Behavioral evidence of oxidative stress derived from haematophagy in the ocellar system of *Rhodnius prolixus* Stål, 1859 red-eyed mutants

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ABSTRACT

The photonegative responses to light stimulation in *Rhodnius prolixus* (Reduviidae, Triatominae) is modulated by compound eyes and ocelli. Screening pigments in the visual system have been shown to protect the cellular structures from oxidative stress damage generated by ingesting blood and light stimulation. Red-eyed mutants of *Rhodnius prolixus* lack screening pigments in compound eyes and ocelli and are exposed to oxidative stress. Experiments with *Rhodnius prolixus* and *Triatoma infestans* red-eyed mutants reared from first nymphal stage have shown a damage in the retina of the compound eyes and a decrease in the photonegative responses to light stimulation. Having in mind that ocelli are only present in the imaginal stages, we designed a group of experiments to test a possible damage of ocelli by oxidative stress mediated by blood ingestion in *Rhodnius prolixus* red-eyed mutants and wild type. We carried out behavioral experiments to evaluate the photonegative responses in adults exposed to different treatments including covering compound eyes or ocelli, and different blood feeding treatments in order to test our hypothesis. Our results show that ocelli in adults of *Rhodnius prolixus* can modulate the photonegative responses in red-eyed mutants better than the compound eyes. In addition, a decrease in photonegative responses was evident when the red-eyed mutants were fed with blood for up to four weeks. The ocelli in *Rhodnius prolixus* can be cataloged as a feedback visual system in the photonegative responses to light stimulation and screening pigments in the visual system are playing an important role to prevent damage by oxidative stress due to blood feeding.

Key words: Rhodnius prolixus, Ocelli, haematophagy, red-eyed mutant, screening pigment, photonegative behavior

1 INTRODUCTION

The visual system in insects is composed of both compound eyes and ocelli to receive the light of the environment (Gullan & Cranston, 2005). Compound eyes are composed by subunits called ommatidia; each one containing a cornea, a crystalline cone, six to nine rhabdomeres and retinular cells, pigmentary cells and screening pigments (Land, 1997). Compound eyes in insects fall in two main groups: apposition

compound eyes observed mainly in diurnal insects and superposition compound eyes found in nocturnal insects (Land, 1997).

The ocelli have fewer structures than the compound eyes, and they are composed by a corneal lens, a layer of corneagenous cells, a retina with a rhabdomeric zone, a medial zone with nucleus and an axonal zone, pigmentary cells and screening pigments (Parry, 1947). The function of the ocelli is varied and relevant in different species of insects (Lazzari et al., 2011). In dragonflies, ocelli can control the course and flight orientation (Cornwell, 1955; Wilson, 1978), and in *Drosophila* and *Calliphora* they can modulate the positive phototaxis (Goodman, 1981).

Triatomines (Reduviidae) are blood-sucking insects with medical importance because some species are involved in the vectorial transmission of Chagas Disease in America (Moncayo & Ortiz, 2006). Two species can be highlighted: *Triatoma infestans* transmitting Chagas disease in southern South America and *Rhodnius prolixus* transmitting Chagas disease in northern South America and Central America (Moncayo & Silveira, 2009).

The visual system in triatomines is formed by two apposition lateral compound eyes and two dorsal ocelli behind the compound eyes on the head (Settembrini, 1984). Functionally, both the ocelli and the compound eyes have been involved in the phototactic negative responses observed in adult triatomines (Lazzari et al., 1998). However, although the function is similar and seems redundant, the control of compound eyes and ocelli is different (Lazzari et al., 2011). An endogen circadian clock controls compound eyes while the ocelli are controlled by the environmental light available *(Lazzari et al., 2011)*. It is well known that the endogen circadian clock cause the following changes in the compound eyes: during the photophase the retinular and pigmentary cells form a "pupil" with a high amount of screening pigments and the rhabdomeres remaining down in the ommatidia; while during the scotophase, the screening pigments in the retinular and pigmentary cells widens, and the rhabdomeres ascend and remain close to crystalline cone (*Reisenman et al., 2002)*.

Although the control of the ocelli is mediated by the environmental light available and no migration in the ommatidia cells is observed, the amount and concentrations of screening pigments change accordingly to the presence of light arriving to the rhabdomeric zone in the ocelli (Lazzari et al., 2011). A large amount of light induces a concentration of screening pigments in the rhabdomeric zone of the ocelli to protect the cellular structures, while a fewer amount of light induces a lower concentration of screening pigments in the rhabdomeric zone of the ocelli to allow the detection of the dim light available under dark conditions (Lazzari et al., 2011).

Compound eyes can suffer damage and loss of sensitivity when the ommatidia are adapted to darkness and receive light (Lazzari et al., 2011). To avoid this damage, the ocelli can modulate the phototactic negative responses and induce in the triatomine to look for shelters in order to protect the compound eyes (Lazzari et al., 2011). Therefore, the ocelli can act as a feedback system of the compound eyes (Lazzari et al., 2011).

To avoid damage in the visual system, all insects (including triatomines) have screening pigments in their compound eyes and ocelli to protect the cellular structures, and this chemical compounds give the characteristic color to compound eyes and ocelli (Stavenga, 1989). The function of screening pigments are various: control of the incident photons that reach each ommatidia, control of the amount of light reaching the rhabdomeres (Stavenga, 1989) and protection of the cellular structures in the visual system by oxidative stress due to UV light and/or the heme-group ingested by blood-sucking insects (Stavenga, 1989; Graça-Souza et al., 2006; Insausti et al., 2013). Then, the screening pigments protect the compound eyes and ocelli for an optimum behavioral performance.

Triatomines exhibit akinesis during the photophase and they remain hidden in the crowns of palm trees or shelters in the walls of the houses and they start their activity by seeking blood sources during the scotophase (Guerenstein & Lazzari, 2009). Therefore the absence of screening pigments in the visual system could affect the behavioral responses to light stimuli (Insausti et al., 2013).

A recessive mutation in the visual system of triatomines due to the absence of screening pigments (Insausti et al., 2013) shows a red color in the compound eyes and ocelli of *Psammolestes coreodes* (Pellegrino & Brener, 1951)*, Triatoma infestans* (Moraes et al., 2005) and *Rhodnius prolixus* (Rosabal & Trejos, 1966).

Red-eyed mutants have been used previously as good models to test the effect of oxidative stress on the compounds eyes (Insausti et al., 2013). Results showed a deterioration of the cellular structures in each ommatidum and a lower performance in photonegative responses to light stimulation (Insausti et al., 2013). Compound eyes suffer more oxidative stress because they are exposed to the action of oxygen radicals since the first nymphal stages, meanwhile the ocelli only appears in adult stage (Settembrini, 1984) and consequently, they only start to suffer oxidative stress damage after the last ecdysis.

Having in mind that the effect of oxidative stress on ocelli of mutant individuals is unknown, the present work aims to evaluate behaviorally under laboratory conditions, the effect of oxidative stress by blood feeding on the ocellar system of individuals wild type and red-eyed mutants of *Rhodnius prolixus* Stål, 1859.

2 MATERIALS AND METHODS

2.1 Animals

Insects used in the experiments were obtained from a laboratory colony maintained in Centro de Investigaciones en Microbiología y Parasitología Tropical (CIMPAT) at Universidad de los Andes. Adults wild type and red-eyed mutants of *Rhodnius prolixus* were reared in the colony at 27 ± 2 °C, 75 ± 10 % relativity humidity and maintained under an artificial 6:00/18:00 h light/darkness illumination regime. Insects were fed *in vivo* weekly with hen's blood.

All individuals used in experiments were adults freshly molted to ensure no damage in the ocellar system (Insausti et al., 2013). All insects tested had in average a weight of 0.0381 g that corresponds to approximately 15 days of starvation (Ortiz & Molina, 2010). The number of individuals for each experimental treatment was 20 and each insect was tested only once during the early scotophase (18:00 h to 20:00 h) (Reisenman et al., 1998).

2.2 Experimental arena

All experiments were conducted in a dark room at 27 °C. The experimental arena was modified from that used by Reisenman & Lazzari (2006). Experiments were carried out in a transparent acrylic rectangular arena (32.5 x 25 x 2.2cm) with four lines (5.5 cm width) and filter paper used as substrate. Half of the arena was kept dark with a black acrylic (2 mm thickness), and the other half remained uncovered (Lazzari et al., 1998). The uncovered half was illuminated by four white light LEDs located 30 cm above the experimental arena (Fig. 1). All LEDs were powered with a 9-volt battery and controlled with a potentiometer to ensure a 6 $\mu W/cm^2$ light intensity (Reisenman et al., 1998) measured with a radiometer-photometer (ILT 1400-A) at the level of the arena.

Insects were transported to the experimental arena in a small dark box where they remained for 40 seconds to reduce the stress for manipulation and then released in the dark half of the arena (Reisenman & Lazzari, 2006). The experiments were recorded for 5 minutes with a camera with infrared night vision (Sony, DCR-SR 200) located above to 35 cm of the experimental arena (Lazzari et al., 1998).

All videos were analyzed with a video Tracker analysis program. At the end, for each individual was registered the time spent in darkness. The orientation of the experimental arena was modified by turning 90° its position after each trial. Also the dark side of the arena was changed after each trial. Paper filters used as substrate in the experimental arena was replaced after ending each trial to avoid chemical cues.

2.3 Experimental procedure

2.3.1 Photonegative responses in insects wild type and red-eyed mutants of *Rhodnius prolixus*

As a control to evaluate the effect of the varnish on the mobility of *Rhodnius prolixus*, we separated insects in two groups: Those with the visual system intact; and 2) Insects with visual system totally covered with varnish. The behavioral responses of groups were evaluated in darkness.

To evaluate the photonegative responses in adults wild type and red-eyed mutants of *Rhodnius prolixus* the insects underwent four treatments: 1) Insects with no treatment in the visual system and no lights on the experimental arena; 2) Insects with no treatment in the visual system and white lights on the experimental arena; 3) Insects with the ocelli covered with black varnish and white lights on the experimental arena; and 4) Insects with the compound eyes covered with black varnish and white lights on the experimental arena.

2.3.2 Oxidative stress effect by blood feeding on the photonegative response of wild type and red-eyed mutants of *Rhodnius prolixus*

To evaluate the oxidative stress effect of blood feeding on the phototactic responses mediated by the ocellar system, wild type and red-eyed mutants of *Rhodnius prolixus* were fed for one, two, three or four consecutive weeks. All insects tested in these experiments had two additional weeks of rest before starting the experiments and all of them had their compound eyes covered with black varnish. Also to evaluate and separate the effect of the blood feeding and aging on the photonegative responses, two groups of *Rhodnius prolixus* wild type were separated, one of them were fed by four consecutive weeks and lost weight for two additional weeks in a total of 45 days, the other group stayed during 45 days without feeding. Also in all these cases the compound eyes were covered with black varnish in all individuals.

The weight of each insect was recorded individually after molting, one day before feeding and after feeding. Insects were weighted with a balance (Ohaus Galaxy G 160). The volume of blood ingested by *Rhodnius prolixus* was calculated by subtracting the weight after - before feeding and using a density of the blood in the hen of 1.0081 g/ml (Mejia-Jaramillo et al., 2009).

2.4 Statistical analysis

The normality of the data was tested in all cases with a Shapiro-Wilk test. For non-normal data a Mann-Whitney test was used to evaluate the differences in photonegative responses measured. The differences in photonegative responses between treatments in the behavioral responses to light stimulation was verified with an ANOVA test in wild type and red-eyed mutants of *Rhodnius prolixus*. Finally, all data were unified in order to analyze them with an ANOVA with interaction term. A test of Tukey was used in all cases to determine which treatments were showing statistical differences.

The normality of the weight data was tested with a Shapiro-Wilk test. Statistical differences in weight between males and females (after ecdysis, one day before feeding and after feeding) were tested with an ANOVA test. A test of Tukey was used to determine statistical differences. A t-student test was used to determine differences between sexes in the feeding of *Rhodnius prolixus*.

The levels of significance used in statistical analyses were $P< 0.05$, $P< 0.01$ and $P< 0.001$.

3 RESULTS

3.1 Photonegative responses in *Rhodnius prolixus* wild type and red-eyed mutants

The effect of covering the visual system with varnish on the mobility of *Rhodnius prolixus* was null (Mann-Whitney test, $P = 0.828$). For this reason, we excluded the varnish as a possible variable that could have an effect on the experiments and modify the time that the insects spent in darkness (Fig. 2).
The time spent in darkness by *Rhodnius prolixus* wild type with different treatments in the visual system showed statistical differences (ANOVA test, $F = 113.4$, $d.f. = 3$, $P = 2e\text{-}16$). Insects with the compound eyes or the ocelli covered were not significantly different between them (Tukey test, $P = 0.852$). However differences were observed between insects without any treatment and the lights on and those insects with lights on and any treatment in the compound eyes or the ocelli (Tukey test, $P = 0.000$). Control insects with visual system not covered and without light showed highly significant differences with the other treatments (Tukey test, $P = 0.000$) (Fig. 3).
In the case of the time spent in darkness by *Rhodnius prolixus* red-eyed mutants with different treatments in the visual system also statistical differences were observed (ANOVA test, $F = 48.91$, $d.f. = 3$, $P = 2e\text{-}16$). All treatments showed also statistical differences between them, except for the insects with compound eyes covered and those insects with the visual system without treatment and lights on (Tukey test, $P = 0.323$) (Fig. 4).
The ANOVA with interaction term comparing the time spent in darkness by wild type and red-eyed mutants of *Rhodnius prolixus* showed significant differences ($F = 8.163$, $d.f. = 3$, $P = 4.49e\text{-}05$). A comparison between wild type and red-eyed mutants with the ocelli covered showed significant differences (Tukey test, $P = 0.011$). No significant differences were observed between wild type and red-eyed mutants in the other treatments (Fig. 5).

3.2 Oxidative stress effect by blood feeding on the photonegative responses of wild type and red-eyed mutants of *Rhodnius prolixus*

3.2.1 Variations in the weight of the insects

Males and females (n= 85 and 75 respectively) of *Rhodnius prolixus* were weighted including wild type and red-eyed mutants. A comparison of the individual body weights obtained showed statistical differences between the three different times (ANOVA test, $F = 131.3$, $d.f. = 5$, $P = 2e\text{-}16$). Males and females after ecdysis and one day before feeding showed significantly differences in body weights in comparison with those insects after feeding (Tukey test, $P = 0.000$). No statistical differences were observed between males and females after ecdysis and males and females one day before feeding (Fig. 6).
A comparison between males and females after ecdysis and one day before feeding showed no significant differences (Student's t-test, $P = 0.819$ and $P = 0.726$ respectively). Only adult males and females shortly fed showed statistical differences in their body weights (Student's t-test, $P = 0.000$) (Fig. 6).

3.2.2 Photonegative responses

The effect of blood feeding on the time spent in darkness by *Rhodnius prolixus* wild type with the different blood feeding treatments showed statistical differences (ANOVA test, $F = 4.464$, $d.f. = 3$, $P = 0.006$). Insects with four consecutive weeks of feeding showed a reduction in the photonegative responses in comparison

to insects with two or three consecutive weeks of feeding (Tukey test, *P* = 0.038 and *P* = 0.005 respectively). The comparisons between the others treatments did not show any significant differences (Fig. 7). The total amount of blood ingested showed that in average *Rhodnius prolixus* wild type obtained 0.034 ± 0.016 ml after one week, 0.079 ± 0.012 ml after two, 0.125 ± 0.016 ml after three, and, 0.129 ± 0.014 ml after four weeks.

A comparison of the effect of blood feeding and aging on the photonegative responses due to the ocellar system showed a reduction in insects with blood feeding and 45 days in laboratory conditions in comparison with insects without blood feeding and the same time in laboratory (Mann-Whitney test, *P* = 0.025) (Fig. 8).

The same treatments applied to *Rhodnius prolixus* red-eyed mutants showed also statistical differences (ANOVA test, *F* = 28.17, *d.f.* = 3, *P* = 2.37e-12). In this case, insects with one week feeding showed statistical differences with insects with three and four weeks of blood feeding (Tukey test, *P* = 0.000 for both comparisons). Also insects with two weeks of blood feeding showed statistical differences with insects with three and four weeks feeding (Tukey test, *P* = 0.000 for both comparisons) (Fig. 9). The total amount of blood ingested by red-eyed mutants showed that in average insects obtained 0.052 ±0.026 ml after one week, 0.074 ± 0.031 ml after two, 0.091 ± 0.030 ml after three, and, 0.114 ± 0.033 ml after four weeks.

Finally, a comparison of the time spent in darkness between wild type and red-eyed mutants of *Rhodnius prolixus* with different blood feeding treatments showed significant differences (ANOVA with interaction term, *F* = 10.88, *d.f.* = 3, *P* = 1.63e-06). Treatments with two, three and four weeks of blood feeding showed differences between wild type and red-eyed mutants (Tukey test, *P* = 0.010, *P* = 0.000 and *P* = 0.000 respectively). No significant differences were observed in insects with one week of blood feeding (Tukey test, *P* = 0.861) (Fig. 10).

4 DISCUSSION

4.1 Photonegative response in wild type and red-eyed mutants of *Rhodnius prolixus*

The results showed that wild type and red-eyed mutants of *Rhodnius prolixus* have a clear photonegative response to light stimulation (Figs. 3-5). In both groups, control insects (no treatment in the visual system and no light) spent approximately the same time in covered and uncovered places in the experimental arena (Figs. 3-5 -VSwoL-). These results confirm that compounds eyes and ocelli have not been stimulated and that phototactic behavior has not been elicited.

The photonegative behavioral responses observed in wild type *Rhodnius prolixus* were similar to those reported previously for *Triatoma infestans* by Lazzari et al. (1998). Covered compound eyes and ocelli spent similar times in darkness (Fig. 3 -CECovers and Ocovers-), but considerable less time in comparison to insects with the compound eyes and ocelli uncovered and with light stimulation (Fig. 3 -VSwL-). These results together confirm that in *Rhodnius prolixus* wild type the photonegative response is mediated by the interaction between compound eyes and ocelli (Fig. 3). Similar results have been reported for *Triatoma infestans* (Lazzari et al., 1998).

For *Rhodnius prolixus* red-eyed mutants the results are different (Fig. 4), and a comparison with the wild type showed statistical differences in the photonegative responses (Fig. 5). The absence of screening pigments in the compound eyes and the ocelli have a direct effect on the behavioral responses to light stimulation. Clearly a reduction in the time spent in darkness was observed in those insects with all the visual system intact (Fig. 4 -VSwL-) and the ocelli covered (Fig. 4 -Ocovers-).

Having in mind that one of the functions of the screening pigments is the protection of the cellular structures by acting as antioxidants to stop the activity of the free radicals e.g. oxidative stress in the visual system (Insausti et al., 2013). It can be suggested that in our experiments the damage caused by oxidative stress to the ommatidia is also taking place and affecting the time that the insects spent in darkness. Something similar was reported by Insausti et al. (2013). A damage in the compound eyes and its effect on the photonegative responses is highlighted by the lost of light detection in red-eyed mutants with the ocelli covered (Fig. 4 -Ocovers-). All together, our results confirm that the ocellar system in *Rhodnius prolixus* is playing an important role together with the compound eyes in the regulation of the photonegative responses (Fig. 4).

However a comparison of individuals only with the ocelli covered and insects only with the compound eyes covered showed statistical differences in the time spent in darkness (Fig. 4 -Ocovers y CECovers-). To explain these differences it is important to consider that the compound eyes in triatomines are exposed to oxidative stress since the first nymphal stages, while ocelli are only present in adult stages (Settembrini, 1984). Insects used in our experiments were tested after the last nymphal ecdysis. In that case, the ocellar system has not suffered damage and the ocelli are intact to get better responses to light stimulation in comparison to the compound eyes (Insausti et al., 2013).

Although the ommatidia in the compound eyes suffer oxidative stress the group with ocelli cover showed a photonegative response to light stimulation (Fig. 4 -Ocovers-). *Rhodnius prolixus* pass through five nymphal stages before to reach the adult stage and after each molt new peripheral ommatidia are added to compound eyes (Insausti et al., 2013). The new peripheral ommatidia added to the compound eyes in the adult stage have not been exposed to oxidative stress and they are the only functional to receive the light *stimulation* (Insausti et al., 2013).

As expected, the presence or absence of the screening pigments modifies the photonegative responses to light stimulation in triatomines (Insausti et al., 2013). Insects with presence of screening pigments have not damage in the cellular structure in the visual system (Insausti et al., 2013). The responses to light stimulation in wild type insects were only modified if any visual structure was covered (Fig. 3). *Rhodnius prolixus* wild type can use either compound eyes or ocelli to receive the environmental light and induce a behavioral response. However, *Rhodnius prolixus* red-eyed mutants have not screening pigments in their visual system and the compound eyes and ocelli are exposed to cellular damage by oxidative stress (Insausti et al., 2013). All treatments realized here in the visual system of *Rhodnius prolixus* red-eyed mutants highlight the relevance of the ocelli and the protective role of the screening pigments in the photonegative responses (Fig. 5). The comparison between treatments in wild type and red-eyed mutant insects showed differences in the group with ocelli covered and the group with the visual system intact and exposed to light (Fig. 6 -Ocovers andVSwL-). In red-eyed mutant insects covering the ocelli left almost blind the adults due to the accumulative damage of the ommatidia during their postembryonic development (Insausti et al., 2013).

Adults of *Rhodnius prolixus* red-eyed mutants are highly dependent on ocelli and not on compound eyes for photonegative behavioral responses. If that is true, oxidative stress by successive blood ingestion should have an effect on the behavioral responses in adults of *Rhodnius prolixus* red-eyed mutants.

4.2 Oxidative stress effect by blood ingestion on the photonegative response in *Rhodnius prolixus* wild type and red-eyed mutants

Our experiments with different treatments of blood feeding showed the important consequences of oxidative stress caused by blood ingestion in the photonegative responses of *Rhodnius prolixus* wild type and red-eyed mutants.

All wild type and red-eyed mutants showed a photonegative response to light stimulation (Figs. 3 to 5), but these responses can be modified by the mutant condition in the visual system and the amount of blood ingested for several weeks. *Rhodnius prolixus* wild type showed a photonegative response through the weeks (Fig. 7) and this response could be attributed only to the ocellar system because the compound eyes were in all cases covered. The ingestion of blood did not produce any damage to the ocellar system during the first three weeks (Fig. 7; 1, 2, and 3 Weeks) because wild type insects have screening pigments to protect the retina (Insausti et al., 2013). However a statistical difference in the time spent in darkness was observed in those insects with four weeks of blood feeding (Fig. 7; 4 Weeks). In this case the photonegative response is reduced in comparison with the other treatments (Fig. 7). Apparently the ocellar system suffered damage in the retina and the time spent in darkness was modified. The damage in the ocelli can be originated by two causes: oxidative stress by blood ingestion and heme-group activity (Graça-Souza et al., 2006) or by aging of cellular structures and reduced body mobility of the insects (Ridgel & Ritzmann, 2005).

The aging is a natural phenomenon that has several effects on all body receptors (Ridgel & Ritzmann, 2005). In insects, it has been reported a reduction in locomotors activity in cockroaches (Ridgel & Ritzmann, 2005) and a reduction in the size of the brain when old adults are compared with young adults (Kern, 2012). We do not know the effect of aging on triatomines, but if this physiological process is having an effect on the mobility of the insects it can only be observed in adults older than 45 days (decrease in activity after four weeks of feeding and two weeks of body weight reduction).

On the other hand, it is possible that the amount of blood ingested by triatomines during their life cycle has an accumulative effect product of the oxidative stress of the visual system even with the presence of the screening pigments. Wild type insects with four weeks of blood feeding has ingested an average of 0.12914375 ml of hen's blood, and it is possible that this high amount of blood ingested affect the cellular structures of the visual system over time. *Rhodnius prolixus* wild type has screening pigments in the ocellar system in order to optimize the behavioral performance in the photonegative responses (Insausti et al., 2013). Although the screening pigments are in the visual system to protect them, the aging and the amount of blood ingested could have a synergistic effect on the photonegative responses in adults of *Rhodnius prolixus*. According with our results, especially the blood ingested. The time spent in darkness in the groups with 45 days under laboratory conditions is only modified when one of these groups has been feeding with blood by four consecutive weeks. Although the aging is a possible precursor of the slow response in the photonegative behavior, the ingested blood adds a possible extra damage to the retina of the ocelli, and then, as a consequence, a slow performance in the photonegative responses (Fig. 8).

Rhodnius prolixus red-eyed mutants present a gradual reduction in the photonegative responses to light stimulus through the four weeks of the experiment (Fig. 9). The amount of blood ingested has in this case a clear effect on the photonegative response of *Rhodnius prolixus*. Visual systems without screening pigments suffer a marked damage by oxidative stress (Insausti et al., 2013). In this particular case the ingested blood resulted in a release of the heme prosthetic group as a byproduct of the Hemoglobin degradation (Graça-Souza et al., 2006).

The heme-group is a lipophilic anion that produces damage at the cellular level in three different ways: inhibition of proteins, disruption of the cellular membranes and different electron transference that generate species of reactive oxygen (Sigala & Goldberg, 2014). This species of reactive oxygen, a kind of free radicals, produced by the ferrous group located in central structure, generate a destabilization into cellular membranes and finally a rupture in the cell (Sigala & Goldberg, 2014). To defend the cellular structures against heme-group by ingestion of blood, some haematophagous species have developed various strategies to reduce the free radicals formation and disability the heme deleterious function (Graça-Souza et al., 2006). The strategies to scavenge the environment from excessive heme-group are antioxidant enzymes, heme-binding proteins and heme aggregation (Graça-Souza et al., 2006). The heme aggregation is the most beneficial strategy of defense against the heme-group. This mechanism produces a simple sterical hindrance known as hemozoin to disable the heme function (Graça-Souza et al., 2006). In *Rhodnius prolixus* has been reported that hemozoin in the middle gut contains the 70% of the heme-group ingested (Oliveira et al., 1999; Graça-Souza et al., 2006). Although *Rhodnius prolixus* have a different ways to defend against heme-group, the body not eliminate all prosthetic groups and the residuals can occupy different places (Graça-Souza et al., 2006) like the visual system. In this particular case, *Rhodnius prolixus* red-eyed mutants without screening pigments and the exposition to heme-group in compound eyes and ocelli may has as a consequence a damage at the level of the cellular structures *(*Insausti et al., 2013*).*

The exposition of the cornea, the pigmentary cells and the retinular cells to the free radicals as a product of the degradation of the Hemoglobin can produce damages in the ocelli and a reduction of the photonegative response observed (Fig. 9). If the retina presents a progressive damage through several weeks by the ingestion of blood, few visual structures can be stimulated by light and the threshold response should be reduced.

The relation between the amounts of blood ingested and reduced photonegative responses are evident through the weeks. Insects with only one week of feeding showed a photonegative response close to 67.23 % (Fig. 9; 1 Week) and an amount of blood ingested not exceeding an average of 0.052 ml. Insects with two weeks of feeding showed a similar photonegative response (Fig. 9; 2 Weeks, 66.11 %)and an average amount of blood ingested of 0.074 ml. Nevertheless, the time spent in darkness and the blood ingested by insects with three and four consecutive weeks of blood ingestion was different in comparison with the previous first two treatments. The time spent in darkness was reduced to 60.59% and 57.905 % respectively (Fig. 9; 3 and 4 Weeks). Also the amount of blood ingested exceeded an average of 0.091 ml. As the feeding was carried out each 8 days, the intervals of time to lose weight were really short. That could be an explanation for the similar photonegative responses observed between one and two weeks of blood feeding, and also between three and four weeks of blood feeding (Fig. 9). The differences in the average of blood ingested between the first and the second weeks were low (0.022 ml); and something similar was observed between the third and the fourth weeks of feeding (0.022 ml). This could happen because in the first feeding all insects ingest a high amount of blood and in the second feeding the insects have not lost enough weight after only eight days. Although all insects ingested blood in the second feeding, the average weight is low in comparison with the first feeding. Fifteen days after the first feeding, all insects ingested a higher amount of blood again, but as the next feeding event happens (fourth week), a similar situation was observed like in the second feeding.

A comparison between wild type and red-eyed mutants of *Rhodnius prolixus* and the effect of blood feeding showed the relevance of ocellar system in the photonegative responses (Fig. 10). Only the first feeding treatment presented in both cases similar results (Fig. 10; 1Week). Apparently until that point,

the ocelli are not presenting a significant damage as a consequence of the amount of blood ingested. However, in the way that the amount of blood ingested and the time for oxidative stress increases, the red-eyed mutants start to show dramatically damages in the ocellar system and their behavioral responses associated (Fig. 10; 2, 3, and 4 Weeks).

The importance of the ocellar system in the photonegative responses of triatomines has been observed again in this work. The function of the ocelli is varied between insects e.g. in dragonflies the modulation of muscular tone in flight, balance control (Rowell & Pearson, 1983) and in flies the walking orientation (Wehrhahn, 1984). Triatomines are predominant walking insects and their ocelli modulate the photonegative responses to light stimuli as well as the compound eyes (Lazzari et al., 1998). The occlusion or damage of the compound eyes in the imaginal stage can be replaced by the ocellar system to modulate the negative photonegative responses as was proved in this work using individuals wild type and red-eyed mutants. The ocellar system can modulated the photonegative responses in both conditions (red-eyed mutants and wild type) although in *Rhodnius prolixus* red-eyed mutants the responses decreased markedly by the absence of the screening pigments and the effect of the oxidative stress generated by blood ingestion.

The haematophagous triatomines uses multiple cues to detect a vertebrate host (Guerenstein & Lazzari, 2009); and visual and chemical cues are used to return to some shelters during the photophase (Guerenstein & Lazzari, 2009). Compound eyes and ocelli are involved in the detection of these visual cues in order to find a place to hide. Without this visual information triatomines are in danger. So, having two visual inputs with different times of postembryonic development could be an advantage for triatomines. Although the compound eyes and ocelli have a similar function in the photonegative responses in triatomines (Lazzari et al., 1998), the ocelli can be cataloged as replacement structures in the perception of light stimuli in triatomines and modulate the photonegative responses in absence of the compound eyes.

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Fig. 1. Experimental arena to evaluate the photonegative responses in *Rhodnius prolixus*. (a) Camera with infrared night vision, (b) LED with white light, (c) Rectangular arena of transparent acrylic with four lines, (d) black acrylic, (e) white filter paper, (f) 9-volt battery, (g) electrical circuit.

Fig. 2. Effect of varnish on the photonegative responses of *Rhodnius prolixus* wild type. The two groups were in darkness during the experiment. **NoVarnish:** Visual system without treatment; **Varnish:** Visual system totally covered.

Fig. 3. Photonegative responses in adults of *Rhodnius prolixus* wild type after ecdysis. **CECovers:** Compound eyes covered; **Ocovers:** Ocelli covered; **VSwL:** Complete visual system with light; **VSwoL:** Complete visual system without light. *** = $P < 0.001$.

Fig. 4. Photonegative responses in adults of *Rhodnius prolixus* red-eyed mutants after ecdysis. **CECovers:** Compound eyes covered; **Ocovers:** Ocelli covered; **VSwL:** Complete visual system with light; **VSwoL:** Complete visual system without light. *** = $P< 0.001$.

Fig. 5. Comparison of the photonegative responses between wild type and red-eyed mutants of *Rhodnius prolixus* after ecdysis. Black symbols correspond to wild type insects and red symbols correspond to red-eyed mutants. **CECovers:** Compound eyes covered; **Ocovers:** Ocelli covered; **VSwL:** Complete visual system with light; **VSwoL:** Complete visual system without light.

Fig. 6. Males and females comparisons of the amount of blood ingested by *Rhodnius prolixus* after ecdysis, before feeding and after feeding. **1.female** and **1.male:** Adults after ecdysis; **2.female** and **2.male:** Adults one day before feeding; **3.female** and **3.male:** Adults after feeding. *** = $P< 0.001$.

Fig. 7. Photonegative responses with adults of *Rhodnius prolixus* wild type after ecdysis. **1Week:** One week with blood feeding; **2Weeks:** Two weeks with blood feeding; **3Weeks:** Three weeks with blood feeding; **4Weeks:** Four weeks with blood feeding. * = $P< 0.05$, ** = $P< 0.01$.

Fig. 8. Comparison of the photonegative responses between individuals with aging and blood feeding treatments in *Rhodnius prolixus* wild type. **Aging:** Individuals with aging from freshly molted to adult stage (45 days); **Feeding:** Individuals with aging and blood feeding by 4 weeks from freshly molted to adult stage (45 days). * = $P < 0.05$.

Fig. 9. Photonegative responses in adults of *Rhodnius prolixus* red-eyed mutants after ecdysis. **1Week:** One week with blood feeding; **2Weeks:** Two weeks with blood feeding; **3Weeks:** Three weeks with blood feeding; **4Weeks:** Four weeks with blood feeding. *** = $P < 0.001$.

Fig. 10. Comparison of the photonegative responses between wild type and red-eyed mutants of *Rhodnius prolixus* with different feeding treatments. Black symbols identify wild type insects and the red symbols identify red-eyed mutants.**1Week:** One week with blood feeding; **2Weeks:** Two weeks with blood feeding; **3Weeks:** Three weeks with blood feeding; **4Weeks:** Four weeks with blood feeding.

Figure 1

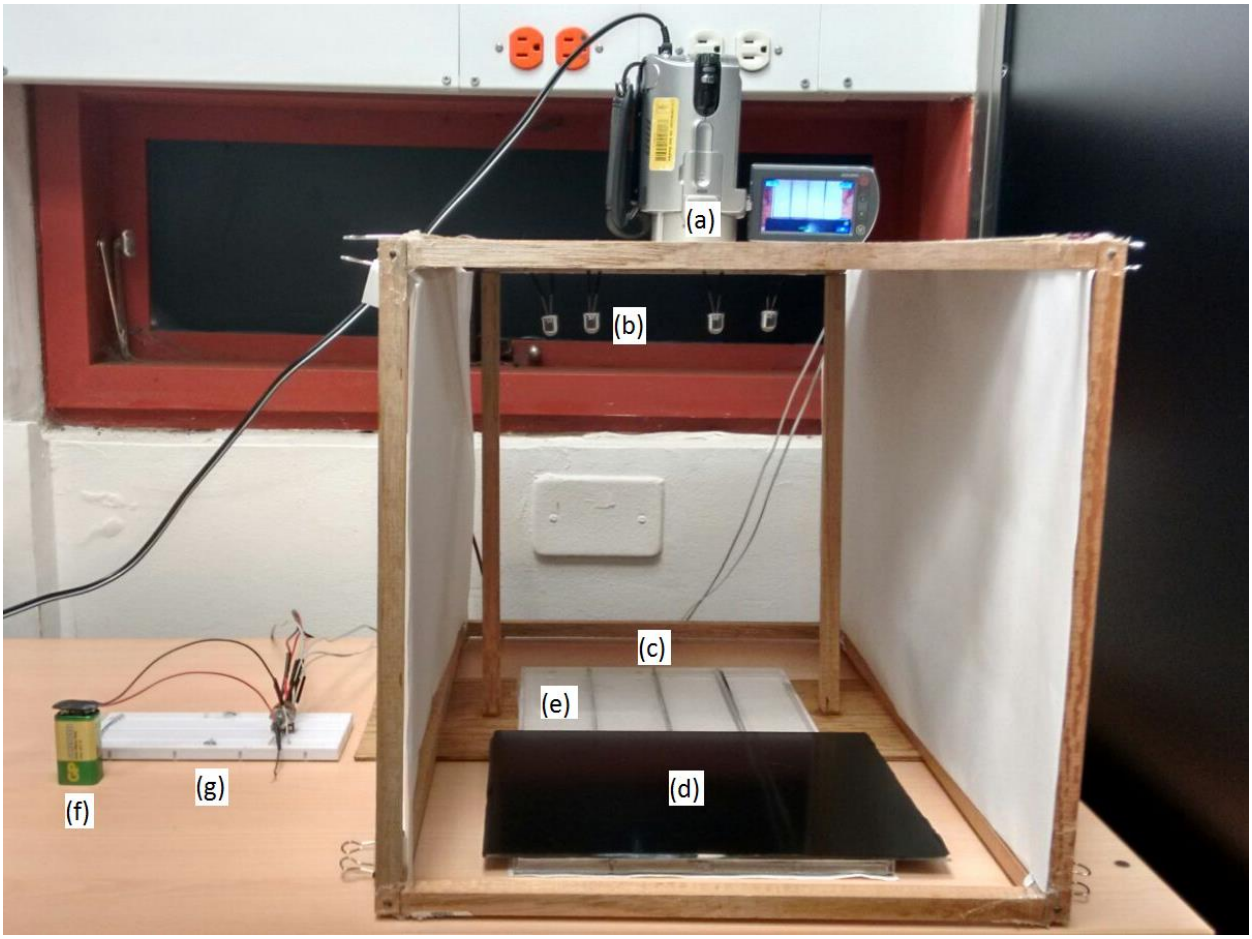


Figure 2

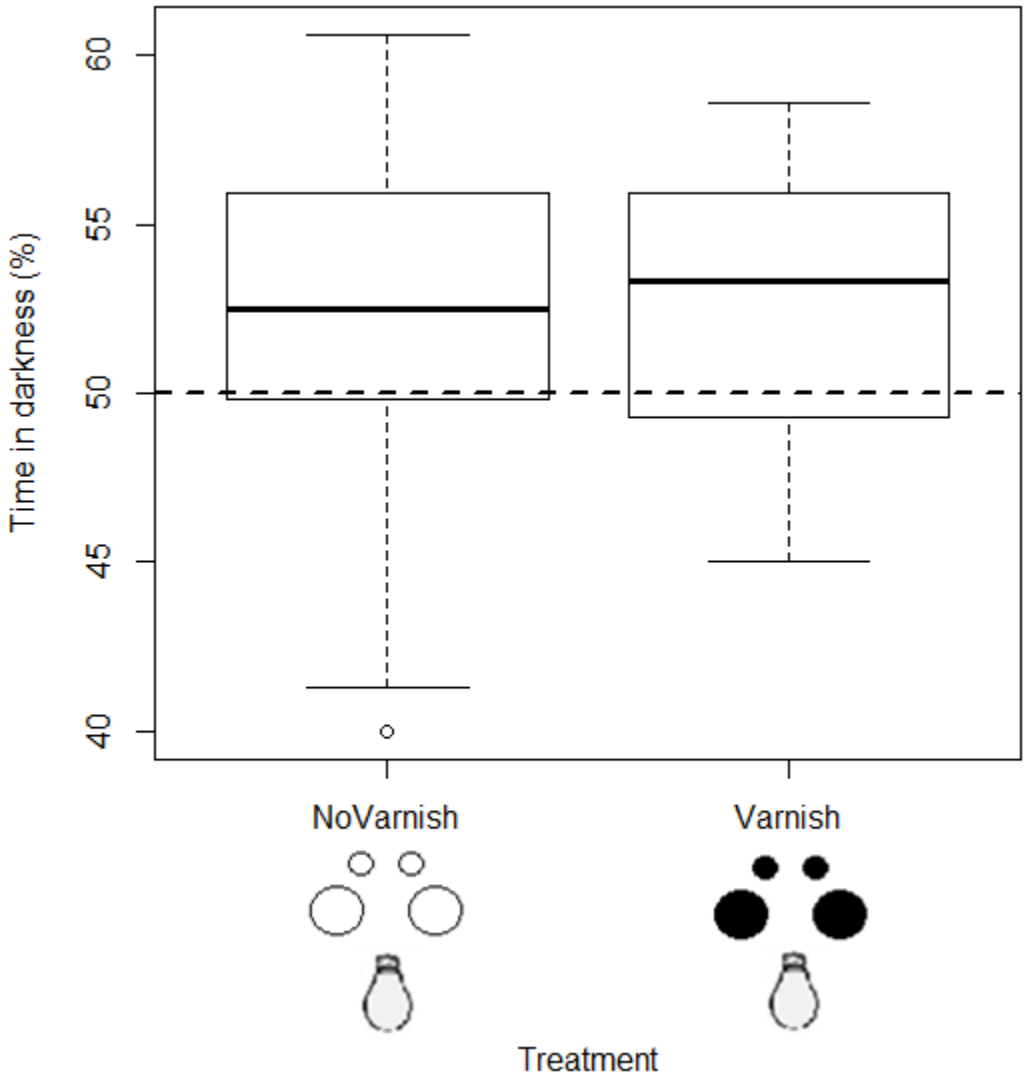


Figure 3

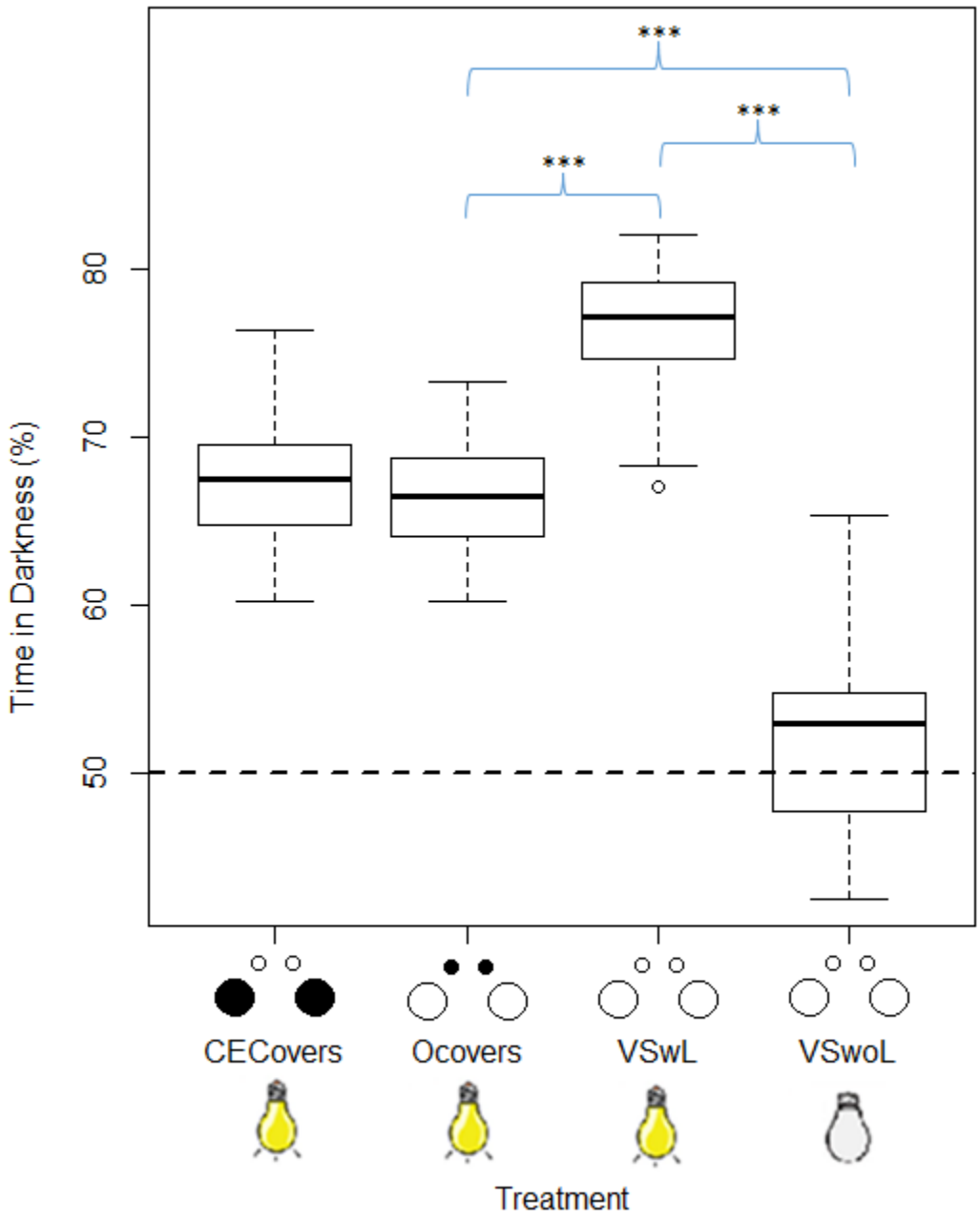
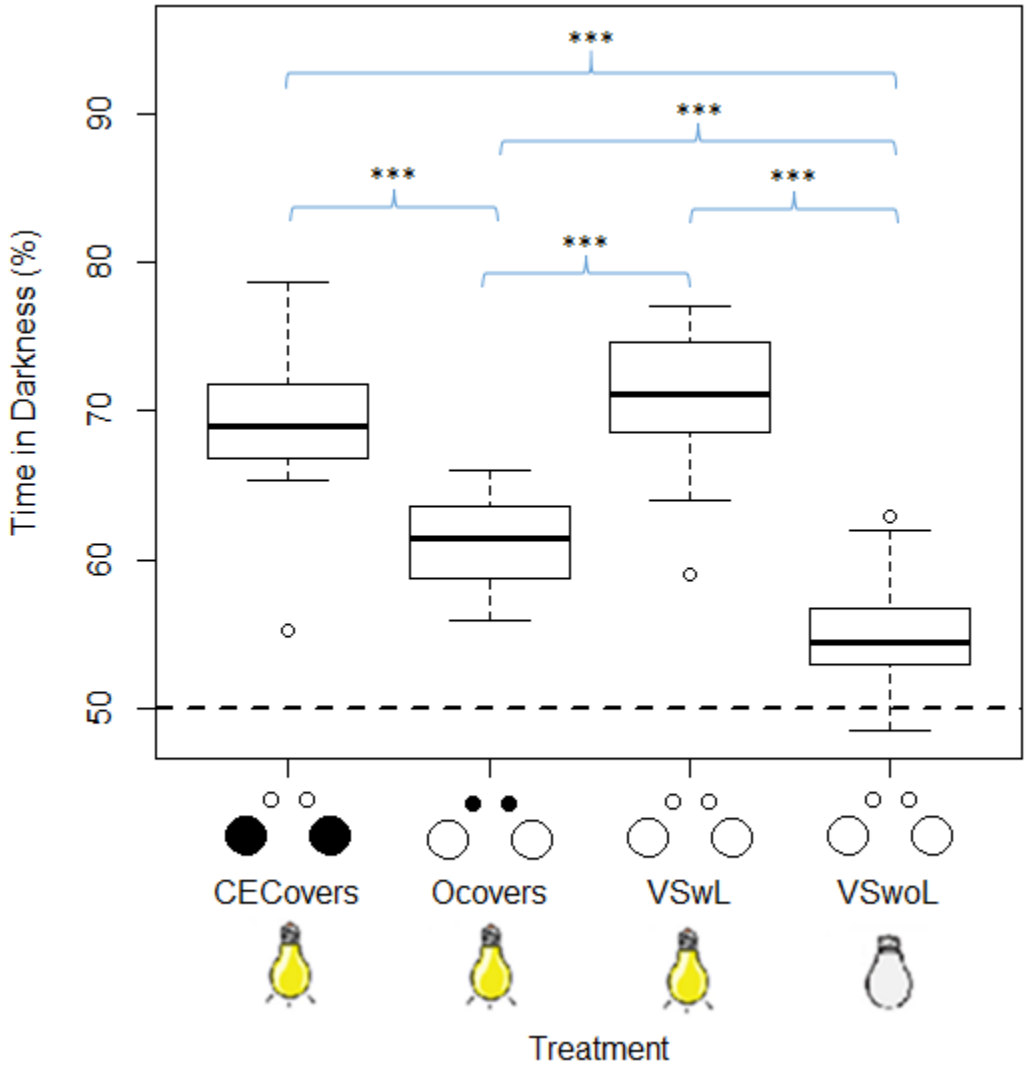

***
***
***
Time in Darkness (%)
50
60
70
80
CECovers
Ocovers
VSwL
VSwoL
Treatment

Figure 4


***
***
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***
Time in Darkness (%)
50
60
70
80
90
CECovers
Ocovers
VSwL
VSwoL
Treatment

Figure 5

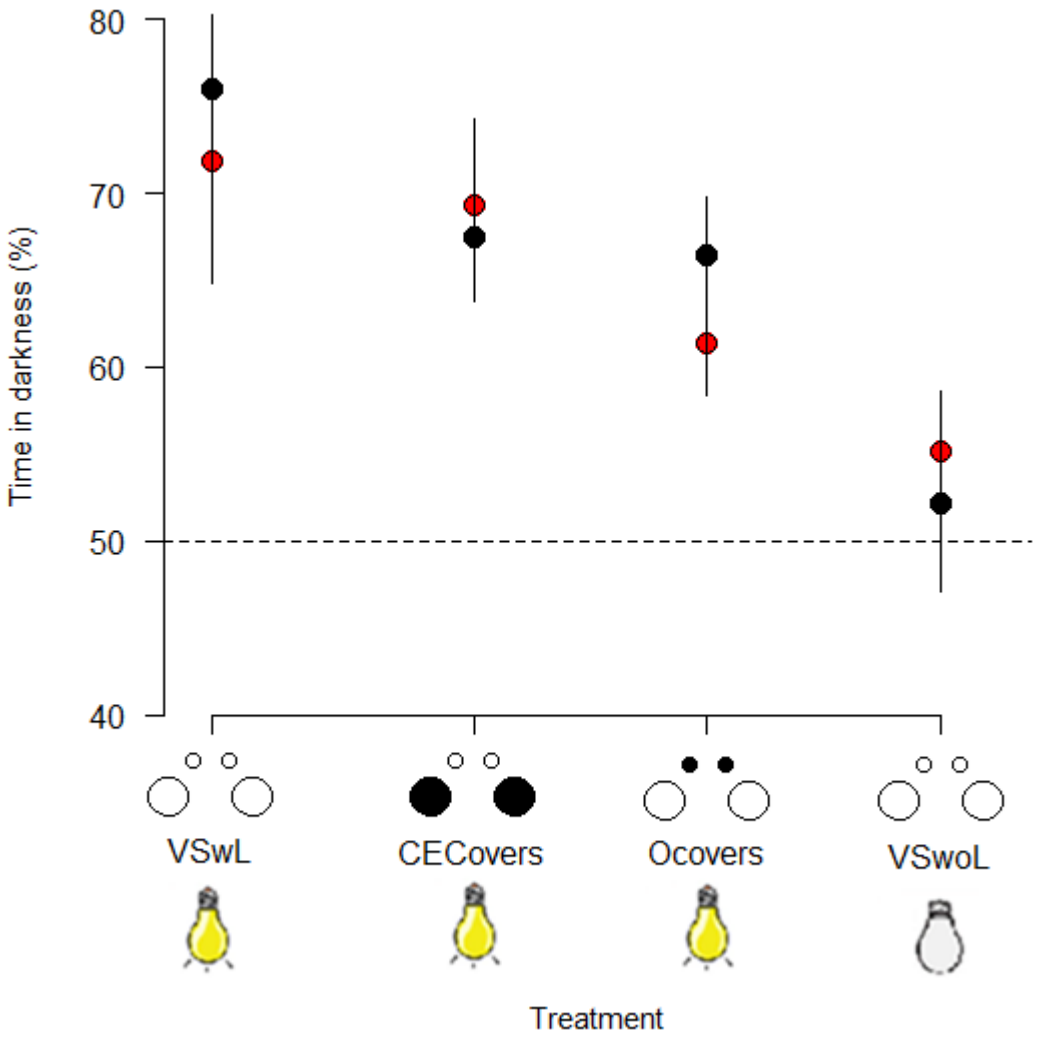

80
70
60
50
40
Time in darkness (%)
VSwL
CECovers
Ocovers
VSwoL
Treatment

Figure 6

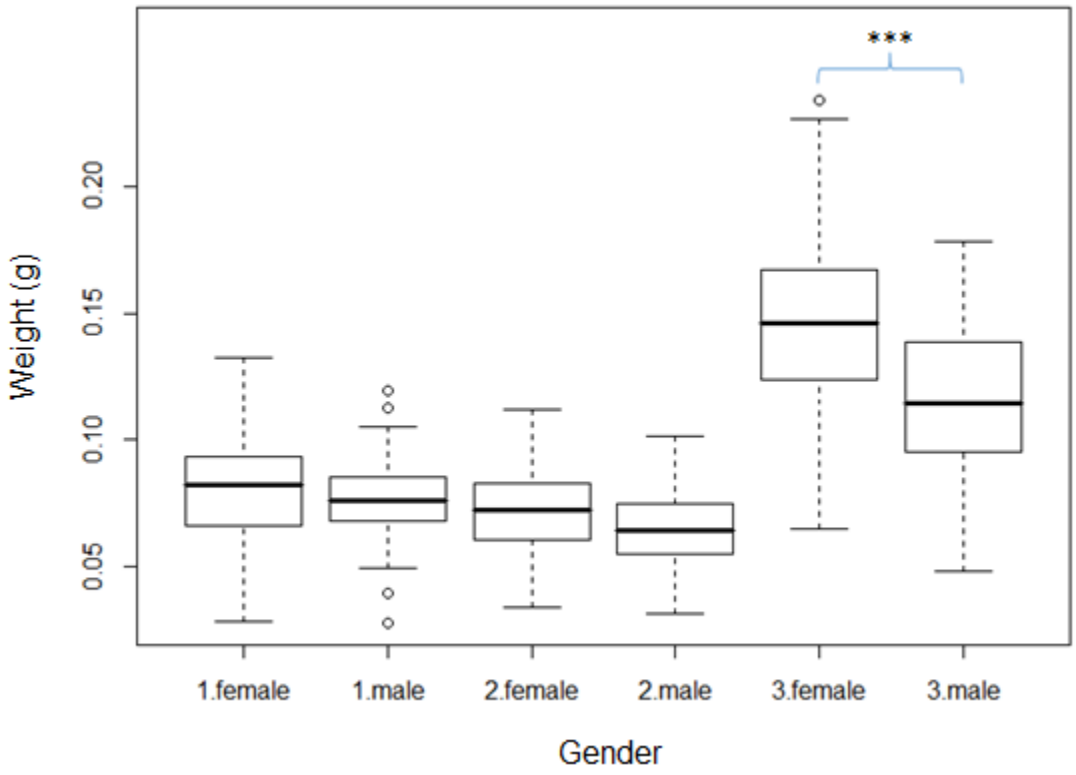

***
Weight (g)
0.05
0.10
0.15
0.20
1.female
1.male
2.female
2.male
3.female
3.male
Gender

Figure 7

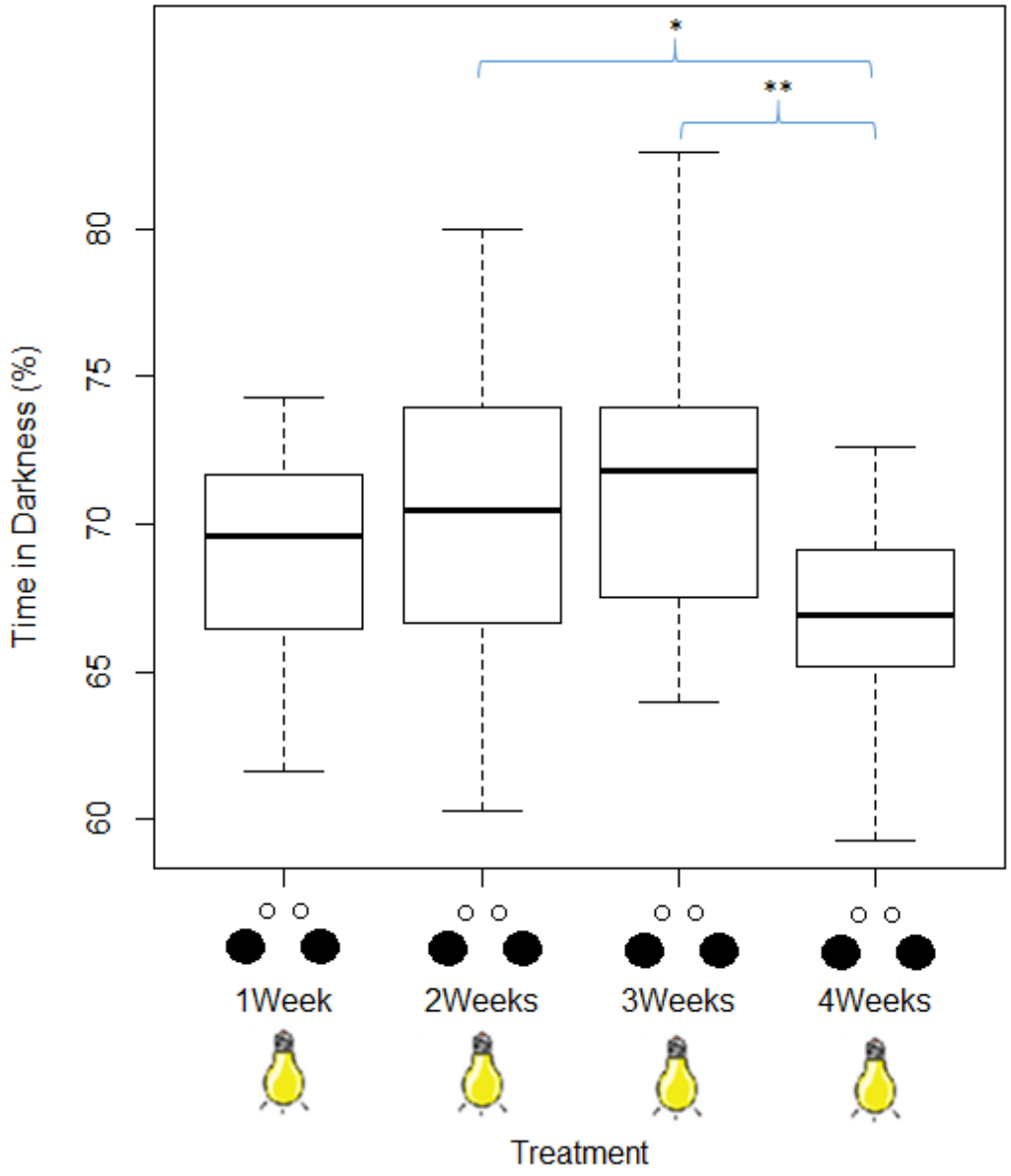

*
**
Time in Darkness (%)
60
65
70
75
80
1Week
2Weeks
3Weeks
4Weeks
Treatment

Figure 8

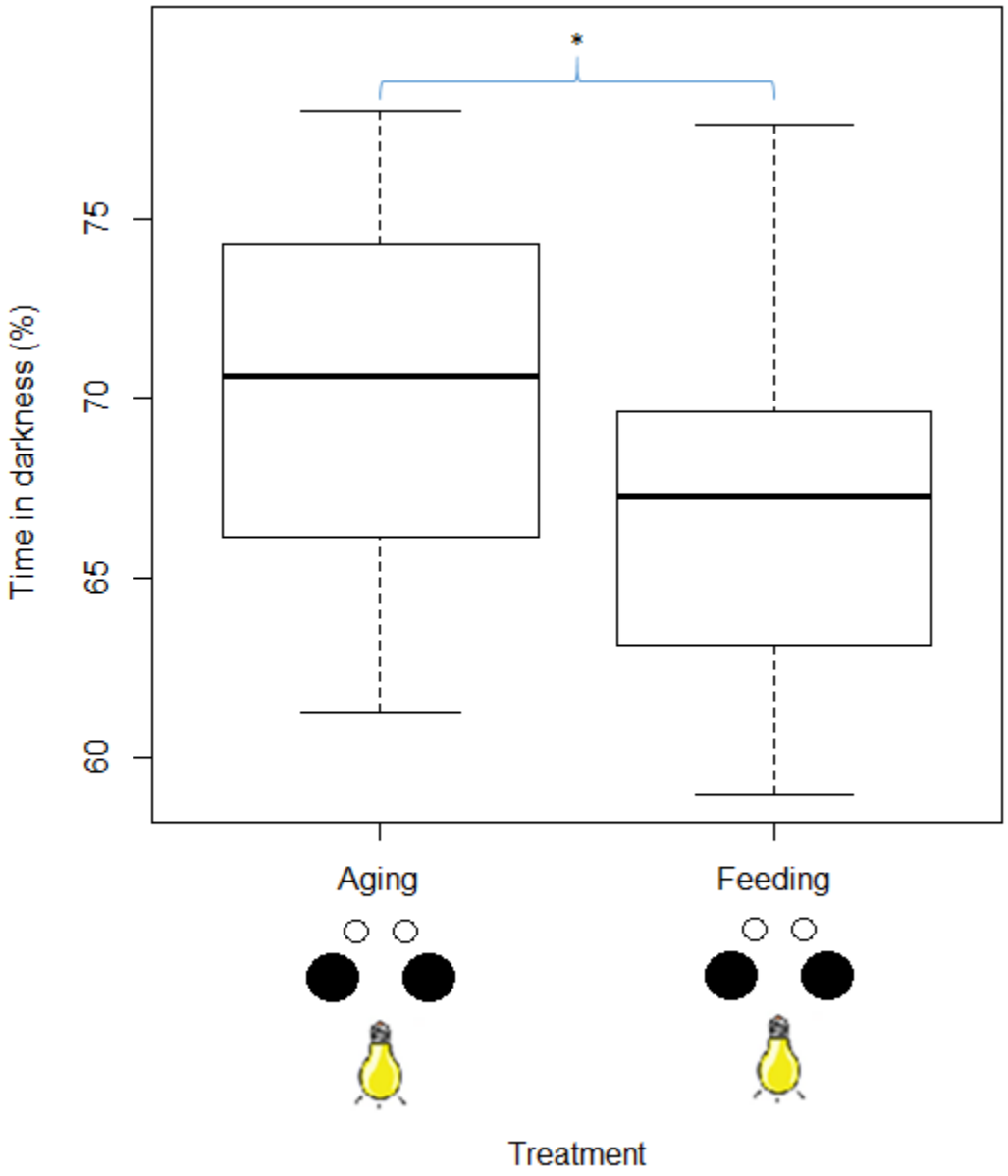


Figure 9

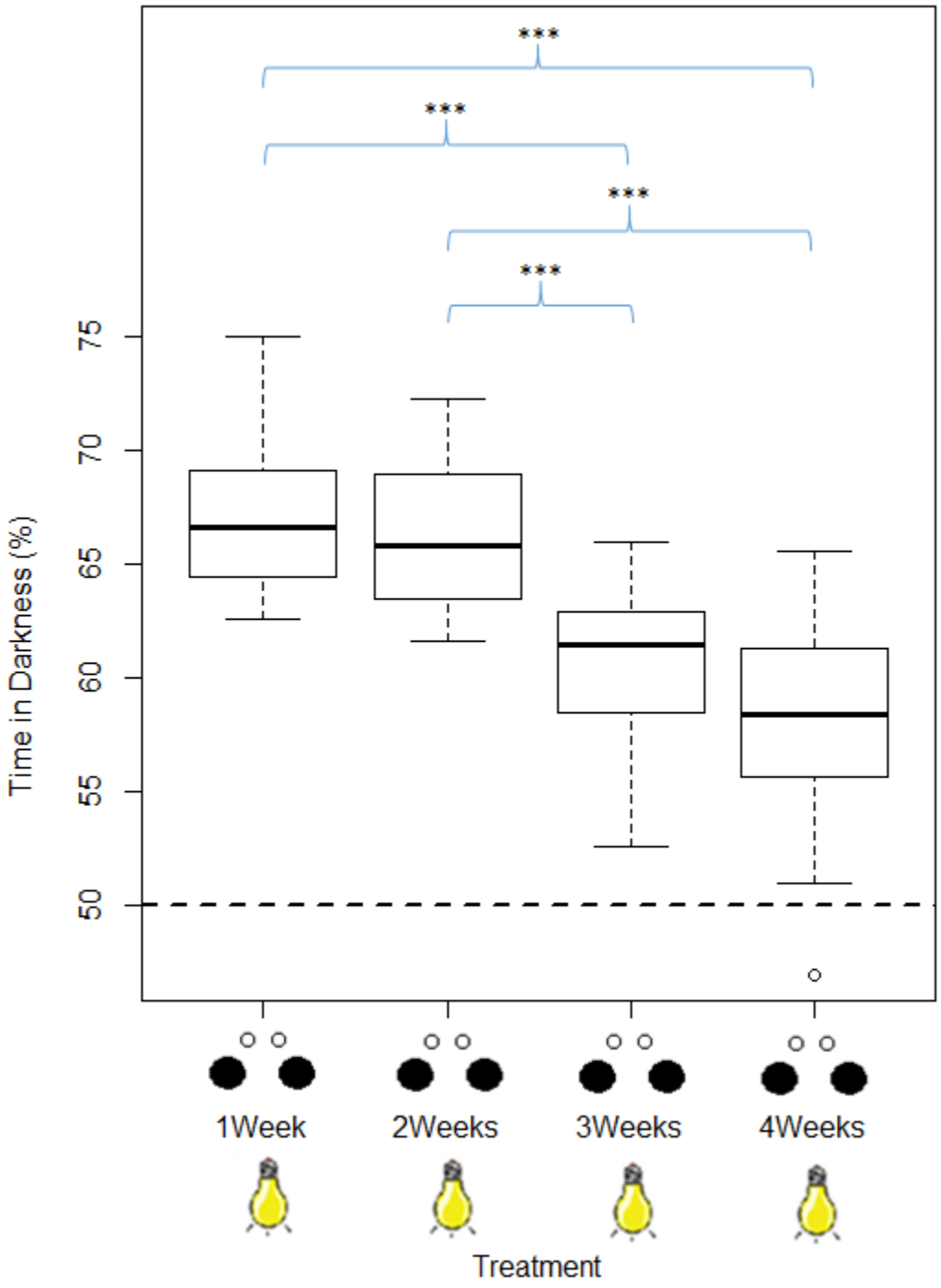
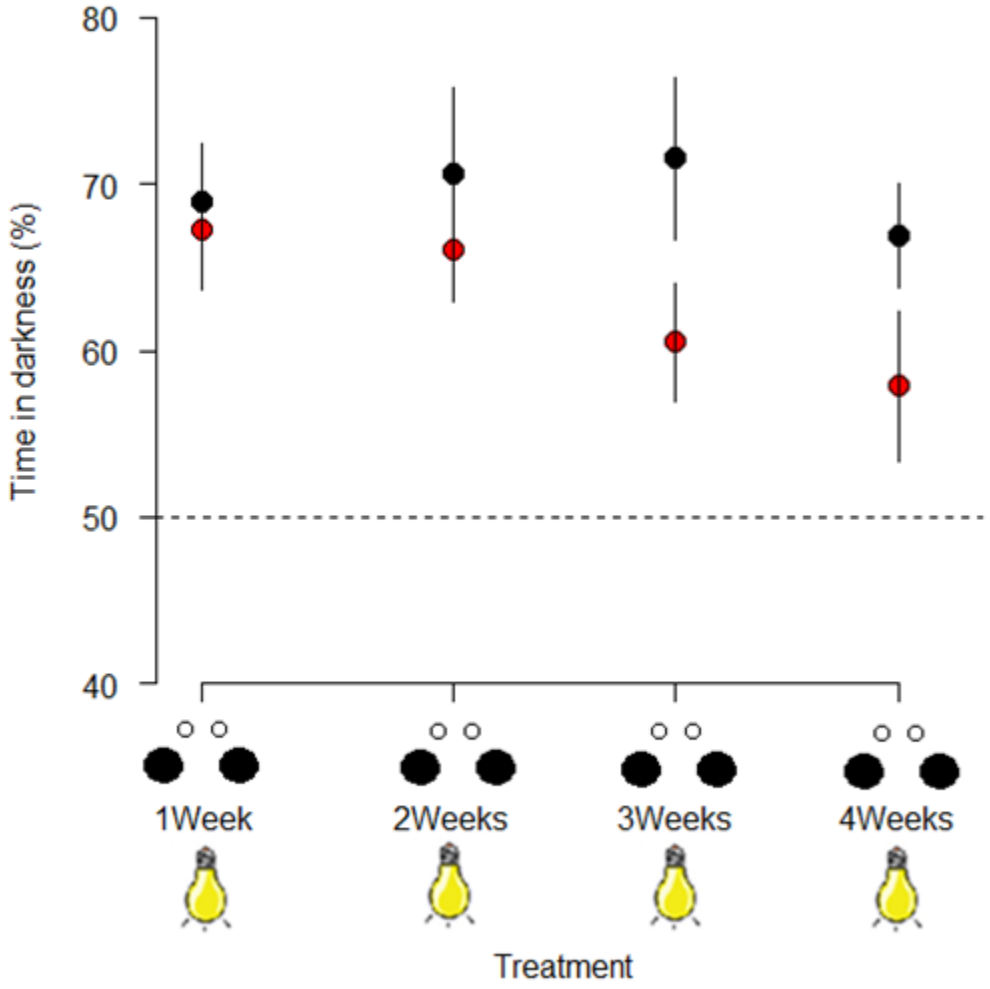

***
***
***
***
Time in Darkness (%)
50
55
60
65
70
75
1Week
2Weeks
3Weeks
4Weeks
Treatment

Figure 10


80
70
60
50
40
Time in darkness (%)
1Week
2Weeks
3Weeks
4Weeks
Treatment