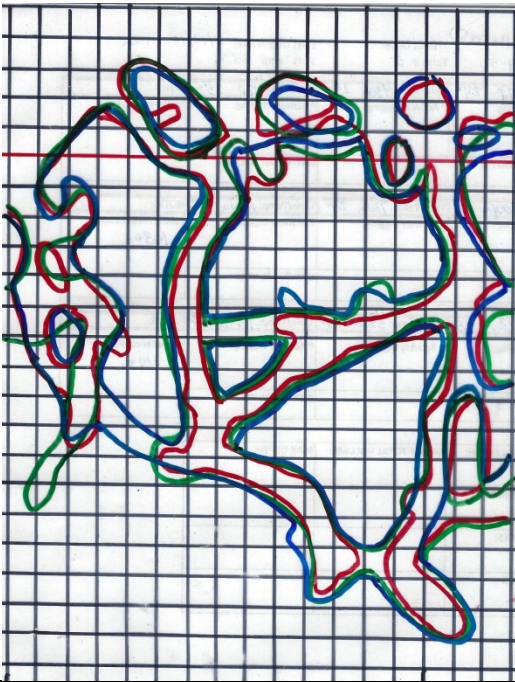
(7)



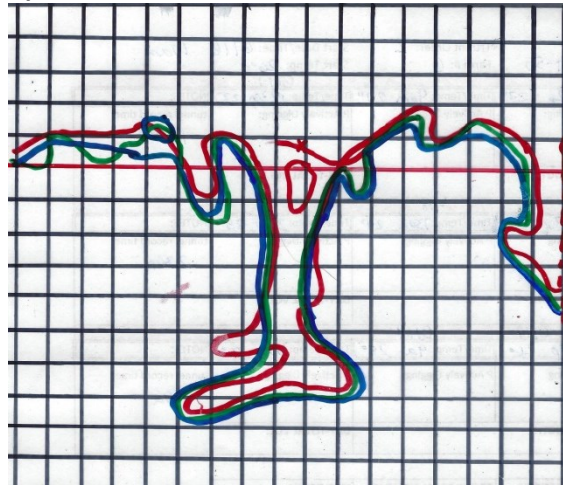
(8)



(9)



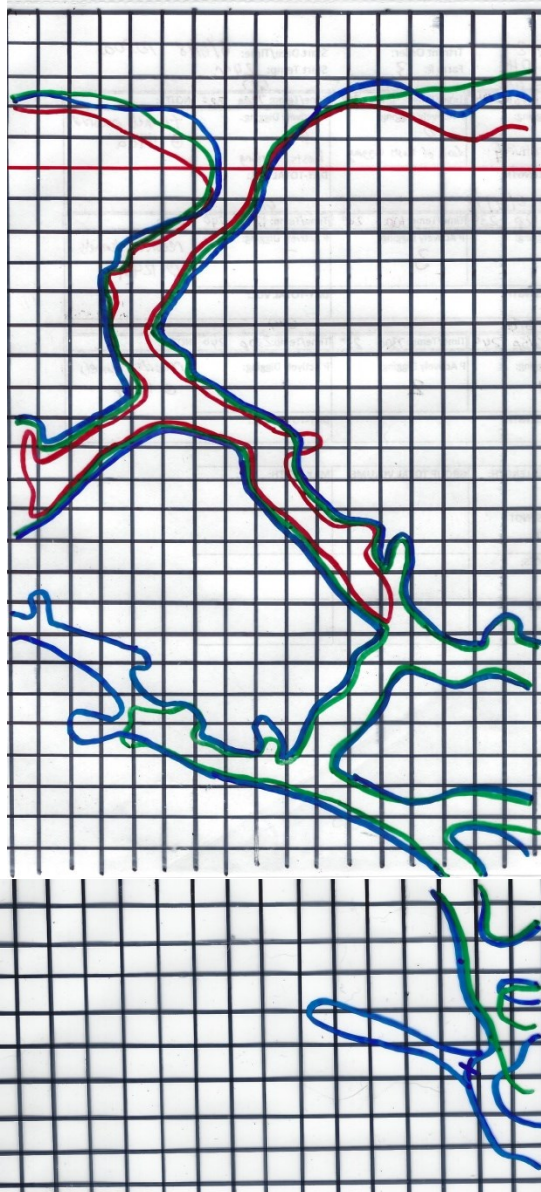
(10)



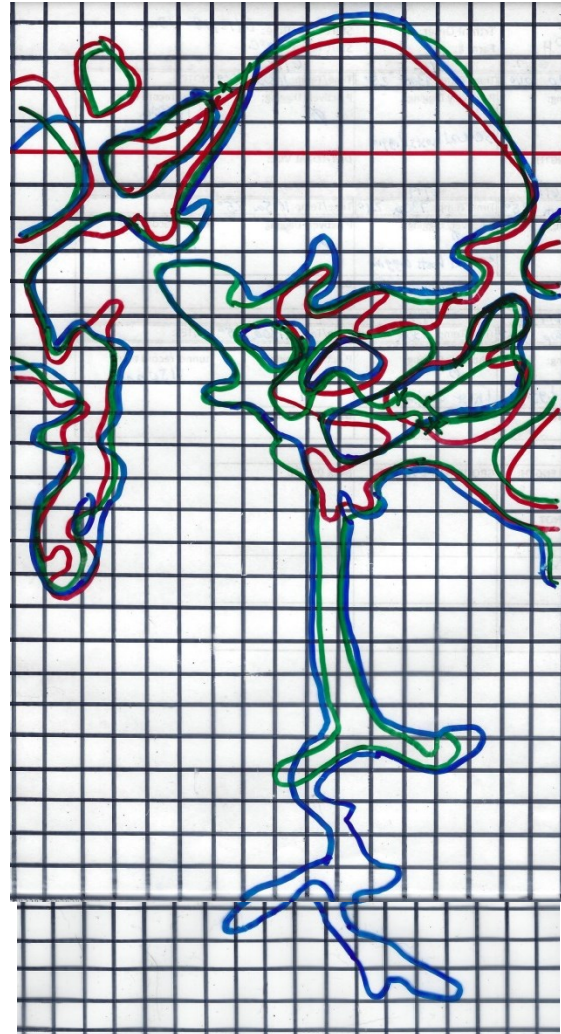


**Mixed Groups (25H:25P):**

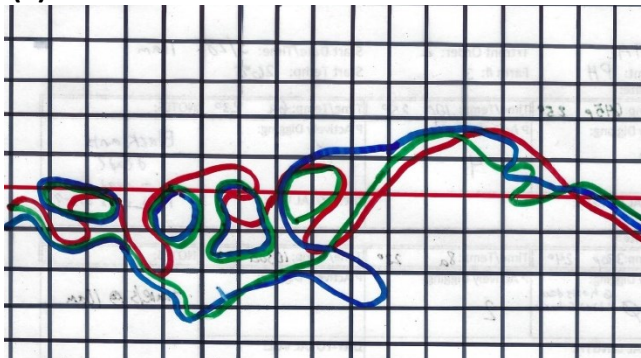
**(1)**



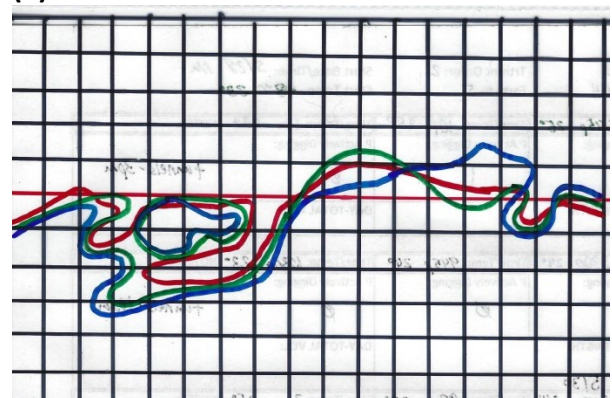
**(2)**



**(3)**

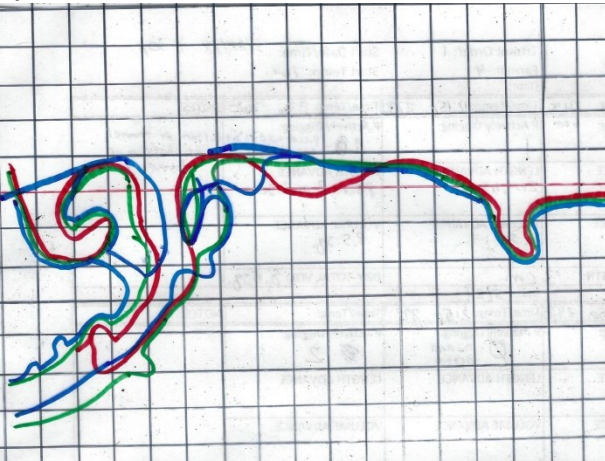


**(4)**

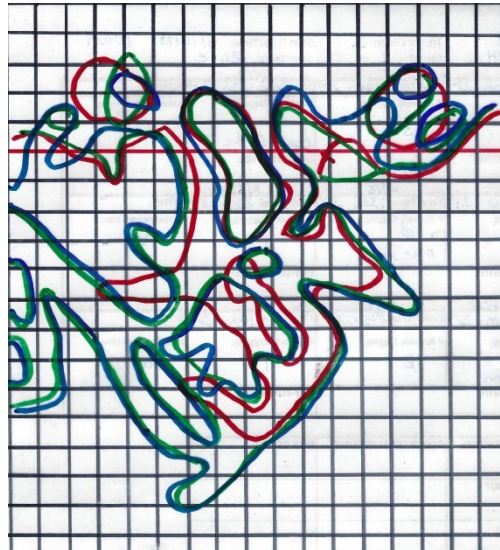




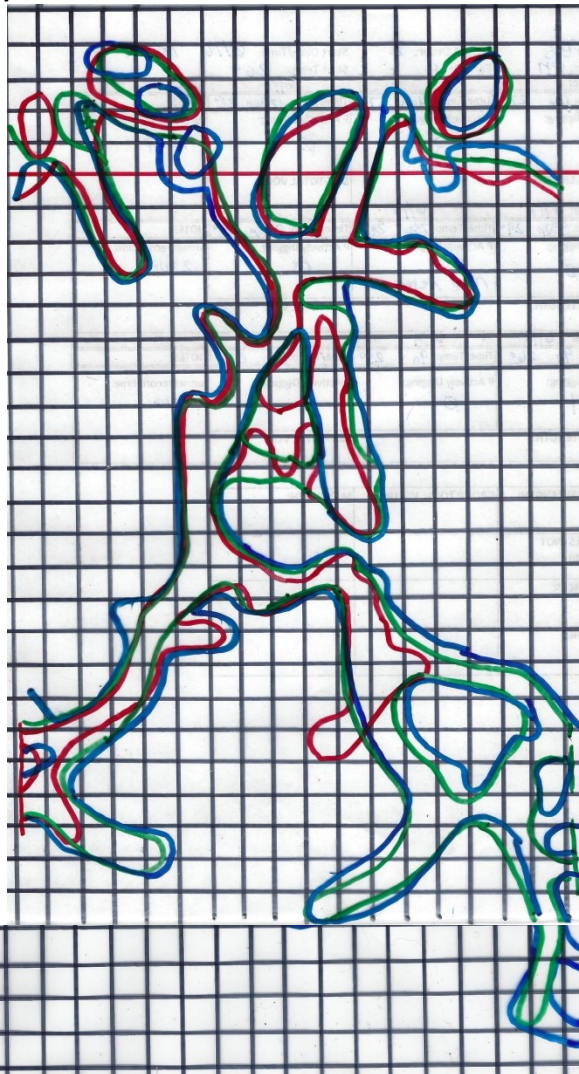
(5)



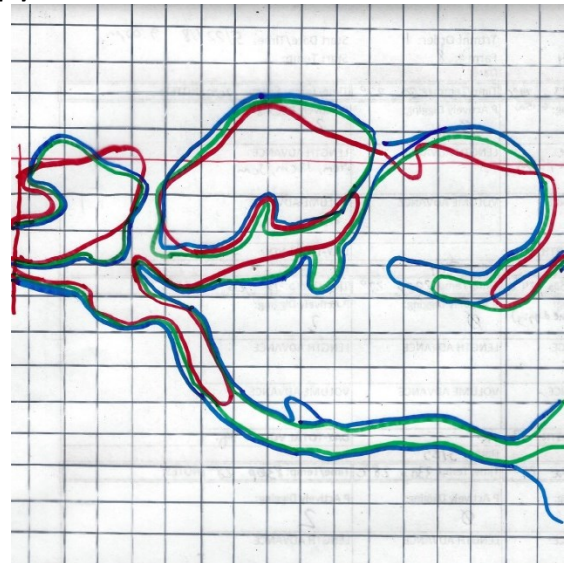
(6)



(7)

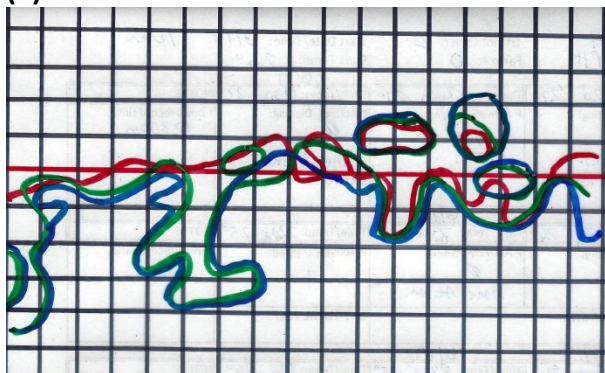


(8)

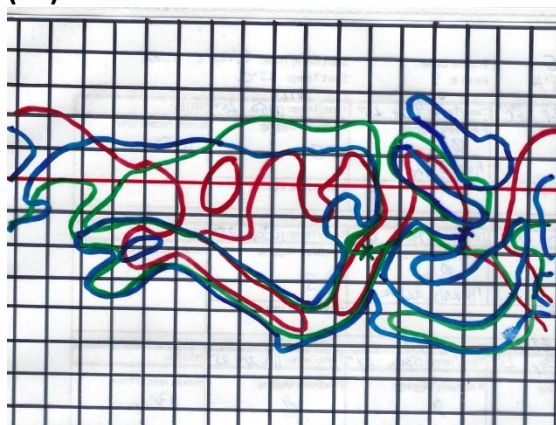




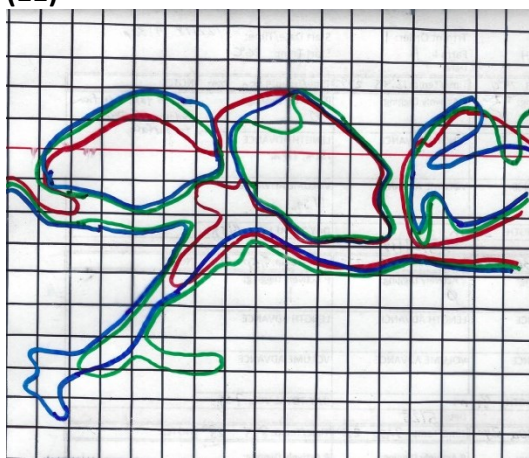
(9)



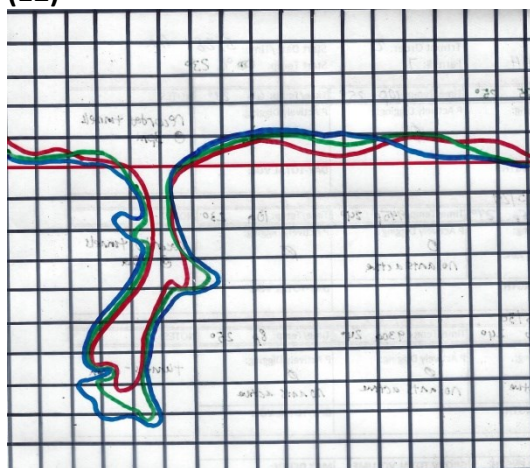
(10)



(11)

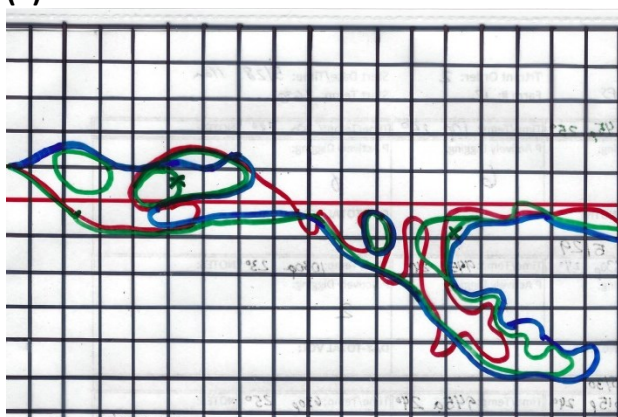


(12)

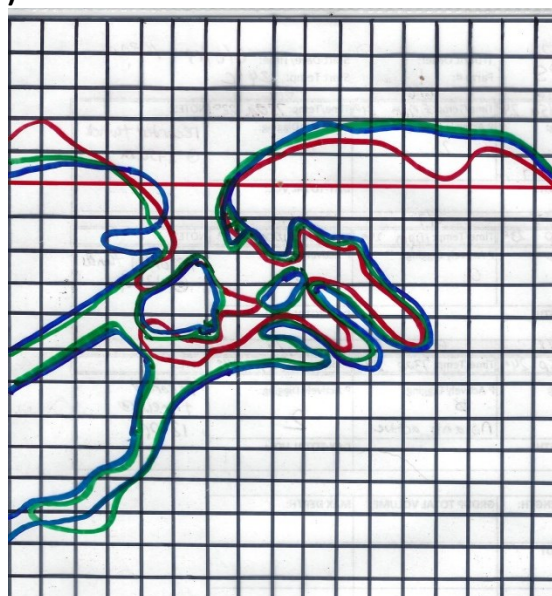


**Parasite-Only Groups (0H:25P):**

(1)

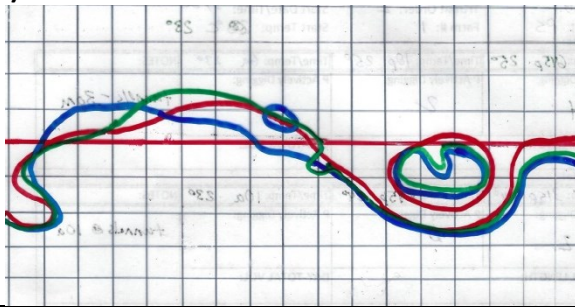


(2)

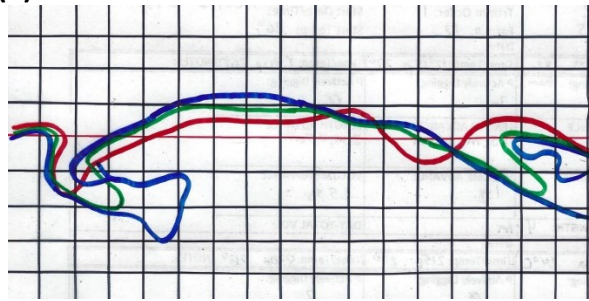




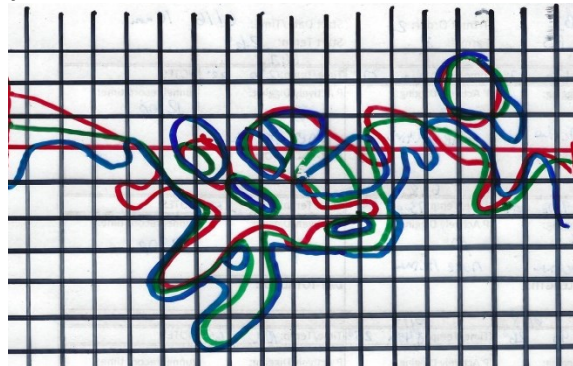
(3)



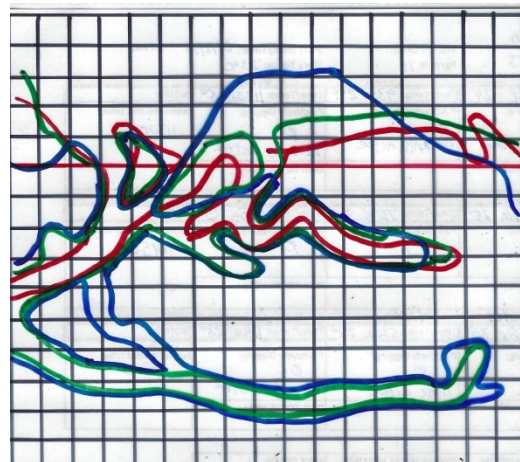
(4)



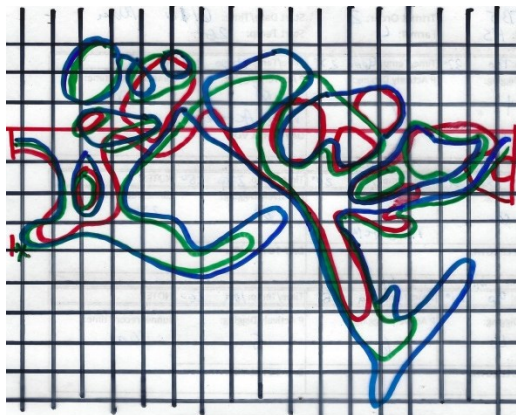
(5)



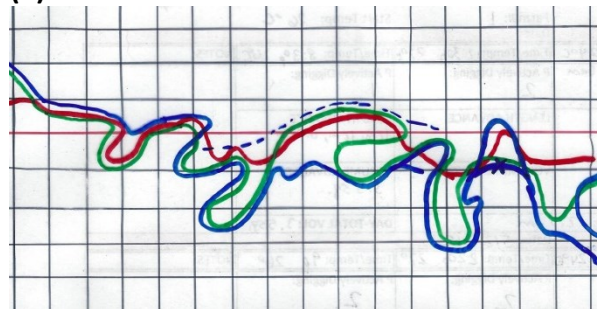
(6)



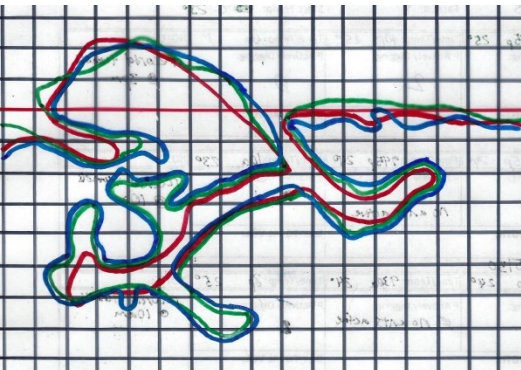
(7)



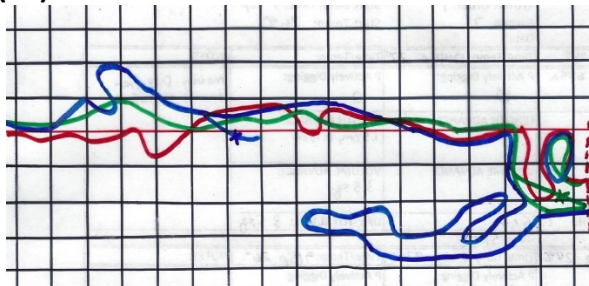
(8)



(9)

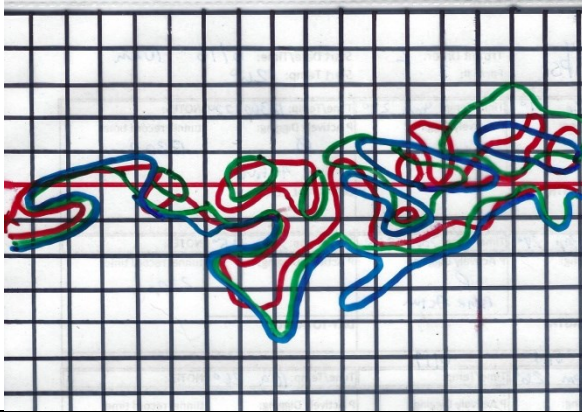


(10)

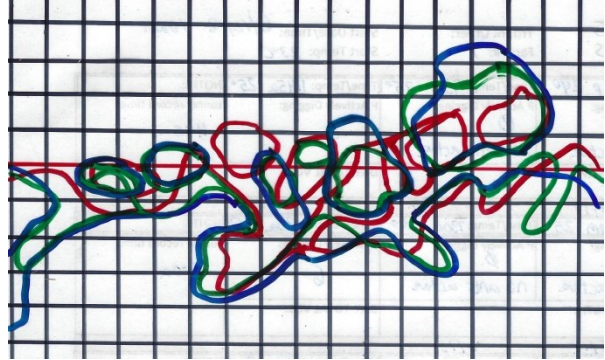




(11)

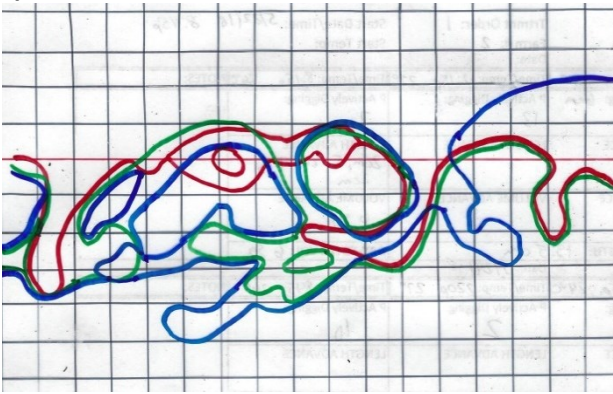


(12)

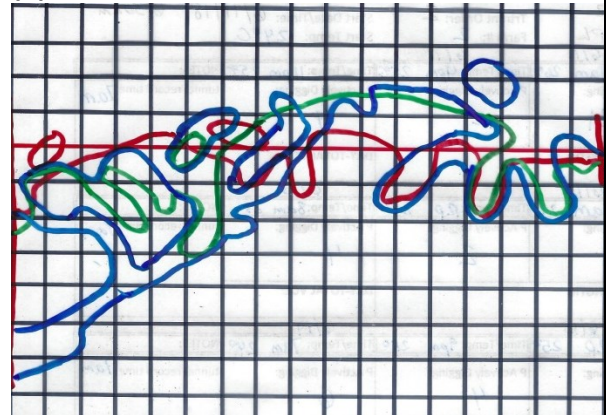


**Parasite-Only Groups (0H:50P):**

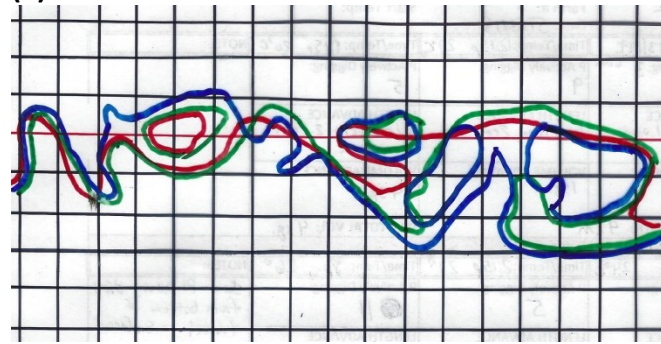
(1)



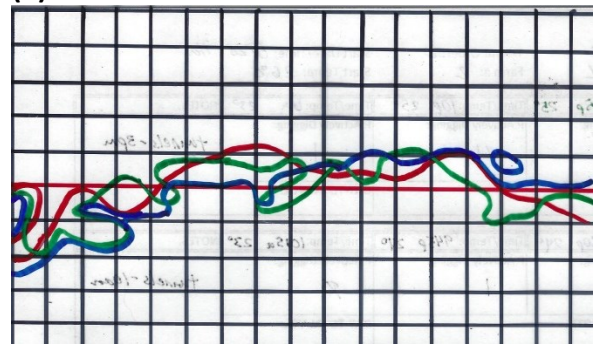
(2)



(3)

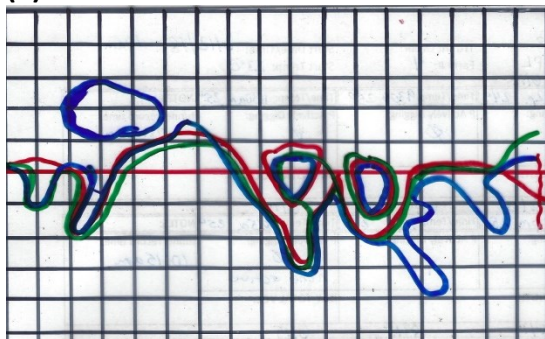


(4)

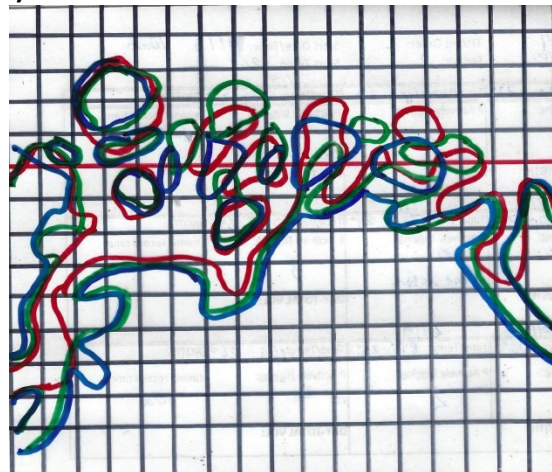




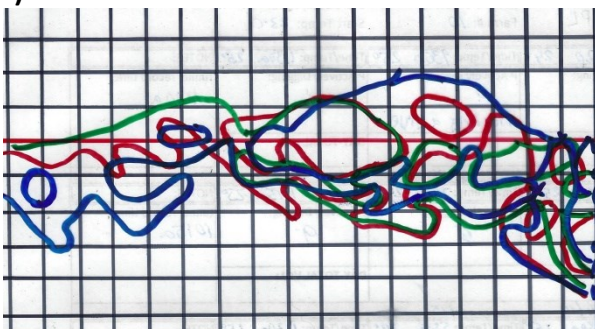
(5)



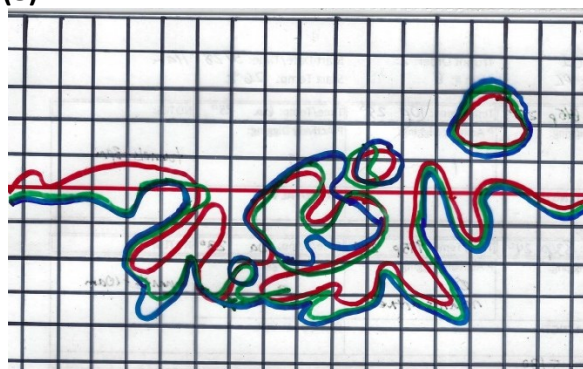
(6)



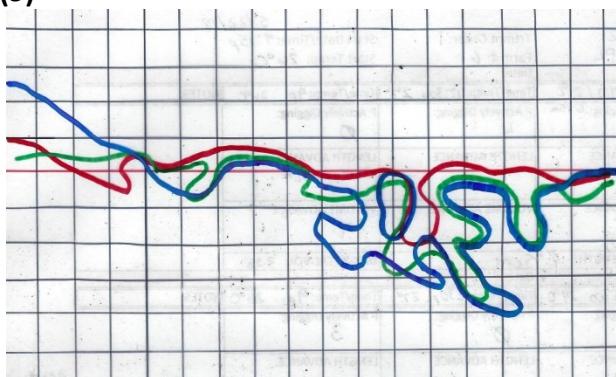
(7)



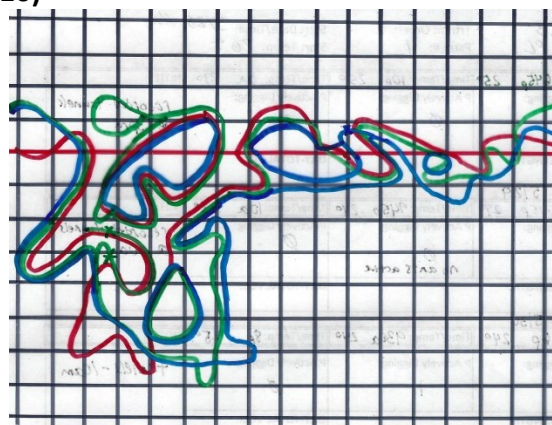
(8)



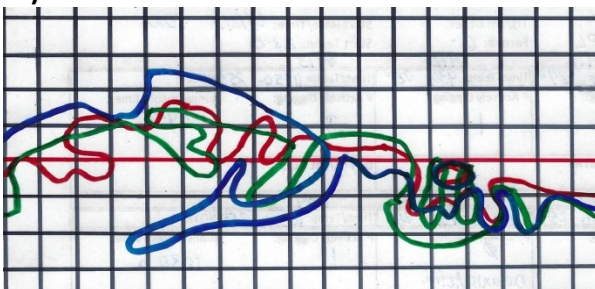
(9)



(10)



(11)



(12)

