Self-fertilization and Outcrossing in the Leech *Helobdella stagnalis*

Figure 1: Eggs developing internally

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Abstract

The relative tendency for the hermaphroditic leech, *Helobdella stagnalis* to outcross (cross-fertilization) or self-fertilize is poorly known. In order to establish whether *H. stagnalis* is capable of self-fertilization and to measure any possible deviations in offspring quality between mating methods, we noted differences in the second generation parent's caudal sucker sizes, time until egg appearance, brood size, progeny development, time until young detach from parent, and offspring survival between isolated individuals and mating pairs. Leeches were collected from Rio de Flag in Flagstaff, Arizona during August 2011 and their young either remained isolated or were paired that November. Statistics showed no significant difference in any of the variables measured between paired and isolated treatments. Results indicate that self-fertilization is a possible reproductive pathway, and suggests that out-crossed offspring do not differ from those produced from self-fertilization. However, the trends produced imply that with increased sample size, significant differences may appear between mating methods. Further experimentation will be conducted to assess parentage of the offspring using molecular markers to verify whether isolated individuals were previously outcrossed or are true self-fertilizers. The importance of research such as this includes not only providing a better understanding of these leeches, but a better comprehension of mixed mating systems, parental care, and their evolution.

Introduction

Helobdela stagnalis is a small freshwater leech known for providing parental care for its young. As described by Kutschera and Wirtz (2001), *H. stagnalis* is a grey two-eyed flat leech belonging to the family Glossiphoniidae. This particular leech species is typically known to reproduce no more than twice a year, influenced by the seasonal changes in temperature (Tillman et al. 1972). Once sexually mature, the hermaphroditic leech will tag all other neighboring leeches with spermatophores (Govedich, 2004). After the body absorbs the spermatophore, an egg-string freely floating within the ovisac becomes fertilized and develops internally until the egg is ready to be secreted (Sawyer, 1986) (Fig. 1). The leech then lays the eggs in a series of soft, translucent cocoons which the leech fixes simultaneously to its ventral side, thus allowing the parent to provide protection while the young continue to develop (Mann, 1957; Sawyer, 1986) (Fig. 2). After hatching, the young leeches attach themselves to the ventral side of the parent where further development occurs (Fig. 3). In this stage the parent will hunt for and feed its young in addition to providing protection and ventilation. The curious life history of *Helobdela stagnalis* has been carefully examined, generally focusing on their phenomenal behavior as a parent. In turn, the results are larger young once detached from the parent and presumably increased survival (Kutschera et. al, 2001). However, a vital characteristic required to completely understand these leeches has been overlooked, self-fertilization.

With any hermaphroditic organism, the occurrence of outcrossing (cross-fertilization) verses self-fertilization becomes a major theme. Govedich (2004) recorded instances of isolated *H. stagnalis* individuals yielding broods and suggested that isolated leeches would only self-fertilize in situations where a reproductive partner was not available after an extended period of time. Similar observations in the production of offspring from isolated laboratory individuals have been seen in *Helobdela papillornata,* after prolonged separation from potential mates (Tan et. al, 2004). However, no further research on this alternative mating behavior has been conducted in order to address its importance in the life history of these leeches.

On the basis of such a lack of information, this experiment was designed in order to establish if *H.stagnalis* is capable of self-fertilization and the consequences of this alternative mating behavior on parental care. The differences tested will be represented by the caudal sucker size of the second generation parent(s), days until the brood appears, brood size, brood development, days until detachment of progeny, and brood survivability. Additionally, investigating the benefits of outcrossing as compared to self-fertilization is greatly important when trying to understand the evolution of mixed mating systems (Christen et. al, 2002; Thornhill, 1993). Self-fertilization, after all, is the most extreme example of inbreeding, comprising of the fusion of gametes produced by the same individual. The relatedness between these offspring and their parent is greater than that between outcrossing young, resulting in dramatically reduced genetic variation (Christen et. al, 2002; Charlesworth, 1980; Charlesworth et. al, 1998; Lively et. al, 1990). Thus, this is depicting the inferiority of self-fertilization to outcrossing broods. Therefore, if *H. stagnalis* uses self-fertilization as a means for reproduction in the absence of mates, then a difference between self-fertilization and cross-fertilization reproductive cycles will be present.

To address these hypotheses, leeches were collected from the ponds of the snowmelt fed Rio de Flag River in Flagstaff, Arizona. This river is formed by the outflow of The Rio de Flag Waste Water Treatment Plant, known to contain endocrine disruptors. *H.* stagnalis can be found in the wetlands near this outflow, typically on the undersides of rocks.

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Basic Research

Methods

Wild leeches with broods were collected from the Rio de Flag River on August 9th 2011. Following their collection they were separated into individual specimen cups and the progeny were allowed to develop until detachment. After a minimum of seven days from their detachment and a change in pigmentation, the progeny were removed and individually separated into specimen cups. On November 14th, the second generation was then reorganized into the treatments. Leeches were randomly placed into two mating treatments, isolated (treatment 1) and paired (treatment 2). Paired mating consisted of a nonrelated mating pair, while the isolated individuals remained solitary and unpaired for the duration of the experiment. All leeches were observed daily until March 1st 2012, recording all observations.

The observations recorded included parental caudal sucker size, the appearance of a brood, the time until egg presentation, brood size, progeny development, and days until offspring detachment from the second generation parent. The caudal sucker size of the second generation leeches were measured by a reticle to the nearest tenth of a millimeter at 3x magnification before pairing occurred. Daily observations allowed for the time until egg appearance to be measured from pairing until the first sighting of egg development. Brood size was quantified by daily offspring counts of attached, detached, removed, and dead progeny. The sum of the removed and dead progeny was used to assess the total brood size for each leech. The developmental stages of the progeny in each brood were separated into eight categories based on size and characteristics. The stages included eggs (stage 1), mature eggs (stage 2), recently hatched progeny (stage 3), very small progeny (stage 4), small progeny (stage 5), normal progeny (stage 6), large progeny (stage 7), and detached progeny (stage 8) (Fig. 4). The sizes referred to in each stage were based off relative sizes in comparison to the parent. The stages of the offspring were assessed daily and average time (days) per stage was calculated. Daily observations allowed for an assessment of time until progeny detached from the second generation parent by measuring the number of days from initial egg development until all the offspring detached. The survivability was evaluated firstly by determining the total number of progeny alive after 32 days, and then survival was assessed through brood counts at different time periods. These brood counts were conducted after hatching, and every week after hatching for nine weeks.

In order to assess the frequency that broods were produced and if self-fertilization occurred, the proportion of broods produced versus no broods produced was determined for each treatment and graphed (Fig. 5). Wilcoxon signed rank tests were used to analyze the time until egg appearance, days until progeny detachment, and brood sizes between paired and isolated leeches. Brood size was also analyzed against the second generation parental caudal sucker size using a regression test in order to eliminate leech size as a determining factor for the number of offspring produced. MANOVA was used to analyze the survival of offspring between paired and isolated leeches. Finally, a Principle Components Analysis (PCA) test was conducted between paired and non-paired individuals to compare the relationship between all variables.

Figure 4: (Right) Each number represents a different stage in the progression of the young while attached to the parent, with the exception of the last stage (8). Here the last stage represents relative general size of recently detached young. Stage one, egg, consists of numerous small to medium sized eggs with a red or dark cream center and a light transparent outer sphere or coating. Stage two, mature eggs, is represented by a large egg that rapidly progresses. A secondary stage in this category is characterized by the elongating and curving of the dark inner mass. It is apparent that this mass is becoming the leech's internal organs. Partial hatching of the cocoon may also occur in the latter part of this stage. In stage three, recently hatched progeny, the cocoon is gone and the young are fixed the mother's ventral side. The young rapidly progress through this stage as the dark organ containing mass continues to elongate along with is outer encasing, or coelom and skin. In this stage the leeches begin to develop their segmentation. In stage four, very small, the leech begins to take a recognizable form and cephalization has taken place with the development of eyespots. In the following stages development continues to occur mainly in the organisms size, coordination, and mobility.

Results

No statistically significant relationships between caudal sucker size and brood size ($F_{[1,0.05]}$=1.29, p> 0.05). There was no significant difference in the time until egg appearance between paired and isolated individuals ($W_{[1, 0.05]}$ = 1.13, p > 0.05)(Fig. 6). Brood sizes yielded no significant difference between paired and isolated individuals ($W_{[1, 0.05]}$ = 0.46, p > 0.05) (Fig. 7). No significant differences in the number of days until progeny detached from the parent were seen between paired and isolated individuals ($W_{[1, 0.05]}$ = 2.32, p>0.05) (Fig.8). Survival of offspring among the treatments also yielded no significant differences ($F_{[1,0.05]}$=0.07, p> 0.05)(Fig. 9). The averages for the observed variables of each treatment were calculated, producing an average standard error of 2.53 across all variables (Fig. 10). The PCA test also generated non-significant results (PCA: p=0.16, p>0.05)(Fig. 11).

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		Mean		Standard Error	
		Treatment 1 (N=3)	**Treatment 2** (N-10)	**Treatment 1** (N=3)	**Treatment 2** (N=10)
Caudal Sucker Size (mm)		9.67	8.70	1.70	1.03
Gestation Period (days)		68.33	65.20	2.40	1.32
Brood Size (individuals)		7.67	17.30	7.25	3.97
Time until Detachment (days)		12.67	20.10	3.51	1.92
Developmental Stages (days):	**1**	0.31	1.69	2.87	2.87
	2	0.09	0.41	0.67	1.10
	3	0.09	0.31	0.00	0.00
	4	0.19	0.38	2.16	1.02
	5	0.31	0.50	2.05	0.87
	6	0.44	0.81	0.94	2.69
	7	0.41	1.56	4.19	5.04

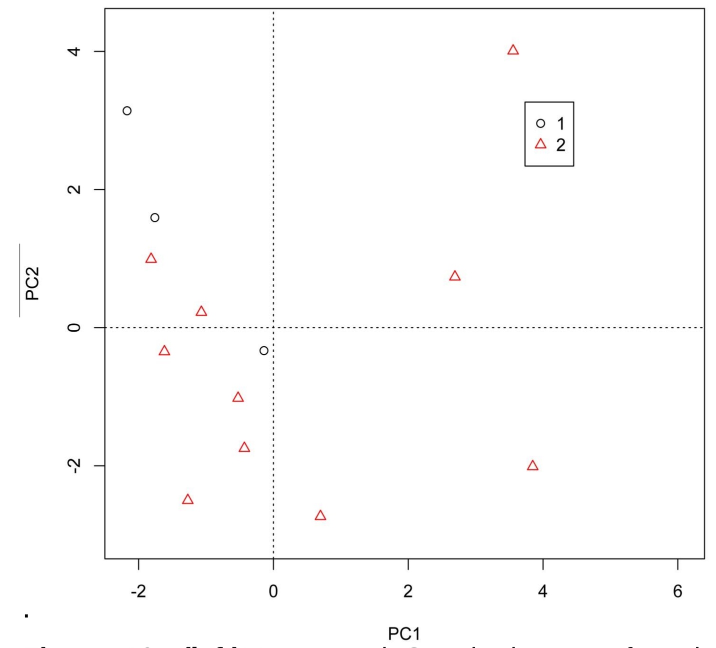


Figure 10: (left) Means and Standard error of each variable for each treatment
Figure 11: (Above)PCA Test against all variables between paired and isolated leeches

Discussion

During the course of this experiment 33% of isolated specimens yielded broods, which in turn supports both the observations of Govedich (2004) and Tan et. al (2004), as well as our initial hypothesis suggesting self-fertilization does occur in *Helobdella stagnalis*. Even though isolated leeches produced broods, paired individuals produced more broods overall. Tan et. al (2004) described that as group size increased so did testisac volume, which may explain the trend found in brood production. It is then inferred that the amount of sperm present increases in groups, allowing for a heightened production of broods in mating pairs.

The establishment of self-fertilization then requires the assessment of differences between mating methods. In analyzing adult caudal sucker size, time until egg appearance, brood size, progeny development, days until offspring detachment, and brood survival, there were no statistically significant differences found. These results not only support our secondary hypothesis but suggest there is no difference in parental care between treatments. Parental care however, is not calculated behaviorally in this case but rather is a preliminary and indirect measure of the parent's investment through reproductive effort and offspring survival. A lack of differences in the time until egg appearance suggests that Govedich's (2004) initial idea of only prolonged separation, relative to those in mating groups, would yield self-fertilized broods, may no longer be an accurate description. Furthermore, this research is promising in furthering the understanding of mixed mating systems, parental care, and the evolution of self-fertilization and parental investment.

Additionally, the graphs developed show subtle trends suggesting that brood size is greater in paired leeches, the latency period is greater for isolated leeches, and the time until progeny detachment is greater for paired leeches. These trends might suggest that with an increased sample size, differences between these traits may be statistically significant. Therefore, further collections of wild leeches and continued observations of all remaining lab specimens will be required in order to more effectively evaluate and compare treatments.

Furthermore, in conducting this experiment it was assumed that paired individuals do in fact outcross and are not self-fertilizing. *H. stagnalis'* tendency to be highly social and clump in nature, especially during breeding periods (Beresic-Perrins, 2010), supports the use of this assumption. In addition, it was assumed that these leeches do not store sperm, and if sperm storage does occur then it was also assumed that they were isolated before sperm storage could occur. Yet no previous literature has investigated the possibility of sperm storage in *Helobdella stagnalis*. However, to verify if true self-fertilization is actually occurring among isolated individuals and mating pairs do indeed outcross, paternity testing will be required. Initially the use of Amplified Length Polymorphisms (AFLP) was suggested due to the lack of any known microsatellites in *H. stagnalis*. Regrettably, AFLPs are highly discouraged for paternity testing and further research is required to find an alternative method. If there is no difference between self-fertilization and cross-fertilization besides genetic diversity, then why do these hermaphrodites outcross?

In the future, behavioral research will be conducted in order to completely and directly address parental care and possible differences between paired and isolated mating treatments. This experimentation includes 24 hour recordings of mating experiments and parent-offspring interactions between mating treatments (Lopez-Oehler, poster 251).

Figure 5: Percent of Brooding and Non-Brooding Individuals Present in Each Treatment

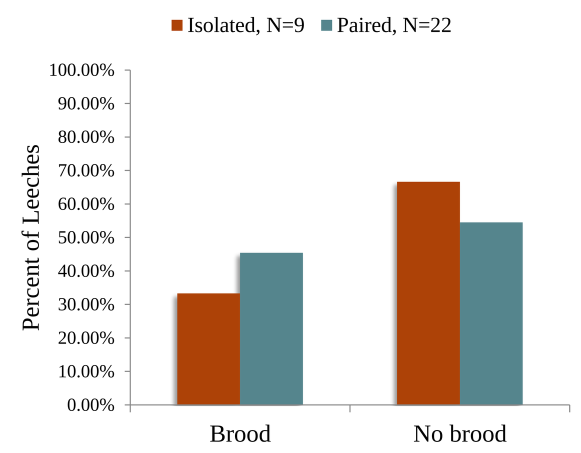


Figure 6: Average Time until Egg Production Between Treatments

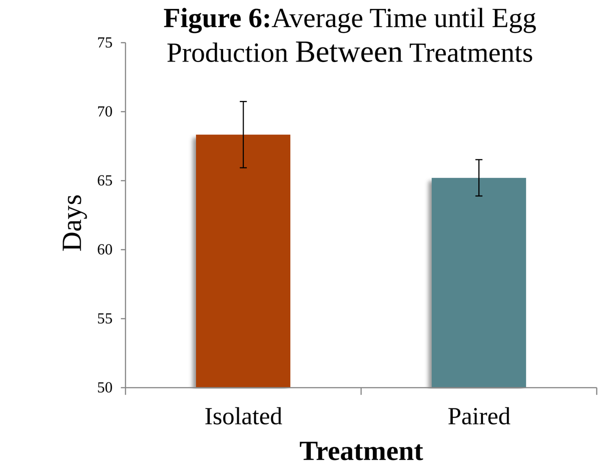


Figure 7: Average Brood Size for Each Treatment

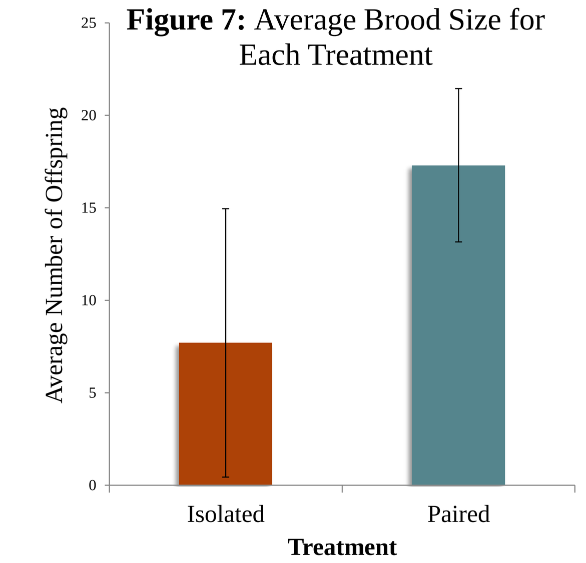


Figure 8: Average Number of Days Until Progeny Detachment

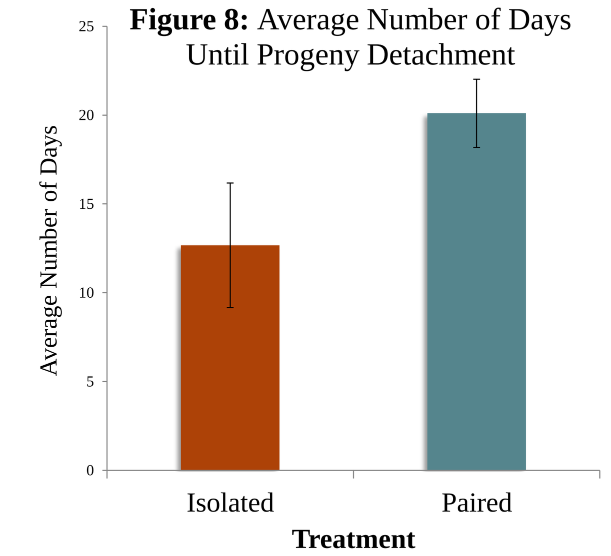
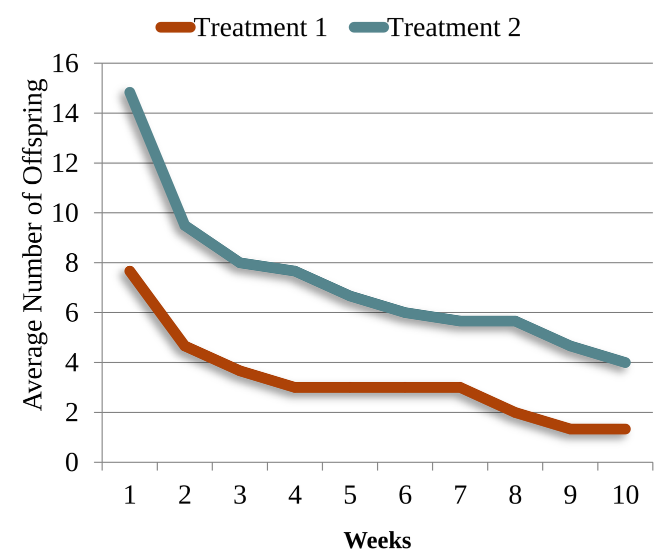


Figure 9: Average Survivorship Between Isolated and Paired Individuals



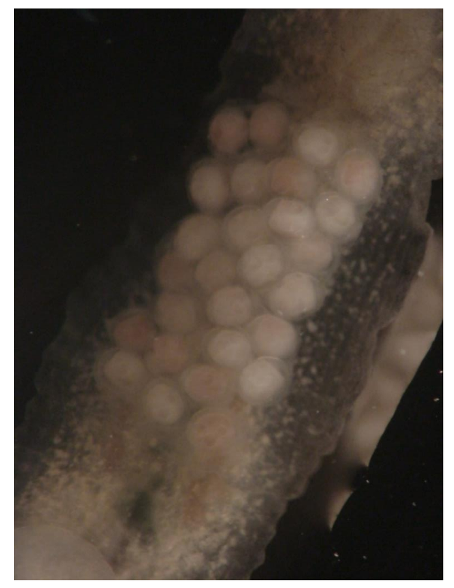


Figure 2: Eggs in cocoons attached to ventral side.

Figure 3: Young progeny fixed to the ventral side.