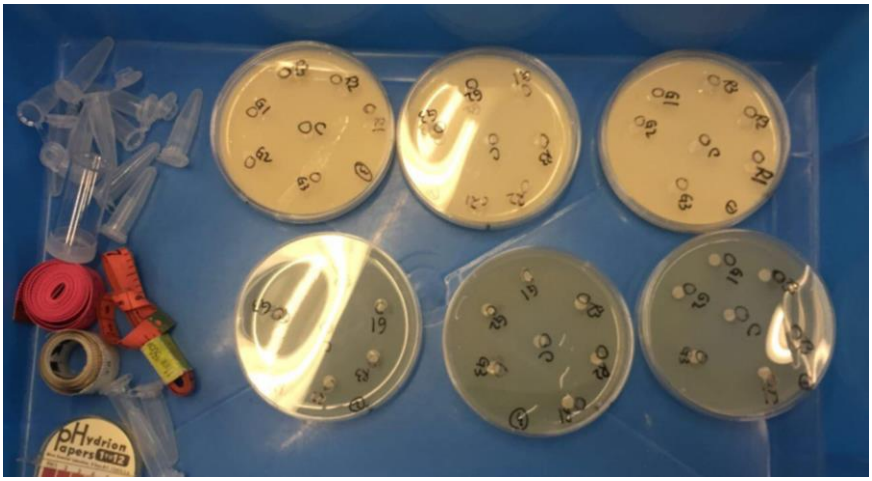

The Mysterious World Inside a Pitcher Plant (*N. mirabilis*)

SEARCHING FOR NATURE STORIES 2018

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CONTENT

1. ABSTRACT

Nepenthes mirabilis (Lour.) Druce is a species of pitcher plants which captures insects for additional nutrients, enhancing its survival at a place with low nutritional value. With a bright colour and alluring nectar, insects are attracted to and trapped inside pitchers with slippery walls. These preys are soon digested by the juice released by the pitcher gland. In contrast to the unique carnivorous behavior though, it is interesting that mosquito is a rare species that can live within the pitchers and lay eggs on the digestive juice without being digested.

The objectives of the current study were to study the digestion of prey by *Nepenthes mirabilis and* the relationship between *Nepenthes mirabilis* and mosquitoes. By measuring the weight change of egg white and crickets placed in *N. mirabilis* for days, as well as observing and estimating the larval density in digestive juice of *N. mirabilis*, it was hoped that more understanding on its digestive mechanism among different pitcher plants and how do the mosquitoes interact with *N. mirabilis* in the environment could be gained.

It was found that the digestion rate of *Nepenthes mirabilis* had no correlation with its developmental stage, pitcher length, number of insects inside and presence of mosquito larvae. The digestion rate of crickets of wild *Nepenthes mirabilis* was found to be 0.0133g/day. Complete life cycles of mosquitoes found confirmed that *Nepenthes mirabilis* is a breeding ground for mosquitoes. It was found that live larvae could survive without being digested but dead larvae would be digested. But the survival rate of mosquito larvae was found to be low, at only 0.24%.

2. INTRODUCTION

2.1 Introduction to the research topic

Nepenthes mirabilis (Lour.) Druce is an insectivorous plant which is commonly known as pitcher plant. Unlike other autotrophic plants, *N. mirabilis* is famous for feeding on insects. The ability to feed on animals which sets it apart from other plants was the result of evolution, enabling it to survive on soil lacking nutrients, especially nitrogen.[1] *N. mirabilis* evolved its special digestive systems many years ago. How *N. mirabilis,* whose distribution being highly localized in Hong Kong, adapt to environmental conditions in where they are found and how efficient its digestive mechanism allows it to gain competitive advantage *in situ* were unknown. Furthermore, despite the ability to digest insects, *N. mirabilis* is well-known for being a site for mosquitoes to lay eggs. Not only is *N. mirabilis* not killing the larvae, it may even provide a safe and nutritious growing environment for them. The current study attempts to find out the relationship between the common pest and the carnivorous plant.

[1]How did some plants become carnivorous? http://www.bioinformaticsbarcelona.eu/news/43/how-did-some-plants-become-carnivorous Retrieved September 8, 2018

2.2 Objectives

1. To study the content of digestive juice of *N. mirabilis*
2. To study the digestive rate of *N. mirabilis* on selected substrates
3. To study the symbiotic relationship between *N. mirabilis* and mosquito

2.3 Research Questions

1. How effective are the protease and chitinase in *N. mirabilis*?
2. How does the digestive capacity of *N. mirabilis* relate to its different developmental stages?
3. Is the environment inside pitchers favorable for growth of larvae of mosquitoes?

2.4 Background information of *Nepenthes mirabilis*

2.4.1 Classification

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Nepenthales
Family	Nepenthaceae
Genus	Nepenthes
Species	*Nepenthes mirabilis*

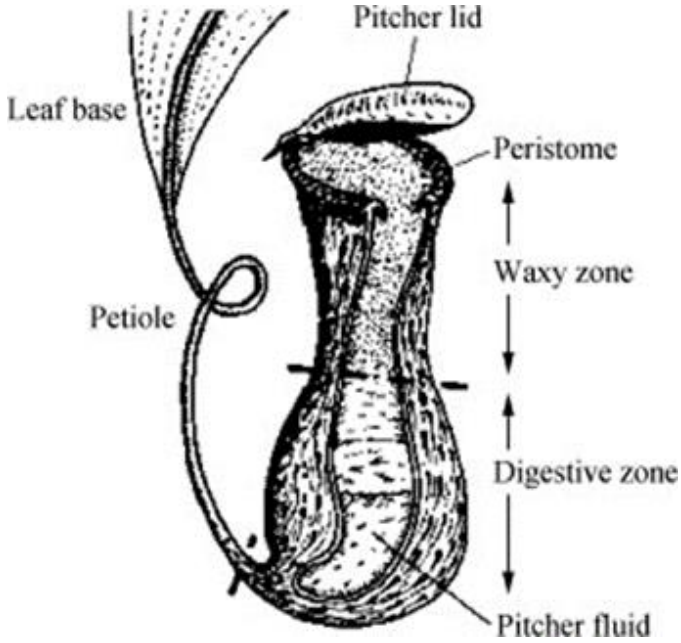

Figure 1 Taxonomy and Structure of *Nepenthes mirabilis*

Nepenthes mirabilis possess specialized leaves forming pouch-like “organs” called pitchers that function as pitfall-traps. They consist of several structures, including a leaf-like lid, a collar-like peristome, a slippery waxy zone and a digestive zone. These pitchers are filled with a digestive fluid that is generated by the plants themselves. *Nepenthes* plants produce various enzymes in order to digest caught prey in their pitchers. Pitchers can be found in the wild, they are also commonly planted indoors. The pitchers can be divided into two groups, red ones and green ones: pitcher with higher intensity of red pigments are more mature ones while those green ones are younger counterparts.

3. METHODOLOGY

3.1 Field Study

Date	Time	Venue	Event
25-03-2018	09:00 – 14:00	Tai Tong Country Park	Search and locate the distribution of *N. mirabilis*
01-05-2018	10:00 - 13:00	Tai Tam Country Park	Search and locate the distribution of *N. mirabilis*
03-08-2018	11:00 - 15:00		Collection of sample of digestive juice of *N. mirabilis.* On-site experiment Collection of larvae found inside the pitchers
05-08-2018	09:00 - 13:00		
22-08-2018	09:00 - 12:30		
23-08-2018	09:00 - 13:00		

A site in Tai Tam Country Park (Fig. 2) was chosen because of the greater number of *N. mirabilis* and its accessibility. It was located near a freshwater stream (Fig.3).

Two series of experiment were done. In each series, six pitchers, including three red ones and three green ones, were chosen for collection of digestive juice and investigation of digestive behaviour. One cricket and a cube of egg white were put in each pitcher. They then were picked up and weighed on the next day. Crickets were used [2]so as to simulate natural prey of *N. mirabilis,* which have high protein level, and considerable amount of chitin in their exoskeleton. Egg white was used to represent the protein of an insect without an exoskeleton, so the digestion of protein can be observed more easily. The insects inside the pitchers were also counted.

Figure 2 The red dot indicates the study site

Figure 3 Overview of the site

[2] Lowering chitin content of cricket.
https://scialert.net/fulltext/?doi=pjbs.2017.523.529

3.2 Objective 1:To study digestion of prey by *N. mirabilis*

3.2.1 Introduction

N. mirabilis secretes scentless nectar to attract insects like ants and beetles. It then glues the insects using the sticky fluid secreted by the small gland at the tentacle.[3] Lastly, the insects are digested by enzymes present in the soup of digestive juice.

Bodies of typical preys of *N. mirabilis* contain high protein contents (e.g. about 197g per kg in house flies[4]). Exoskeletons of these preys are rich in the modified polysaccharide chitin. In order to obtain nitrogen from protein, it is necessary for *N. mirabilis* to digest the exoskeleton and body tissues of insects by the action of proteases and chitinases. Therefore, we would also like to investigate the digestion rate of *N. mirabilis* on protein and chitin. We are curious about the difference in digestion rate between young and mature *N. mirabilis*.

3.2.2 Hypothesis

Young *N. mirabilis* produce and secrete a higher level of digestive enzymes than more mature ones, so as to facilitate the digestion of insects at a higher rate, allowing a higher growth rate among younger pitchers.

3.2.3 Principles of experimental design

Experiments were carried out to study the digestive rate and effectiveness of enzymes of *N. mirabilis*. The experiments were carried out both in the site and in the school laboratory.

1. Determining the effectiveness of enzymes by measuring the size of clear zones on agar plates by addition of digestive juices

Chitin agar plate is used for studying the effect of chitinase in *N. mirabilis* digestive juice while milk agar plate is used for studying the effect of protease in *N. mirabilis* digestive juice. Chitin and protein can be broken down in digestion of *N. mirabilis* by chitinases and proteases present in digestive juices respectively. Agar plates containing chitin and protein could therefore be used to investigate the effect of digestive enzymes of different pitcher plants in digesting chitin and protein. Presence of clear zones indicates chitin and protein in agar are broken down by chitinase and proteinase. With a greater diameter of the clear zone, the higher effectiveness of the chitinase or proteinase presence in the digestive juice is.

Three red pitchers and three green ones were chosen based on their visual signs of healthiness and the amount of digestive juice inside. Wilted pitchers are not chosen because it dehydration might hinder the secretion of digestive juices and that digestive juices in wilting pitchers might actually contain denatured enzymes. Collected digestive juices are stored on ice while being

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[3] Passion for plants- animal eating plants (paragraph5)
http://www.abc.net.au/local/stories/2010/02/01/2806994.htm Retrieved September 8, 2018

[4]Why Insects Should Be in Your Diet https://www.the-scientist.com/thought-experiment/why-insects-should-be-in-your-diet-39838

transferred from the field to laboratory to minimize denaturation of digestive enzymes present in digestive juices due to temperature changes in external environment.

2. Determination of the rate of digestion of *N. mirabilis* by comparison of dry mass of crickets and egg white

The digestion rate is obtained by finding the change in weight of crickets and egg white before and after being put in the N. mirabilis for 48 hours. Yet, it is uncertain whether water inside the aqueous digestive juice solution might have entered the egg white and crickets by processes like osmosis and imbibition, which may cause unwanted rises in their masses. Therefore, we weighed the fresh mass of egg white and crickets respectively after 48 hours in *N. mirabilis* and then incubate them for 4 hours at 99℃ in an incubator to remove the water content inside to obtain the dry mass. By comparing the dry masses, there was a more accurate indication on the rate of digestion.

3.2.4 Trials

Three trials were carried out, the first two in Tai Tam Country Park with wild *N. mirabilis* (3-5/8 and 22-23/8) and the last one in the school laboratory with bought *Nepenthes mirabilis* (4-7/9).

The objectives of the first trial were to measure the length of pitchers and collect digestive juice for detection of enzymes.

The objectives of the last two trials were to measure the length of pitchers, collect digestive juice and investigate digestion rate.

The last trial is used to compare any difference in digestive rate between wild *N. mirabilis* and the one grown indoor.

Only the last trial involved the measurement of dry mass due to technical considerations, the dry mass of crickets from the second trial was estimated by data from the third trial.

3.2.5 Procedure

1. Three red pitchers and three green ones were chosen based on their visual signs of healthiness and the amount of digestive juice inside. They were labelled as R1, R2, R3 for the red ones and G1, G2, G3 for the green ones by attaching paper with labels on pitchers. Only pitchers that looked healthy and had more than half of the pitcher filled with juice were chosen.
2. Photos were taken and their size measured. We defined the length of a pitcher as the distance between the pitcher's' lid pivot and the pitcher's bottom. (Fig. 4)

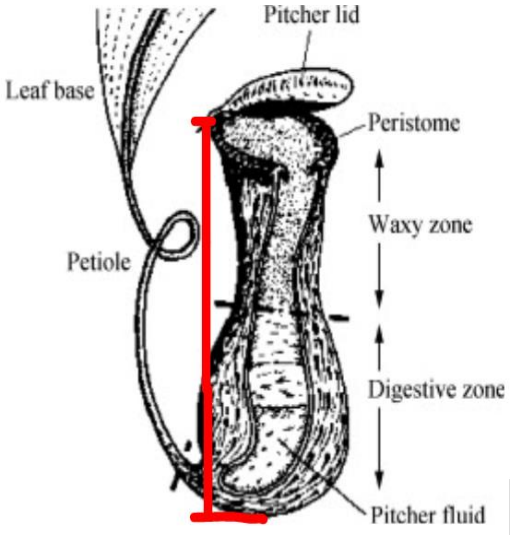


Figure 4 The definition of the length of a pitcher in this study

3. The number of mosquitoes on the inner wall of the pitchers and the number of dead insects in the digestive juice were counted. (Fig. 5)

Figure 5 Inner wall of a pitcher

4. The chosen pitchers were labelled by tying strings at the tendrils.
5. About 2 ml of of digestive juice was collected from each pitcher and stored at 0℃ by a dropper, (Fig. 6) to avoid the enzymes in juice from being denatured due to heat.

Figure 6 Storage of digestive juice samples on ice

6. A dead cricket and about 0.6g of cooked egg white were put into each pitcher. The crickets were bought from Natural Aquarium (天然水族).
7. The crickets and egg white were collected the next day and weighed.

8. They were put into an incubator at 99℃ for four hours and weighed again in order to obtain their dry mass.
9. The percentage of water mass of crickets after putting inside digestive juice was calculated.
10. Three other crickets were weighed and put into an incubator for four hours before weighing again, in order to find out the percentage of water mass originally present in crickets, so that the dry mass of crickets before putting in pitchers could be calculated.
11. A piece of filter paper was immersed into the digestive juice of each pitcher. The filter paper dipped with digestive juice was placed into protein and chitin agar plates separately.


Figure 7 The milk agar plate

12. In order to enhance the reliability, step 11 was repeated three times.
13. The position of each filter paper with different pitcher juice is marked on the plates (Fig. 7) and the agar plates are incubated for 24 hours at 35℃
14. The diameters of clear zones were measured and recorded after incubation.

3.2.6 Assumptions

1. Four hours of incubation was enough to remove all water content of a cricket. This is to ensure the dry mass obtained is accurate to represent the amount of organic matter inside crickets and egg white which has not been digested by pitchers.
2. All crickets had the same increase in water content after putting inside digestive juice.
3. The percentage of water mass in each cricket was more or less the same.

3.3 Objective 2: To study the relationship between *N. mirabilis* and mosquito

3.3.1 Introduction

Mosquitoes, being different from other insects, are suspected to be able to survive inside the pitchers without being digested (Fig.8). According to scientific literature[5], mosquitoes have a mutualistic symbiosis with *N. mirabilis*. Not only does fluid in pitchers allow mosquitoes to hatch in, it also provides nutrients for larvae to grow into adults. We are curious about this phenomenon, since pitcher juice should be able to digest insects, thus preventing mosquito larvae from surviving. The objective of the following investigation is to observe whether mosquito and their larvae could survive in *N. mirabilis*, and to preliminarily suggest possible mechanisms involved.

[5]Food web and fluid in pitchers of Nepenthes mirabilis in Zhuhai, China

https://www.tandfonline.com/doi/abs/10.1080/12538078.2005.10515467#aHR0cHM6Ly93d3cudGFuZGZvbmxpbmUuY29tL2RvaS9wZGYvMTAuMTA4MC8xMjUzODA3OC4yMDA1LjEwNTE1NDY3P25lZWRBY2Nlc3M9dHJ1ZUBAQDA= Retrieved September 8, 2018

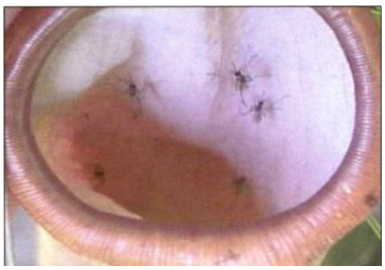


Figure 8 Mosquitoes landing on the pitcher wall

3.3.2 Hypothesis

Mosquito larvae can survive and develop in the pitchers of *N. mirabilis.* Mosquito larvae have a higher chance of survival in pitcher juice than in water.

3.3.3 Principle of experimental design

Experiments were designed to find out the growing and survival situation of mosquitoes and larvae inside fluid of *N. mirabilis.* We observed the living situation of mosquitoes inside pitchers during our field trips to Tai Tam Country Park. Fluid samples of *N. mirabilis* are taken in Tai Tam Country Park on 3/8 and 23/8 for further investigation in school laboratory.

Through counting and observing the change in number and morphology of mosquito larvae (Egg → Larva→ Pupa→ Adult) in regular time intervals, we can suggest the relationship between the digestive juice and the mosquitoes. If a complete life cycle was witnessed in the experiment, it could be concluded that mosquitoes could bred in pitcher. Besides, estimation on the density of larvae allow us to study the survival rate of the larvae as well as to see if there is any difference in the growing condition between young and mature *N. mirabilis* for the larvae.

3.3.4 Trials

Two trials were done by collecting the digestive juice from Tai Po Country Park and observing the growth of the mosquito larvae in school laboratory. (3/8 and 22/8)

The objective of the first trial was to observe whether the mosquito larvae can grow or will be digested in the juice.

The objective of the second trial was to investigate the growth and development of the mosquito larvae.

3.3.5 Procedure

1. Digestive juice that contained mosquito larvae (Fig. 9) was collected from pitchers using droppers and stored in plastic containers at room temperature.

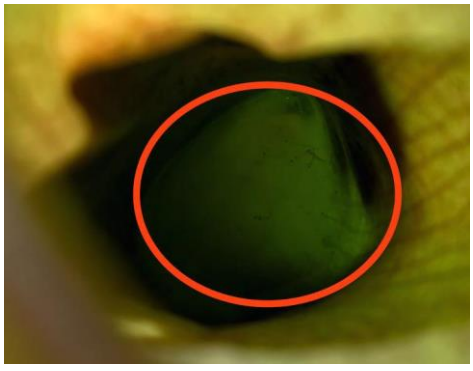


Figure 9 Mosquito larvae found in the pitcher

2. The density of mosquito larvae was estimated by observation with naked eyes.
3. The lids of the containers were opened for a few hours a day to provide sufficient oxygen for the larvae, but when there were grown mosquitoes found, the containers were kept closed to prevent grown mosquitoes from escaping. Small holes weren't punched so as to prevent unwanted entry of nutrients and oxygen continuously.
4. The larvae were closely monitored for 4 - 8 days until mosquitoes were found in the containers or all the larvae had died.
5. The number of adult mosquitoes were counted and the survival rate of mosquito larvae was calculated.

Method of estimation of larvae density

1. About 1.5 ml of digestive juice was added into a 1.5ml Eppendorf tube.
2. A photo was taken and an area with ten larvae was estimated, then it was divided by the total area of digestive juice on that photo. Based preliminary trrials, the densities of mosquito larvae collected from different pitchers were fairly consistent. Therefore it was assumed that the density of larvae throughout the juice and among different pitchers was the same.

4. RESULTS

4.1 Result on Objective 1:To study the feeding behaviour of *Nepenthes mirabilis*

Table showing the percentage of weight of dry mass of crickets (Trial 3)

Cricket label	Fresh mass (g)	Dry mass after being incubated for about 4 hours (g)	Percentage change
A	0.1569	0.0362	-76.93%
B	0.2458	0.0516	-79.01%
C	0.1895	0.0405	-78.63%
			Average:

Table 1

Table showing the percentage of weight of water of crickets after being in digestive juice (Trial 3)

Fresh mass after experiment	Dry mass after incubation	Percentage of weight of water
0.2643`	0.0281	89.37%
0.2309	0.0233	89.91%
0.2438	0.0313	87.16%
0.2381	0.0252	89.42%
0.1617	0.0161	90.04%
0.2108	0.0162	92.31%
		Average: 89.70%

Percentage of weight of water after experiment=
(1 - Dry mass after incubation/Fresh mass after experiment) x 100%

Table 2

Table showing the change in dry mass of crickets and no. of other insects inside the pitchers(Trial 2)

Label	Length of pitcher (cm)	Initial fresh mass (g)	Fresh mass after experiment (g)	Estimation of initial dry mass (g)	Estimation of dry mass after experiment (g)	Percentage change in dry mass	Change in dry mass (g)	No. of other insects inside
G1	10.8	0.2693	0.29	0.05680	0.030	-47%	-0.0268	0
G2	12.7	0.2118	0.17	0.04467	0.018	-60%	-0.0267	3+
G3	9.5	0.2080	0.18	0.04387	0.019	-57%	-0.0240	1
R1	8.0	0.1419	0.11	0.02993	0.011	-63%	-0.0290	2
R2	9.5	0.3010	0.25	0.06348	0.026	-59%	-0.0376 (discarded)	2
R3	11.4	0.3254	0.41	0.06863	0.042	-39%	-0.0266	3+

Table 3

Remark: All pitchers in Trial 2 contain mosquito larvae.

Table showing the change in mass and dry mass of egg white (Trial 3)

Label	Fresh mass before experiment (g)	Fresh mass after experiment (g)	Dry mass after incubation (g)	Percentage of weight of dry mass	Change in mass (g)
G1	0.67	0.53	0.07	13%	0.14
G2	0.62	0.50	0.10	20% (discarded)	0.08
G3	0.64	0.59	0.05	8.5%	0.05
R1	0.63	0.63	0.07	11%	0.00
R2	0.66	0.65	0.06	9.2%	0.01
R3	0.63	0.58	0.06	10%	0.05

Table 4

Remark: The mean of the percentage of weight of dry mass of egg white is about 10%, which is the same as fresh egg[6], it can be assumed that the water content of egg white has not changed after being submerged in pitchers. The change in fresh mass can represent the change in mass.

Table showing the change in fresh mass of egg white and no. of other insects inside the pitchers(Trial 2)

Label	Egg white fresh mass(g)		Change in mass (g)	No. of other insects inside
	before	after		
G1	0.60	0.47	0.13	0
G2	disappeared		/	3+
G3	0.60	0.52	0.08	1
R1	0.60	0.38	0.22	2
R2	disappeared		/	2
R3	0.60	0.52	0.08	3+

Table 5

[6] Chemical Composition Of Eggs http://indianchickens.com/consultancy%20content/Hatchery%20unit/Chemical%20Composition%20of%20Egg.html Retrieved 11/9/2018

Table showing the change in mass and dry mass of crickets (Trial 3)

Label	Initial fresh mass (g)	Estimation of original dry mass (g)	Fresh mass after experiment (g)	Dry mass after incubation (g)	Change in dry mass (g)
G1	0.2762	0.06024	0.2643`	0.0281	-0.0321
G2	0.1293	0.02820	0.2309	0.0233	-0.0049
G3	0.2001	0.04364	0.2438	0.0313	-0.0123
R1	0.2186	0.02768	0.2381	0.0252	-0.0025
R2	0.1946	0.04244	0.1617	0.0161	-0.0263
R3	0.1288	0.02809	0.2108	0.0162	-0.0119

Table 6

Table showing the no. of mosquitoes and insects in correlation to pitcher color (Trial 1, 3/8)

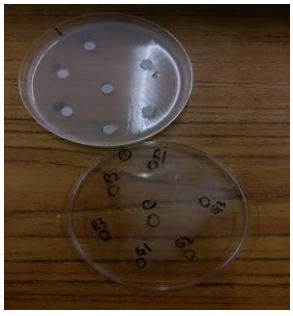
Label	No. of mosquitoes inside	No. of insects inside
G1	4	0
G2	2	0
G3	1	1
G4	0	1
R1	0	2
R2	0	0
R3	0	0
R4	0	3+

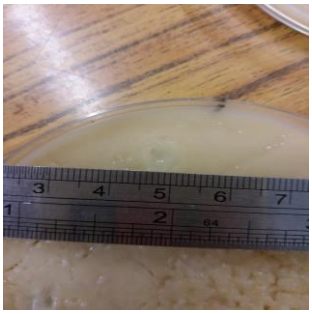
Table 7

Table showing the no. of mosquitoes and insects in correlation to pitcher color (Trial 1, 4/8)

Label	No. of mosquitoes inside	No. of insects inside
G1	2	0
G2	1	3+
G3	1	1
G4	0	2
R1	1	2
R2	0	2
R3	0	1
R4	0	3+

Table 8





Figures 10 (a – c) After the incubation for 24 hours, no significant clear zones could be found in either trial 1 or 2. The agar plates even formed clumps and lured some house flies after two days.

4.2 Result on Objective 2:To study the relationship between *N. mirabilis* and mosquito

One sample of digestive fluid which was marked “Sample A” was collected from *N. mirabilis* in trial 1 (3/8) to investigate the survival situation of mosquito larvae.

Table 9: Growth and development of the larvae collected on 3/8 (Sample A)

 3/8: Digestive fluid of *N. mirabilis* was collected from the Tai Po Country Park. More than 10 larvae were found inside fluid.	 8/8: A mosquito was emerged from a pupa.	 14/8: The mosquito fell into the digestive juice and was found disappeared.	 27/8: Number of larvae reduced. About 7 larvae were found.	 7/9: Only 3 developed larvae were left in the fluid.

Two samples of digestive fluid from a young and a mature pitcher containing mosquito eggs were collected from Tai Po Country Park in trial 2 (23/8). They were marked as G1 and R1 respectively. (The samples are taken from the same pitchers marked G1 and R1 using which we also performed experiment on objective 1)

Table 10: Growth and development of the larvae collected on 23/8 (G1)

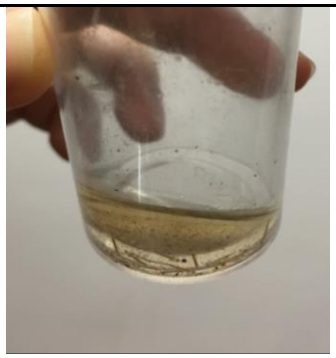
 23/8: Sample with alive larvae was collected	 25/8: more than 10 larvae were found with a round head and siphon tube at the tip of its abdomen	 27/8: larvae were found with a larger head size and darker in colour.	 31/8: Number of larvae reduced while the remaining larvae (around 5) grew bigger and darker.

Table 11: Growth and development of the larvae collected on 23/8 (R1)

 23/8: Sample with alive larvae was collected.	 25/8: Pupa were found.	 27/8: Mosquitoes emerged from the pupa. Remaining larvae grew to bigger size and darker colour.	 31/8: More than 8 mosquitoes were found.	 7/9: All grown mosquitoes disappeared, leaving behind larvae which had not yet developed into pupa or adult mosquitoes.

	Figure 11 This sample of digestive juice was collected for some time before the photo was taken, so the mosquito larvae had sedimented. But when mosquito larvae were collected, all samples were from the bottom of pitchers, so it can be assumed that the density of mosquito larvae in each sample is about the same. This sample can be divided into three regions of different densities. Each black box indicates the volume which contains 5 larvae. Region 1 contains about 6 black boxes, region 2 contains about 6 black boxes and region 3 contains about 3 black boxes. Therefore, there are about 75 larvae per ml.

5. DISCUSSION

5.1 Discussion on objective 1: To study the feeding behaviour of *N. mirabilis*

The dry mass of a cricket is about its original mass times (1 - 78.19%) = 0.2181, and that of a cricket that has been in a pitcher is about its measured mass times (1- 89.70%) = 0.1030. These findings were used to predict the dry mass of crickets in Trial 2 and Trial 3.

From Trial 2 (Table 3), the mean of the change in dry mass of the crickets is about 0.0266g. The standard deviation is about 1.59 x 10^{-3}g, so the change in mass was more or less consistent. The percentage change in dry mass of the crickets varies from about -30% to about -60%. This implies that pitchers cannot detect the size of prey; or that pitchers would not change the composition of digestive juice in order to digest preys of different sizes. The change in dry mass of crickets has no apparent correlation to pitcher size, pitcher color and number of insects inside. The rate of digestion of crickets is 0.0266 / 2 = 0.0133g / day.

From Trial 3 (Table 6), the change in dry mass of the crickets varies from 0.0025g to 0.0325g. The mean is 0.015g and the standard deviation is about 1.08 x 10^{-2} g, which is 579% higher than that from Trial 2. This shows that the digestion rate is very inconsistent among different pitchers. The variation in change of mass seems to have no correlation to pitcher color and pitcher length. The digestion rate of crickets in the wild is higher and more consistent than that in the laboratory. The large variance in digestion rate may be due to the lack of insects and microorganisms that are present in a wild environment (Trial 2) but absent in a laboratory. Therefore, insects and microorganisms could play a role in helping pitchers to digest food. The difference in temperature and amount of sunlight received by the pitchers may also be a factor. The pitchers were put in an air conditioned room in Trial 3. Since chemical reactions and enzymatic activities varies drastically with temperature, the lower availability of sunlight and lower temperature may slow down the digestion rate of pitchers. Other mechanisms related to the digestion of crickets may also be affected, such as the detection of prey, secretion of digestive juice, and rate of active transport of digested food.

The experiments about the digestion rate of egg white are inconclusive. The digestion rates in Trial 2 and 3 were inconsistent. The standard deviation of change in mass in Trial 3 is 0.0465g and that of Trial 2 is 0.572g. The percentage change also varies from -1.5% to -21% and -13% to -37% in Trial 3 and Trial 2 respectively. The variation of change in mass has no correlation to pitcher size, pitcher color or insects inside.

From the photos of chitin and milk agar plate, only a few small clear zone can be seen, and their diameters are too small to be measured by apparatus available. Judging from the experiments concerning crickets and egg white, the change in mass of crickets and egg white after being put in the digestive juice for days are not significant. It is thus expected that the concentration of enzymes in *N. mirabilis* digestive juice are low which leads to a very low digestion rate. It is thus believed that the breakdown of protein and chitin required long time for complete digestion since there is in fact digestion present in the experiment, which can be proved by the drop in dry mass of crickets. Hence, large clear zone cannot be found within 24 hours.

Moreover, there are clumps on the agar plate after addition of digestive juice for several days. Reproduction of mosquitoes and consuming the nutrients (e.g. protein) in the agar might account for the formation of clumps.

5.2 Discussion on objective 2: To study the relationship between *N. mirabilis* and mosquito

From Trial 1 (Table 7 and 8), green pitchers had more mosquitoes inside while red pitchers had more insects in side. There are 1.75 mosquitoes found in average in green pitchers while there is no mosquito found in average in red pitchers. Thus, green pitchers seem to attract more mosquitoes to rest inside. In addition, there are 0.5 insects found in green pitchers while there are at least 3 times of insects found in average in red pitchers so that red pitchers are said to attract more insects as prey. From Trial 2 (Table 3), pitchers of all colors contain mosquito larvae. Therefore, mosquitoes may choose younger pitchers to lay eggs in, so when the eggs hatch the larvae would have more time to mature. As the larvae grow, the pitchers would also grow and turn red, so the larvae would have more food source when growing. As all pitchers in Trial 2 contains mosquito larvae, *N. mirabilis* is a regular breeding spot for mosquitoes.

This is to clarify why we do not perform the investigation on objective 2 during our field trip outdoor, instead we chose to do observation on relationship between mosquitoes and *N. mirabilis* by collecting fluid sample back to school laboratory. As a complete life cycle of mosquitoes usually takes 10-14 days, it is difficult for us to observe the development of a mosquito from its larval to adult stage during the short period in our field study. Besides, the opening of pitchers is usually

small in size, only allowing small amount of light to pass through, which makes observation by naked eyes or putting a camera inside the pitcher to monitor development of mosquito larvae inside a dull pitcher unfeasible. Therefore, we decided to collect *N. mirabilis* fluid samples in transparent containers for better monitoring of the changes inside the fluid samples with larvae.

During the investigation of objective 2, a complete life cycle was found after a larva had grown to adult and died inside the container.

From table 9, only one larva successfully grew into adult after 5 days of trial 1 (on 8/8). The only one mosquito fell into digestive fluid and disappeared, and after which no mosquitoes were observed to have emerged from pupa anymore. Yet, the number of larvae inside digestive fluid kept decreasing as described in table 9 (from >10 to only 3 larvae left). The same case also holds for sample G1 in trial 2 (refer to table 10), in which number of larvae decreased from >10 to around 5 left while no mosquitoes were found emerged in G1.

Out of our expectation, while *N. mirabilis* fluid provide a breeding place for larvae, above findings suggest that not all larvae will be able to grow into adult mosquitoes. It may be due to the lack of oxygen in the containers, and insufficient nutrients inside digestive fluid as the container lids were closed for most of the time especially when there were mosquitoes emerged, so no extra insects in outside environment can be captured and digested by the *N. mirabilis* fluid or act as food to provide nutrients for growth of larvae. Scientific research[7] suggests that larvae live as filtrators next to the surface of *N. mirabilis* fluid and feed on small particles of decaying prey and organisms. As prey cannot be captured by *N. mirabilis* fluid in our experiments to feed the larvae, larvae may be dead in a poorly aerated and malnourished environment. Besides, some larvae die before metamorphosis. According to the scientific findings, there is selective effect of *N. mirabilis* fluid which only allows larvae that are able to adapt to certain living environment in *N. mirabilis* fluid to survive. The disappearance of larvae is suggested by decreasing larvae count in both samples. It could be concluded that digestive fluid of *N. mirabilis* selectively kills a portion of larvae but at the same time provides a growing place to some larvae when there is sufficient nutrients in *N. mirabilis* fluid provided to larvae.

Referring to table 11, more than 8 mosquitoes were found to have emerged from pupa (31/8). Afterwards, all grown mosquitoes fell back into *N. mirabilis* fluid and were found disappeared one week later (7/9). By comparing the photo taken on 7/9 with that on 23/8, the colour of Nepenthes fluid on 7/9 was apparently deeper than that on 23/8 with many black spots inside the fluid. This proves that after mosquitoes fell into *N. mirabilis* fluid, they would be digested by the fluid so they were found disappeared. The deeper fluid colour and black spots inside fluid further correlate with this proof. The black spots are the evidence that *N. mirabilis* fluid had not yet finished digesting the dead mosquitoes, so some body parts of mosquitoes was still remain to be digested. It could be concluded that digestive fluid of *N. mirabilis* also digests mosquitoes when mosquitoes fall prey to it.

Figure 12

[7] Traps of carnivorous pitcher plants as a habitat: composition of the fluid, biodiversity and mutualistic activities https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3025736/ Retrieved December 15, 2010

Moreover, the above figure shows that some larvae are attached to some black spots (dead body parts of mosquitoes) and seems like the larvae were consuming them. This shows that the dead mosquitoes may become food of larvae to provide nutrients for their growth as there were once 8 mosquitoes emerged in sample R1. It explains why there is less significant decrease in larvae number in R1, properly owing to the nutrients provided by dead mosquitoes are still sufficient to allow continuous growth of larvae so they would not die due to malnutrition.

Different life stages of mosquito were observed throughout our investigation, from larva, pupa to adult mosquito. However, eggs of mosquitoes are not observed in our study. It is properly due the small size of eggs which may not be observed by naked eyes. Eggs are usually attached together to form 'raft' and float on the surface of water[8], while all of our *N. mirabilis* fluid samples were extracted from the bottom of pitchers using droppers, so eggs of mosquitoes may not be collected in our samples. Nevertheless, our hypothesis is proved to be partly correct. Our experimental results has already proved that *N. mirabilis* provide a breeding place for larvae of mosquitoes to grow into adults. As discussed in the above, *N. mirabilis* fluid have selective effect to allow the growth of some larvae, but not all of them will be able to survive and mature in the pitchers of *N. mirabilis.* Our study also points out that *N. mirabilis* only allow mosquitoes to hatch eggs on fluid surface. Meanwhile, when a mosquito, no matter it is living or dead, falls into and sinks down in the fluid, it will then be digested by *N. mirabilis* fluid.

From Tables 9 - 11, a total amount of 50 ml of digestive juice containing mosquito larvae was collected. At the end, a total of 9 adult mosquitoes were found. Therefore, the survival rate of mosquitoes is 9 / (50 x 75) = 0.24%

We can compare the survival rate we obtained with a research done by Jannelle Couret, Ellen Dotson, and Mark Q. Benedict.[9] : The diet mixture used was comprised of beef-liver powder, tuna meal, and vitamin mix in water and was tested at 1%, 2%, 4%, and 8% concentrations (10 mg/ml, 20 mg/ml, 40 mg/ml, and 80 mg/ml of diet mixture in deionized water respectively)

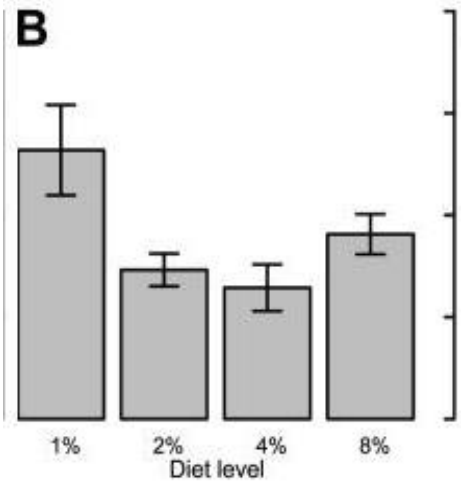


Figure 13 Mortality rate across diet concentration in the study by J. Couret *et al*

[8] The American Mosquito Control Association. Mosquito info: Life cycle. https://www.mosquito.org/page/lifecycle Retrieved September 8, 2018

[9] Temperature, Larval Diet, and Density Effects on Development Rate and Survival of Aedes aegypti (Diptera: Culicidae) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3911954/ Retrieved September 7, 2018

According to another study done by Wadaka Mamai, corresponding author Rosemary Susan Lees, Hamidou Maiga, and Jeremie R. L. Gilles,[10]larval mortality rate is 1.66 ± 0.37 % in clean water.

The survival rate of mosquito larvae in *Nepenthes mirabilis* is much lower than those in the above studies. This may be due to insufficient oxygen provided to the larvae. Or errors in handling the larvae, such as unwanted shaking, heat, or light intensity. Another possibility is that the pitchers of *N. mirabilis* happen to be a possible breeding ground for mosquitoes, and some mosquito larvae do survive but the *N. mirabilis* actually tries to consume them.

5.3 Further Investigation

1. Since the digestive rates of different sizes of crickets are the same, what are the factors affecting the secretion rate of digestive enzymes of *N. mirabilis*? Can *N. mirabilis* detect the entering of prey thus secreting more enzymes? How does the pitchers detect and release corresponding enzymes after catching a prey? Or are the enzymes density constant throughout its life?
2. From the results, wild *N. mirabilis* have a relatively more consistent and higher digestion rate than indoor grown *N. mirabilis.* What are the factors or features allowing wild *N. mirabilis* to achieve a higher digestion rate than indoor grown *N. mirabilis*?
3. It was found that live mosquito larvae could survive in pitcher liquid but the dead ones would be digested. How can live mosquito larvae survive and grow into adult in the pitcher without getting digested?
4. Since pitcher fluids is also inhabited by microbes[11], besides the relationship between mosquito larvae and pitcher plant, does the microbes living inside the pitcher enhance the efficiency on breaking the prey into inorganic matter?

6. CONCLUSION

From the experiments in section 4.1, wild *N. mirabilis* digested crickets at 0.0133g/day, while the digestion rate of crickets of bought *N. mirabilis* could not be determined. The digestion rate of egg white from both types on *N. mirabilis* could not be determined. Pitcher color, pitcher length, number of insects inside pitchers, and the presence of mosquito larvae did not have effects on digestion rate of *N. mirabilis*. The presence or amount of proteinase and chitinase could not be determined.

From the observations in section 4.2, mosquitoes were able to lay eggs in *N. mirabilis.* The eggs would hatch and the larvae could grow into adult mosquitoes. Therefore *N. mirabilis* is an available breeding ground for mosquitoes. However, from our observations, the mortality rate of mosquito larvae is much lower than that studied by other researchers. The reason to this is uncertain.

[10] Reusing larval rearing water and its effect on development and quality of Anopheles arabiensis mosquitoes https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4793705/ Retrieved September 9, 2018

[11] Lam, W. N., Chong, K. Y., Anand, G. S., & Tan, H. T.Dipteran larvae and microbes facilitate nutrient sequestration in the Nepenthes gracilis pitcher plant host.(2017, March 01) http://rsbl.royalsocietypublishing.org/content/13/3/20160928 Retrieved September 11, 2018

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