Immunity, Sex and Parasites:

Does Sex of Sand-Field Cricket (*Gryllus firmus*) Affect Immune Response to Eugregarine Parasites?

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There is controversy about the effects gut-dwelling eugregarine parasites have on their invertebrate hosts. If crickets (*Gryllus firmus*) apportion resources to reproduction differently in males *vs.* females, then resources used to mount immune responses to parasites may also differ – especially if the parasites are pathogenic. I investigated the possible differences in immune response between male and female crickets and attempted to determine whether these differences are related to intensity of parasitic infection. To do this, pieces of nylon filament were implanted into the hemocoel of crickets which tested the immune response where hemocytes surround the filament (encapsulation). These responses were compared to intensity of parasitic infection. No statistically significant relationship between sex and melanisation, or sex and parasite load were found. I found that the duration of melanization was negatively correlated to parasite abundance and that there was a positive correlation between body size and parasite number. This result suggests the existence of a relationship between the parasite and host that could be conflicting with sexual selection theory, such as host manipulation by the parasite.

Introduction

Sexual Selection Theory

Sexual selection theory states that male fitness is limited by the amount of potential mates they can obtain (Shuker, 2014). There is an increase in male competition for mates that results in elaborate ornaments such as the sage grouse, in most species, female choice is the driving force of such ornamentation. Male sage grouses have elaborate feathers and enlarged chest cavities that bloat when filled with air. These characteristics not only make the sage grouse more conspicuous towards predators but are an example of sexual dimorphism driven by sexual selection theory. The enlarged chest cavity and feathers are examples of ornaments female sage grouses look for in a potential mate, and therefore selection favours the production of these characteristics in male sage grouse (Gibson and Bradbury, 1985).

Gryllus Firmus

Sand-field crickets (*Gryllus firmus*) are commonly found throughout the Southeastern United States and are considered a model organism in research for topics such as flight musculature and calling behaviour. *G. firmus* crickets have a single generation per year (univoltine) and males are territorial when competing for available burrows and calling locations (Saleh, N., Larson, E., & Harrison, R., 2014).

Eugregarines

Gregarine parasites are Apicomplexan protozoa of the class Sporozoa and Order Gregarinida (Zuk 1987). Apicomplexan protozoa have a key morphological characteristic, an apical complex structure (called an epimerite) that allows the parasite to attach to or penetrate the host. Gregarine parasites are closely related to Toxoplasma and Plasmodium. These two parasites are known to cause toxoplasmosis and malaria respectively. Although there is little comprehensive information regarding apicomplexans, their lifecycle has been described in depth. Septic gregarines or intestinal gregarines are ingested as occysts, which travel through the hosts' body before attaching to the epithelium tissue of the mid-gut. Then they feed off the hosts minerals and nutrients before forming into a trophozoite, which defines the feeding stage of the apicomplexan lifecycle. The trophozoite detaches before finding another detached trophozoite that subsequently pair and form a cyst. The cyst is eventually released through the host's feces. These parasites have been shown to have detrimental effects on their hosts including decreased longevity, lowered weight, and death (Zuk 1987).

Insect Immunity and Knowledge Gaps

When the immune response of the cricket identifies a pathogen, haemocytes, which are an insect's blood cells, will surround the pathogen or immune effect and become melanized or darkened by enzymes and chemicals released by the humoral system. One such enzyme released by the humoral system includes phenol oxidase (Siva-Jothy *et al.,* 2001). This response is an act of defense that isolates the pathogen and represents an organized and co-ordinated cellular response by these insects. Another interesting way insects may avoid the lethal affects of parasitism is to simply avoid being infected; one method for aversion of infection was examined within this study. Sheridan and colleagues (Sheridan et al. 2000) suggest that males are not more prone to parasitic infections than females, despite the greater intensity of sexual selection acting on males. This conclusion will be compared with the results of this study.

Rationale

Insects are not studied often in regards to parasitic or reproductive research, however there are a wealth of benefits to doing so, including short generation times and a large amount of previous information on their life cycles, mating behavior, etc. (Adamo *et al.* 2001). Invertebrates in general, such as *Gryllus firmus* can be housed in large numbers, just like they were within this experiment. Invertebrates also have simple nervous systems which are easily studied. It is thus important to note that, under ethical reason, conducting research on invertebrates may provide a number of opportunities and information that would otherwise not be available.

Objectives

The objectives of this experiment were to determine whether a difference in immune response exists between male and female *G. firmus* crickets and to investigate the relationship between immune responses and parasite loads. According to sexual selection theory, there should be a differential investment in energy in regards to defense against parasites between males and females. Expected differences in immune response would be recognized by females showing a higher level of melanization than males and the assumption that females will allocate more energy towards reproduction and survival in comparison to males (King *et al.*, 2011). Differential allocation of resources assumes that the more energy spent towards one trait (e.g. reproduction) will take away energy used for another trait (e.g. parasitic resistance). This experiment provides insights on the potential impacts of eugregarine parasites on invertebrate hosts, future studies on possible control of eugregarine parasites, and treatment plans for the hosts.

Materials and Methods

To thoroughly explore the possibility of differential immune responses between male and female *G. firmus* crickets, this experiment began with their capture and containment. The insects collected for this experiment were living in containment and not in their natural habitat. Before the crickets were extracted for this experiment, they were maintained in a 25°C room in large plastic containers with the entirety of their population. The majority of this experiment took place in a room temperature (25°C) basement lab (room 5-080) at MacEwan University.

Collection of G. firmus Crickets

Gryllus firmus crickets were collected from their large communal containers and placed into separate containers.

Incision and Isolation of G. firmus

An adult cricket (either male or female) was randomly chosen from a bin and was pierced with an insect pin (0.19mm gauge) through the plural membrane on the right side of their abdomens. After, a nylon filament line (0.23mm gauge), which was approximately 2mm long, would be inserted into the incision. These filaments lines were previously roughened with sandpaper due to the assumption that haemocytes would adhere to the filament more effectively (Zuk *et al.*, 2004). In addition, the nylon line was also knotted because this reduces the likelihood that the filament line would fall out of the incision. This decision was made after previous trials of crickets had a large fallout of unknotted lines. After the filament line was placed within the incision, the cricket was released into a separate individual plastic container. This container consisted of an individual cell from an egg carton (provides shelter and a dark space), a centrifuge tube filled with water, 3 pieces of rabbit food and 3 pieces of cat food ab-libitum. An equal number of adult male and female crickets would be selected and left in the containers for various time durations. These time durations were: 24 hours, 48 hours, 72 hours, 96 hours, and 96+ hours.

Extraction of Implant

After the specified time period, the cricket was decapitated with micro-scissors and weighed. The filament line was extracted and dabbed with a kimwipe (thin cleaning tissue) before being mounted on a cover slide using Permount®. The melanized line was placed beside a un-melanized line of approximate equal length on the cover slide, the un-melanized lines act as the control.

Dissection of G. firmus

The body of the cricket was transferred into a clear dish before adding insect saline to barely submerge the cricket's body. The mid-gut was dissected out, and with an insect pin (0.19mm) the mid-gut was opened and the epithelium tissue was teased out. Using a clicker, the gregarine parasites was manually counted. One finished, the remains within the dish were discarded and the dish was cleaned for future use.

Quantifying Data

After the slides dried, they were photographed using an under Infinity Capture Program® with an infinity capture camera and its associated program (Lumenera, Ottawa, Ontario). These photographs were then evaluated using ImageJ (National Institutes of Health, Bethesda, Maryland, USA) which produced a histogram. This histogram provided numbers for different statistical categories (e.g. mean, mode, etc.) which therefore allowed for the intensity of melanization to be quantified and used for data analysis.

Statistical Analysis

An ANCOVA test was used to test the relationship between two variables and how they change between one another. Melanization and sex are the two considered variables and the intensity of melanization in relation to sex was tested. There were 3 continuous variables (melanization, body size, and time period) and 1 categorical variable (sex of cricket). Scatter plots were also used to visualize the correlation or correlations between all variables. The independent variable was the sex of the cricket and the dependent variable is the melanization of the nylon filament. The control is the un-melanized nylon line which is mounted beside the melanized line on the cover slides for each cricket tested.

Results

Anticipated results include a statistically significant correlation between parasite number and melanization intensity and for there to be a statistically significant relationship between parasite number and sex. The sample consisted of 60 adult crickets, with 30 males and 30 females. The gregarine data was log transformed in Figures 1, 2, and 3 to homogenize data. Also the time periods 72 hours, 96 hours, and 96+ hours were collapsed into one category (72+ hours) due to their small sample sizes. A total of 14 specimens tested after 24 hours, 18 specimens for 48 hours, and 28 specimens tested collectively for 76+ hours. Sample size limits were due to time constraints of the study.

Three t-tests were performed to compare males and females on three different dependent variables: body mass, log gregarine numbers and mean melanization score (Table 1). For body mass, a t-test for unequal variances was used (t=-5.456, df=37.606, p<0.001). Body mass was the only statistically significant dependent variable with a p value <0.05 (p=0.016 for the test for equal variances). For log gregarine numbers, a t-test for equal variances was used (t=-1.199, df=53, p=0.238). For mean melanization score, a t-test for equal variances was used (t=-0.326, df=50, p=0.746). There was no difference between the variances in the population for the log gregarine numbers or the mean melanization score.

As represented in Figure 1, there is no statistical significance between melanization and sex. However, there is a statistically significant decrease in the number of parasites at 48 hours and longer (Figure 2). An ANOVA test was done (F=6.085, df=2,52 p=0.004). Tukey's post-hoc test results are indicated by the letters over the data points. No letters remained the same over the data points indicating that these values are different (Figure 2). Lastly, there is a significant positive correlation between body size and parasite numbers for males and females combined (r=0.296, p=0.033), but not for males or females on their own (Figure 3). However, taking account of this correlation by dividing parasite numbers by mass (to get a parasites per unit mass measure) did not reveal any significant relationship between parasites and melanization, nor did the sexes differ on this new corrected measure of parasite numbers (Figure 3).

In addition, Figure 4 shows that there was no relationship between melanization intensity and duration of time. An ANOVA test was done (F= 1.583, df=2,49, p=0.216). Figure 5 indicates that there is no statistically significant correlation between parasite number and melanization score for either sex (p>0.273).

To conclude, as shown Figures 1 – 5 respectively: there is no significance difference between melanisation intensity and sex; there is a significant decline in the number of gregarine parasites at 48 hours and over; there is a significant positive correlation between body size and parasite numbers for males and females combined (but not for males or females on their own); melanisation intensity is lighter >48 hours however it was not statistically significant; and there is no statistically significant relationship between melanisation intensity and gregarine numbers. Additionally, there is no statistically significant relationship between parasites and melanization.

Discussion

Males and females do not have any significant differences in immunity responses. Since there was no significant different in melanization between sexes, this suggests different implications in regards to sexual selection theory. There are many studies that show immune responses having differential intensity towards parasite burden. These studies also suggest that there may be many confounding variables that directly affect the allocation of resources towards the trade-off between parasitic resistance and reproduction (Bailey *et al.*, 2011; Siva-Jothy *et al.*, 2001 and Zuk *et al.*, 2004). This leads to three different, but related, theories regarding the relationship between sex, the number of parasites and immune response.

The first theory examined co-evolution/co-adaptation between parasites and their hosts. Parasites cause significantly detrimental pathologies which will cause stronger immune responses in hosts. Therefore, there should be a direct relationship between immune function and parasites in which a stronger immune response will decrease the number of parasites. This experiment found that the number of parasites decreased significantly within its host at 48 hours or more but did not find a significant relationship between melanization intensity and parasite number. Siva-Jothy and colleagues (Siva-Jothy *et al.* 2001) studied field-population of damselflies and found that the magnitude of the encapsulation response to an immune challenge (such as a nylon insert) was not affected by chronic parasite burdens. A conclusion that can be made from this study indicates that maintenance of an adequate encapsulation response is an important life-history requirement. This conclusion can be made based on the fact that the immune response is maintained regardless of parasite burden or immune challenge (Siva-Jothy *et al.*, 2001).

Second, different sexes may invest more energy into immunity as a trade-off with reproduction. Adamo and colleagues (Adamo *et al.* 2001) explain that males expend more energy on mate competition than females, and males also fuel the competition for mates by investing less energy into other traits, such as immunity. Even though a decrease in immunocompetence will increase the risk of death, as long as males live long enough to produce a large amount of offspring (or more than their rivals), this strategy will result in increased reproductive fitness (Adamo *et al.*, 2001). Bailey and colleagues (Bailey *et al.* 2008) reflect on the cost of immune defence against parasitic infections and suggests that sexual selection may favour individuals that optimize their allocation of resources to immunity, especially in environments where parasite transmission is high (e.g. high population densities). They also suggested that the more prevalent a parasite is, the higher the parasitic resistance of the host subsequently becomes. The results from this experiment seems to contradict this conclusion, as eugregarine parasites are in fact highly prevalent in *G. firmus* populations, where nearly all the crickets studied were infected by eugregarine parasites at varying degrees. Resistance of the host, in this study, is associated with resistance to infection which leads to a multitude of pathologies.

Zuk and colleagues (Zuk *et al.* 2004) suggest that where male and female investment in resources is relatively equal, the difference between the sexes in immune response/disease susceptibility may be minimal. Their study also found a male-biased, rather than female-biased, superiority in immune ability. Their study specimens (*Teleogryllus commodus*) are expected to concentrate their allocation of resources toward reproduction in a shorter time period than their comparison species, *Teleogryllus oceanicus*. This is because crickets becoming sexually mature later in the season will be at a disadvantage unless they can reproduce quickly. Selection should thus favor early reproduction with a demand for rapid investment of resources into reproductive activity. This distinction should be particularly pronounced for the females, which put a larger proportion of their body mass into reproductive tissue (Zuk *et al.*, 2004). This study also suggests that the sex under the most constraints for reproductive effort might also be expected to show a decreased investment in immunity (Zuk *et al.*, 2004). Adamo and colleagues (Adamo *et al.* 2001) also mentioned trade-offs could exist between immunity and other traits in insects such as increased foraging behaviour. The crickets in this experiment were given food ad-libitum. Therefore it is assumed that trade-off of energy investment between increased immunity and increased foraging behaviour is minimal. Siva-Jothy and

colleagues (Siva-Jothy *et al.* 2001) discuss the relationship between investment in immune function within and across different areas of an insect's body as being poorly understood. Thus, it will subsequently need to be carefully assessed if we are to understand how life-history decisions (e.g. immunity vs. reproduction) affect parasite resistance (and therefore fitness) as well as how parasite burdens affect subsequent life-history decisions.
Lastly, females may consider to reproduce with males with lower parasite loads, these males would resultantly have better immune systems. However this is only a possibility if immunity is heritable. Adamo and colleagues (Adamo *et al.*, 2001) suggest that in crickets, choosing males on the basis of body length or weight will not increase the chance of mating with a more disease resistant male. I found that larger males did not show a decrease in parasitic resistance, suggesting that they can invest in both growth and immunity. This could therefore be the better investment for the female. A hypothesis by Hamilton and Zuk (1982) suggests that females could base their choice of mates on heritable resistance to parasites. This resistance is suggested to be generated in a process of co-adaptational cycles of hosts and their parasites and also advertised in mate's display of sexual traits. Co-adaptational cycles refer to the interaction between host and parasite which ensures a continual source of fitness variation in genotypes of both the host and parasite (Hamilton and Zuk 1982). However, there is a debate on the existence of co-adaptational cycles, how often it occurs, and if this affects mate choice (Møller 1990).

An observation made in this experiment was that once the nylon implant was inserted into the abdomen of the cricket, the individual would start to kick and the filament line. This kicking behaviour was assumed to be mimicking grooming behaviour. This behaviour would, more often than not, successfully remove a part of the filament line from the incision or the entire line. Vincent and Bertram (Vincent and Bertram 2010) state that parasites (tested *Ormia ochracea*) can be extremely costly and they can affect several aspects of an insects' life (tested *Gryllus texensis*) including retarding the development/maturation rates, reducing mating success, fecundity, and survival. However, host behaviour can influence ability of the parasite to reduce its host's fitness. For example, hosts can groom to reduce the number of parasites that attach to them. One way crickets avoid or reduce lethal costs of parasitism is to remove parasites before they have a chance to enter the body. This study found that crickets that died as a result of parasitism groomed significantly less often than crickets that did not die, and therefore crickets should groom when parasite levels are high. Consistent observations of grooming behaviour in crickets (e.g. over a specified time period) was not conducted. This was due to the grooming behaviour being an observation along the duration of the experiment and this behaviour lacks background information that would allow for inferences to be made.

There a few general conclusions that can be made by this experiment, these conclusions may be applicable to broader scientific fields apart from simply invertebrate research or parasitology. These conclusions relate to parasite-host relationships and differential investment in relation to sexual selection theory. Sheridan and colleagues (Sheridan *et al.* 2000) suggest that males are not generally more prone to parasite infections than females despite the greater intensity of sexual selection acting on males. This supports the findings of this experiment where there was no significant difference in immune response between males and females. Zuk and colleagues (Zuk *et al.* 2004) found that the difference between male and female encapsulation abilities became larger in the field sample compared to laboratory sample. This could be directly related to this experiment, since all crickets were in a laboratory setting (habituated in containment). Zuk and colleagues (Zuk *et al.* 2004) also found that the presence of gregarine parasites were unrelated to the encapsulation ability of the crickets *Teleogryllus commodus* and *Teleogryllus oceanicus.* This finding can thus be compared to the findings of this experiment where there were not significant differences between encapsulation and parasite number. In addition, Shoemaker and colleagues (Shoemaker *et al.* 2006) studied *Gryllus texensis* females and found that mating cues caused females to alter the distribution of their immune resources. Thus increasing resistance to some pathogens, but possibly decreasing resistance to others. Therefore, a conclusion of this study could be that female *G. firmus* crickets may have a higher resistance to other pathologies or symptoms caused by eugregarine parasites which are not related or identified by their encapsulation response. The last theory is related to host behaviour modification due to parasitic infection. Dobson (1988) states that changes in the behavior of infected hosts are most commonly recorded in the intermediate hosts of parasites with complex life cycles. All the changes in the host's behavior are therefore created to increase the rates of transmission of the parasites between hosts. However, the results of this study did not reveal any indication that the behaviour of the cricket was modified by the eugregarine parasites to increase its transmission between hosts.

This experiment faced many logistical issues that could be rectified in future studies. A larger sample would have a greater chance of revealing any patterns that may show a link between sex and immune response. A total of 8 slides were destroyed, no data was calculated for these melanized lines and were subsequently exempt from the results of this experiment. Other difficulties that arose during this experiment include sex differences in immune response or parasite infection is highly context dependent. Thus there is variation in immune response under different social or environmental circumstances including geography, distribution of parasite, age, social situation, and nutritional status. Therefore, any generalization made about sex differences in immunity is difficult. Additionally, it is important to study immune responses in a variety of different vertebrate and invertebrate species with different life histories and under various conditions to gather as much information as possible.

The results of this study suggests that more research needs to be done on eugregarine parasites and the impacts they have on their hosts *G. firmus.* Perhaps another unknown factor is affecting immunity responses in crickets, such as physiological modification due to parasitic infection or the immunity assay. As gregarines are intestinal parasites that do not roam freely in the body cavity, perhaps there is a better way we could test immunity response. Siva-Jothy *et al.* (2001) conducted a similar experiment where they also evaluated the immune responses of field-populated damselflies to eugregarine tophozooites. They used a phenol oxidase (PO) assay, which tested the insects haemolymph (blood) for the PO enzyme. Phenol oxidase is an enzyme released by an insect's humoral system that melanizes the haemocytes surrounding the immune challenge or pathogen. The amount of PO found and measured within the insect's body was subsequently related to parasitic infection. Therefore it is assumed that a higher PO level would indicate a greater infection of eugregarine parasites. Thus, a PO Assay may be the next step of this experiment. As stated by Adamo and colleagues (Adamo *et al.* 2001), insects are underused model systems for study of trade-offs between immunity and reproduction. Due to their short generation time, simpler immune systems, and a more straightforward relationship between reproductive effort and reproductive fitness, insect model systems can be extremely helpful in investigating links between immunity, behaviour, and reproduction. In addition, this experiment may provide insights for future studies on the possible control of eugregarine parasites and for treatment plans for the hosts. For example, previous studies suggest that the oral ingestion of chemical substances by the host may rid them of eugregarine parasites (Clopton and Smith, 2002). However, more research will need to be done on eugregarine resistance and host tolerance to chemicals and medication.

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Table 1. Comparison of independent sample tests of variances between males and females of Sand-field Crickets (*Gryllus firmus*) over a time period of approximately three months.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Mass (g)	Equal variances assumed	6.146	.016	-5.456	54	.000	-.28114	5.1530E-02	-.38445	-.17783
	Equal variances not assumed			-5.456	37.606	.000	-.28114	5.1530E-02	-.38549	-.17679
LG10(gregnum+1)	Equal variances assumed	3.194	.080	-1.199	53	.236	-.24266	.20240	-.64862	.16330
	Equal variances not assumed			-1.220	50.946	.228	-.24266	.19897	-.64212	.15680
MMEAN	Equal variances assumed	.319	.575	-.326	50	.746	-2.1897	6.7183	-15.6838	11.3045
	Equal variances not assumed			-.326	46.632	.746	-2.1897	6.7183	-15.7080	11.3287

Figure Legends:
Figure 1. Analysis of melanization intensity between male and female *G. firmus* crickets. A scatter plot graph shows that there is no significant difference between melanization intensity and sex.

Figure 2. Analysis of parasite loads between male and female *G. firmus* crickets. There is a significant decline in the number of gregarine parasites at 48 hours and over.

Figure 3. Analysis of correlation between eugregarine parasite number and body mass of male and female *G. firmus* crickets. Scatterplot depicts a significant positive correlation between body size and parasite numbers for males and females. Dividing parasite numbers by mass (to get a parasites per unit mass measure) did not reveal any statistically significant relationship between parasites and melanization, nor did the sexes differ on this new corrected measure of parasite numbers.

Figure 4. Analysis of melanization intensity over specified periods of time. The melanization intensity was calculated after being extracted from a *G. firmus* cricket after a set period of time as indicated on the horizontal axis. Melanization was lighter after 48 hours or more, however this was not statistically significant.

Figure 5. Analysis of the correlation between gregarine parasite load and melanization intensity between male and female *G. firmus* crickets. Male and female crickets are shown to have no significant relationship between melanization intensity and gregarine numbers.

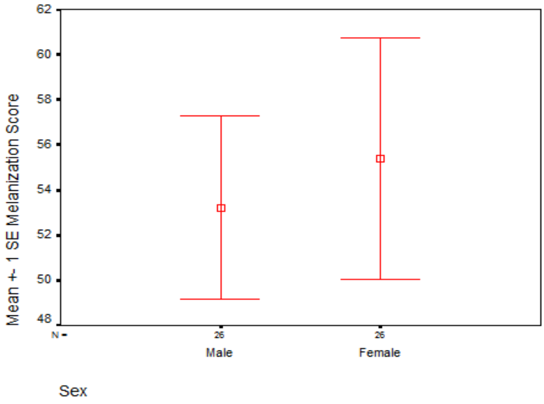

Figure 1. Analysis of melanization intensity between male and female *G. firmus* crickets.

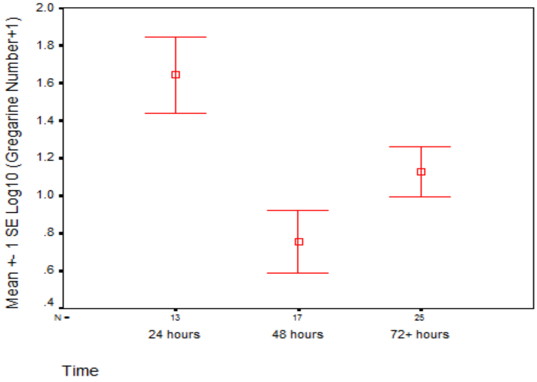

Figure 2. Analysis of parasite loads between male and female *G. firmus* crickets.

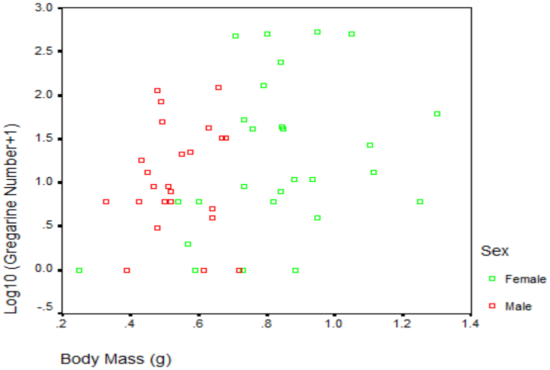

Figure 3. Analysis of correlation between eugregarine parasite number and body mass of male and female *G. firmus* crickets.

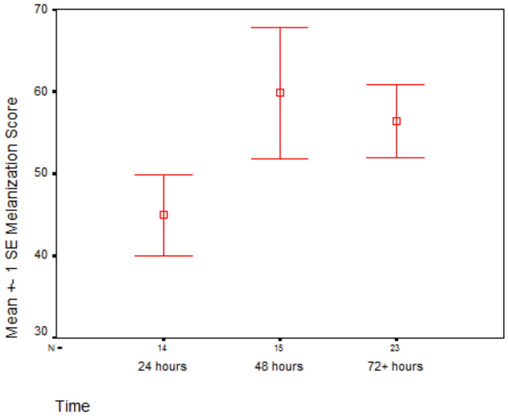

Figure 4. Analysis of melanization intensity over specified periods of time.

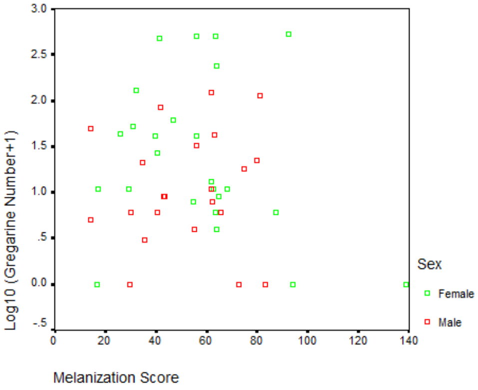

Figure 5. Analysis of the correlation between gregarine parasite load and melanization intensity between male and female *G. firmus* crickets.