How the spectral composition of artificial light at night effects the hatching of *Mamestra brassicae* (Lepidoptera: Noctuidae) eggs

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Bachelor thesis

How the spectral composition of artificial light at night effects the hatching of *Mamestra brassicae* (Lepidoptera: Noctuidae) eggs

Experimentally testing the effect of different wavelengths of artificial light at night on *M. brassicae* eggs

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Study program: Applied biology

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Preface

This report is one of the results of my internship at the Netherlands Institute of Ecology. Before this internship I had made the decision that I wanted to pursue a master in evolutionary biology after my bachelor. I wanted to choose my internship carefully to find a good place to prepare myself for my master. I am very happy with my decision to intern with Gabriel Charvalakis and Kamiel Spoelstra at NIOO. I learned how to conduct research, how to write a scientific report and how to work with R statistics on a much higher level than before and I feel like I am much more prepared to follow a master.

I also wanted to explore research that is a little more applied than the previous research I had done. Looking into the effects of artificial light at night on moths was thus exactly what I wanted. Also because of my interest in insects as a study species. I want to thank Kamiel and Gabriel for their guidance and the opportunity to do this internship. I would like specially to thank Gabriel for his one-on-one feedback, contagious enthusiasm, and patience with me. I would also like to thank Mike Scheper. He was another student also working on moths with Gabriel and I was able to discuss problems I ran into whilst writing my thesis.

I would also like to thank Floris Keizer. Floris is my supervisor from Aeres University of Applied Science. I think he goes further than others to help his students and I felt like I could rely on him as a supervisor.

Pelle van Hilst

12 June 2023

Wageningen

Samenvatting

Licht vervuiling is mogelijk een van de oorzaken van de afname van de motten populaties. Eerder onderzoek op motten heeft al negatieve effecten aangetoond. Licht met lange golflengten heeft vaak een significant kleiner negatief effect dan licht met korte golflengten. Deze onderzoeken zijn niet uitgevoerd op eieren.

Effecten op het ei kunnen mogelijk gevolgen hebben in de volgende fases. Dit onderzoek is uitgevoerd op *Mamestra brassicae*. Deze soort legt op verschillende momenten in het jaar eieren. Het is voor de embryo's daarom belangrijk om te weten wanneer ze gelegd zijn en wanneer ze uit moeten komen.

De effecten van licht vervuiling zijn getest door de eieren een 10L:14D fotoperiode te geven in de controle. De vier experimentele groepen kregen per groep blauw, groen, rood en wit kunstlicht wanneer de controlegroep de scotofase had. Deze kleuren om het effect van de golflengten te testen.

In dit onderzoek is geen dagelijks patroon in het uitkomen van de eieren gevonden. Er was geen significant verschil tussen de patronen van verschillende groepen. De eieren onder rood en blauw licht startte wel eerder met uitkomen. Deze resultaten zaten tegen het significante aan. Mogelijk waren met een grotere steekproefgrootte wel significante effecten gevonden. Er was een groot effect van de repetitie op het patroon van uitkomen. Dit kan betekenen dat er genetische basis is voor de tijd van uitkomen. Er zaten geen verschillen tussen de gemiddelde tijd van uitkomen van de groepen. Er kwamen niet meer eieren uit in de scotofase en de kunstlicht fase vergeleken met de fotofase dan werd verwacht door de grotere lengte van de scotofase. De proportie gestorven eieren was significant lager dan de controle in de groep met blauw licht en significant hoger in de groep met wit licht. Dit kan komen doordat de blauwe groep een hogere totale bestralingssterkte had. Het is mogelijk dat de totale bestralingssterkte te laag was voor een fotoperiodische reactie omdat de LEDs niet de bestralingssterkte van echt zonlicht konden na te bootsen. Deze resultaten zijn mogelijk beïnvloed door het met de hand tellen.

In dit onderzoek worden verschillende vervolgonderzoeken voorgesteld om de genetische basis van de tijd van uitkomen, het effect van totale bestralingssterkte en de voorkeur voor uitkomen in de scotofase of fotofase te onderzoeken. Het advies is om te mitigeren met rood licht.

Contents

1 Introduction

Long term studies in central Europe show a decline in moths. In Britain a 442-species abundance indicator shows a 25% decline in moths from 1970 to 2016 (Hayhow et al., 2019). The Netherlands also shows significant declines in moths (Groenendijk and Ellis 2011; Hallmann et al. 2020). Moth adults and larvae are important for the ecosystem. Specifically, moths are key prey-species for bats, birds, and spiders (Vaughan, 1997; Wickramasinghe et al., 2004; Wilson et al., 1999; Pekár & Toft, 2015; Nyffeler et al., 1994). Additionally nocturnal Lepidoptera (moths) are important pollinators. At least 289 plant species or wider taxa within 75 families of plants are pollinated by moths (Macgregor et al., 2015). Although rare, there are plants completely dependent on moths and even plants completely dependent on a single or few species of moth species (Johnson et al., 2004).

Some or most of the decline in moths could be the result of climate change (Conrad et al., 2002; Martay et al., 2017) and habitat fragmentation (Fox, 2013; Fox et al., 2014). Another driver could be artificial light at night (Boyes et al., 2021B; Macgregor et al., 2017; Knop et al., 2017; Van Langevelde et al., 2018). Artificial light at night (ALAN) is known to be an anthropogenic stressor which impacts ecosystems and biodiversity (Hölker et al., 2010). ALAN can make the night a lot lighter than it would naturally be. Natural moonlight doesn't get much stronger than 0,3 LUX and is much lower on most nights (Kyba, Mohar, & Posch, 2017). Under streetlights at night in the Netherlands the average light strength at ground level is 8,2 LUX (s.e. = 0,3) (Spoelstra et al., 2015). Any effects of ALAN affect a large area that is still expanding. In 2016 23% of the night sky in the world between 75N^{o} and 60S^{o} and 88% of the European sky was polluted with light (Falchi *et al*., 2016). This is still expanding. Kyba et al. (2017) calculated the artificially lit outdoor area grew non-exponentially by 2.2% per year between 2012 and 2016. There are some countries where the artificially lit area did not grow, surprisingly this included some of the brightest countries like the Netherlands (Kyba et al., 2017). Still the large and still growing area affected by ALAN makes studying the effects of ALAN important.

There has been an increasing amount of research on the effect of ALAN. For example, on bats, birds, and moths. The research on bats has shown negative effects on roosting, commuting, foraging, drinking, predation risk (Salinas-Ramos et al., 2021). The research on birds also shows negative effects. Birds are attracted to light. This has some negative effects like birds crashing into illuminated buildings and windows and seabirds stranding of seabirds that are attracted to shore by the lights. Some other effects of ALAN on birds are alterations in reproductive physiology, changes in flight behavior, disorientation, and disruption of circadian rhythms (Cabrera-Cruz et al., 2018). Moths are a logical study species given their ecological importance and nocturnal lifestyle. Previous studies of moths have shown that ALAN has several negative effects on moths, such as suppressed activity and feeding, increased predation, disrupted pheromone production and reduced copulation (Boyes et al., 2021A).

These negative effects make sense because light can be important information for organisms. For example, to entrain their circadian rhythm (Moore-Ede, 1982). Circadian rhythms are changes in organisms that follow a 24-hour cycle. Organisms have biological clocks that allow them to follow the rhythm. A biological clock is an internal system that regulates the cycle of rhythms. The circadian clock is the biological clock regulating the circadian rhythm. This rhythm can fall out of sync with day and night if the if external cues are absent (Sweeney, 1963). One cue for day and night is the daily cycle of light and dark also known as the photoperiod.

One possible way to mitigate the effect of ALAN is to change the spectral composition of the light. Agee (1973) showed moths are less sensitive to longer wavelength lighting in two adult moth species by testing their sensitivity using an electroretinogram. Later research on the attraction of moths when using different wavelengths supports this finding with moths being less attracted to longer

wavelengths (Van Langevelde et al., 2011; Van Grunsven et al., 2014). Although there might be some differences between species groups. Somers-Yeates et al. (2013) found moths from the Geometridae family are not differently attracted by longer or shorter wavelengths. Light with longer wavelengths that is still usable for humans is red.

For our study we chose *Mamestra brassicae* (Lepidoptera: Noctuidae) as a representative of the Noctuid family. The noctuid family is the largest family among moths. Aside from that *M. brassicae* is a commonly studied moth which makes it easier to compare. *M. brassicae* has four life stages an egg stage, larval stage, pupal stage and pharate adult stage (De Vlinderstichting, n.d.).

There have already been some studies looking into the effect of ALAN on *M. brassicae*. Van Geffen et al. (2014) found that male caterpillars reach a lower larval mass, pupate earlier, and reach a lower pupal mass when they were exposed to white and green ALAN. Red ALAN did not have these effects. Both sexes of pupae exposed to green and white light before pupation also emerged earlier than the pupae under red light and in the dark control. These effects could have a big effect on the survival of the moths. In a following study Van Geffen et al. (2015) found that the amount of sex pheromone produced by adult females is reduced when they are exposed to ALAN. The pheromone blend was also different. These effects were significant with all spectral compositions that were tested, although the effect was the smallest when red light was used. The red light did however not differ significantly from the other spectral compositions. This disruption of the pheromones could have a negative effect on reproduction.

This information on the effects of ALAN on moths and other wildlife is useful for governments, municipalities, land management organizations and anybody interested in the conservation of nature. Governments can regulate artificial light and mitigate the effect of light pollution.

Eggs are the only life stage of moths that has yet remained unstudied in relation to ALAN. The eggs are important as apart from the pupae which are underground this is the only stage where the moth cannot move away from the light. This is also the first interaction the moth has with light which could cause trickle down affects throughout the rest of its life.

There has been some research on the effect of photoperiod on moth eggs. Which could give us an indication of possible effects ALAN might have. Minis & Pittendrigh (1968) have reported that eggs of the *Pectinophora gossypiella* moth may be photosensitive once they reach the midway point of their development. This same study also showed its hatching time was based on the start of the photophase and that a 15-minute exposure to light can be enough to start a circadian rhythm (Minis & Pittendrigh, 1968). Du Merle (1999) found that different photoperiodic regimes do not affect the hatching date or the needed cold requirements for diapause in *Tortrix viridana* (Lepidoptera: Tortricidae). A different study though did link shorter photo period in the egg stage with a lowered fecundity in the pharate adult stage (Deseó & Sáringer, 1975). Nevertheless, our current understanding on how photoperiod affect egg development is quite limited.

We hypothesize that the daily hatching time of *M. brassicae* eggs may be regulated by a light sensitive clock mechanism. This is because *M. brassicae* lay their eggs from May to October having two generations in this period (De Vlinderstichting, n.d.). There is only a small temperature difference between the start and end of the egg laying period. The average temperature in the Netherlands between 1991 and 2020 was 12.2-14.0C^{o} in May, 14.1-16.1C^{0} in September and 10.2-12.4C^{o} in October (KNMI, n.d.). The photophase length in these months is different though. The photophases in May are longer and are also increasing while the photophases in September and October are shorter and decreasing. *M. brassicae* could thus take their cues to hatch from photoperiod.

For *M. brassicae* eggs it is important to know when they are because eggs laid in May have a warm period with plenty of food ahead of them. Where eggs laid at the end of the laying period have limited time to pupae and be ready for winter. This is because although larvae do overwinter, they mostly overwinter as pupae (De Vlinderstichting, n.d.). This might be because the larvae cannot withstand lower temperatures as well as pupae (Johansen, 1997). This would mean it could be useful to hatch earlier for eggs laid at the end of the season. For caterpillars early in the season it would make sense to hatch a little later so more plants are available.

We hypothesize that ALAN could make a short photophase seem like a longer photophase making caterpillars hatch later. This would mean caterpillars late in the season might hatch too late and not be in time to pupate which causes a higher mortality.

Based on the findings of Minis & Pittendrigh (1968) we also hypothesize that *M. brassicae* eggs hatch at the onset of the photophase in a photoperiodic regime. If this is the case embryos could putatively mistake the onset of ALAN for the onset of the photophase. Such a scenario could potentially be a false trigger which could lead to a synchronicity mismatch for the emerging larva. The larvae would hatch at night when the temperature is colder which could cause a higher mortality. ALAN is, however, a weaker light source than sunlight and might not be strong enough to trigger hatching.

The main question of this study was,

what is the effect of different wavelengths of artificial light at night on *Mamestra brassicae* eggs?

This question will be answered by the sub questions,

what hatching pattern do *M. brassicae* eggs have,

what affect do different wavelengths of ALAN have on a potential hatching pattern of *M. brassicae* eggs,

what affect do different wavelengths of ALAN have on the hatching date and hatching time of *M. brassicae* eggs and,

what affect do different wavelengths of ALAN have on mortality of *M. brassicae* eggs?

If negative effects of ALAN are found this study can be used to argue for mitigation of light pollution. If different spectral compositions have differing effects this information can be used to find a spectral composition that has the smallest negative effect on moths. This spectral composition can then be used to mitigate some of the negative effects of ALAN on moths.

2 Materials and methods

In this chapter the materials and methods used to perform the experiment and the statistical analysis used to analyze the results from the experiment are described.

2.1 The experiment

Eggs

The *Mamestra brassicae* eggs used in the experiment came from Wageningen University. These eggs were used for all research questions. The WUR's rearing is kept under a constant temperature of 20 C° ± 1 and a photoperiod of 16L:8D. The moths lay their eggs on filter paper. The eggs were laid between 36 and 6 hours before the experiment. In this period, they were kept in a 20 C° room with a 16L:8D photoperiod. The experiment used 6294 eggs in total. There were four treatment groups and one control group. Each group contained between 1072-1423 eggs. Each treatment had three replications of 275-572 eggs in about five to ten clutches. For each group the number of total eggs was counted using a stereoscope and in cases of large patches also a manual counting program called DotDotGoose was used (Ersts, 2023) (Appendix B). The eggs were kept in ⌀ 9 cm petri dishes with a lid, there was one petri dish per repetition. The filter papers with the eggs were kept in place by double sided tape.

Methods

For the experiment the petri dishes with the eggs were placed into specialized lightproof boxes where the experiment took place (Figure 1). Great care was taken to make sure every box was the same. The light in the experiments was produced by two LED-strips inside the boxes. Each box contained 20 LEDs, nine to eleven LEDS per strip. The boxes were kept in a dark room at 13 C° ± 1. This temperature is similar to the temperature the eggs would have at the start and egg laying season in the Netherlands. This lower temperature also extends the time it takes for the eggs to hatch which increases any differences and extends the time the eggs are exposed to ALAN. Fans installed via lightproof tubes with a 90° bend provided ventilation to make sure the LEDs didn't increase the temperature in the box. One box of every treatment was equipped with a temperature and relative humidity logger (Appendix A). The relative humidity fluctuated between 37% and 94%. The fluctuations were the same in all treatments. when the white light during the photophase was on the LEDS increased the temperature consistently with between 0.4 C° and 1.0 C°. When the ALAN was on the temperature did not change.

Figure 1

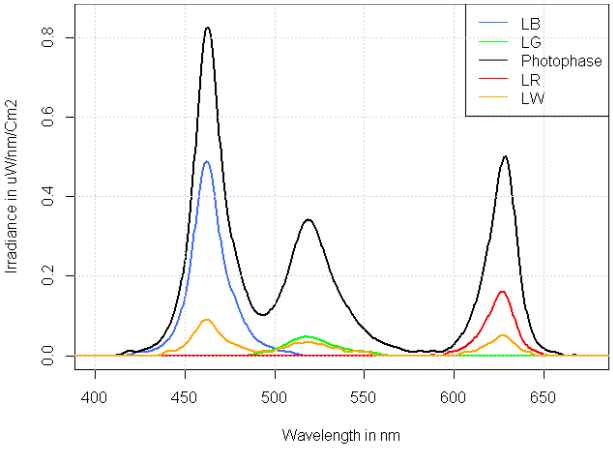
The light boxes used for the experiment

Note. The light boxes used for the experiment.

The effect of different colors of ALAN was tested on the eggs. During the entire experiment all groups had a 24-hour cycle with photophases of 10L. The light during the photophases started at 9:00 and was white light (414 nm – 659 nm, peaks 463 nm and 628 nm, 92 ± 1 LUX) (Figure 2). A higher LUX would have been preferred but could not be achieved with the LEDs. The spectral composition of the light was measured using a QE Pro Series Spectrometer. The control group only received light during the 10L photophases and had 14D scotophases which started at 19:00 giving them a 10L:14D photoperiod for the entire experiment. The four other groups received ALAN when the control group had their 14D scotophases (Figure 3). Giving them a 10L:14ALAN photoperiod. The ALAN had a strength of 8 ± 1,2 LUX which is comparable to that of streetlight at ground level (Spoelstra et al., 2015). Four different spectral compositions of ALAN were tested as research has shown moths to be less sensitive to longer wavelengths (Agee 1973; van Geffen et al., 2014, 2015). The tests ran with red (596 nm – 649 nm, peak 627 nm, 7,5 ± 0,1 LUX), green (487 nm – 560 nm, peak 518 nm, 7,7 ± 0,4 LUX), blue (419 nm – 513 nm, peak 462 nm, 7 LUX) and white light (438 nm – 554 nm, peaks 518 nm and 627 nm, 9,1 ± 0,1 LUX) (Figure 2). The LUX is not the same for all treatments because of limitations of the LEDs. One replication of each group was together in the same box when they received the 10L photophases. When the scotophases of the control group started each petri dish of the four ALAN groups was moved to its own box where they received their respective colors of ALAN for 14 hours. The control group remained in the box. After 14 hours the normal photophase started again and the petri dishes from the ALAN groups went back into the boxes with the control group for the photophase. The repetitions switched randomly between boxes of the same treatment to mitigate any effect of the box.

Figure 2

The spectral composition of the light



Note. The spectral composition of the light the eggs received during the photophase and during the ALAN treatment. On the X-axis the wavelength of the light. On the Y-axis the irradiance. The total irradiance is the surface area on the graph per treatment.

Figure 3

The timeline for 7 days of the treatment the eggs had during the entire experiment

	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
Control														
LB														
LW														
LR														
LG														
Start phase	09:00	19:00	09:00	19:00	09:00	19:00	09:00	19:00	09:00	19:00	09:00	19:00	09:00	19:00
Phase length (hours)	10h	14h	10h	14h	10h	14h	10h	14h	10h	14h	10h	14h	10h	14h

Photophase = Scotophase = Blue ALAN = White ALAN = Red ALAN = Green ALAN =

Note. The representation of 7 days of the treatment the eggs had until the hatching was over. In yellow the 10L photophase at the same time for all groups. Then after the 10L photophase the control group receives a 14D scotophase in black. The ALAN groups receive a 14 hour ALAN treatment. All ALAN groups get their own color ALAN. After the 14 hours are over the ALAN and control groups receive the same 10L photphase again and the cycle repeated until the experiment was over.

Counting hatching

To answer our questions about the timing of hatching and the effect of ALAN on timing the hatching was manually monitored every two hours between 9:00 and 19:00. Meaning caterpillars counted at 9:00 hatched somewhere between 19:00 of the previous day and 9:00 of the day they were counted. One by one petri dishes were taken out of the box. During this time white light was used to see the eggs and caterpillars (spectral composition). The caterpillars were removed using a brush or by flicking the petri dish making the caterpillars fall on white paper. Caterpillars that had their entire head out of the eggshell, but were not fully out yet, were removed from the eggshell using the brush and also counted. All hatchings were counted until no eggs hatched for an entire day. After that the leftover eggs were kept in a fridge.

Mortality data

Our questions about mortality were answered by assessing the mortality after all the hatching was over. After the experiment the hatched eggs and unhatched eggs were counted making use of a stereoscope and DotDotGoose (Ersts, 2023) (Appendix B). If it was unclear whether an egg had hatched it was opened using sharp tweezers to see if there was still an embryo inside.

2.2 Statistical analysis

The data was analyzed using R statistics (R Core Team, 2023) (Appendix C).

The data will be tested analytically using the Kolmogrov-Smirnov test, the Shapiro-Wilk test and graphically using a Quantile-Quantile plot to see if the data is parametric. The distribution was analyzed further to find the best distribution fit for the data.

Hatching pattern

A survival analysis was performed to see if there were any differences in hatching pattern between the treatments. The cox proportional hazard model was used. A random effect was added for the petri dishes to prevent pseudo replication.

Mean hatching time

The mean hatching time was calculated. The Kolmogrov-Smirnov test and the Shapiro-Wilk test both came back unsignificant and the Quantile-Quantile plot confirmed this, so the data was not non-parametric. Further analysis showed a normal distribution fit the data best. A two-way ANOVA was then used to see if there were any significant differences. If necessary, a Tukey honestly significant differences test was used to see where the differences lied.

Hatching phase

The proportion of eggs hatched during the scotophase and photophase was calculated. The Kolmogrov-Smirnov test and the Shapiro-Wilk test both came back unsignificant, and the Quantile-Quantile plot confirmed this so the data was parametric. Further analysis showed a normal distribution fit the data best. A general linear model was then used to test for differences between the treatments. A pairwise t-test was used to test if the proportion of eggs hatched in the scotophase was significantly higher than the proportion of eggs hatched in the photophase. A Bonferroni correction was applied to take in account the large number of tests.

Mortality

The proportion of unhatched eggs for each repetition was calculated. The Kolmogrov-Smirnov test and the Shapiro-Wilk test both came back unsignificant and the Quantile-Quantile plot confirmed this, so the data was not non-parametric. Further analysis showed a beta distribution fit the data best. A general linear model with a beta distribution was then used to test for differences between the treatments.

3 Results

In this chapter the results of the experiment and the statistical analysis described in the materials and methods are described. In the figures the control will be revered to as C and the blue ALAN treatment as LB, green as LG, red as LR and white as LW.

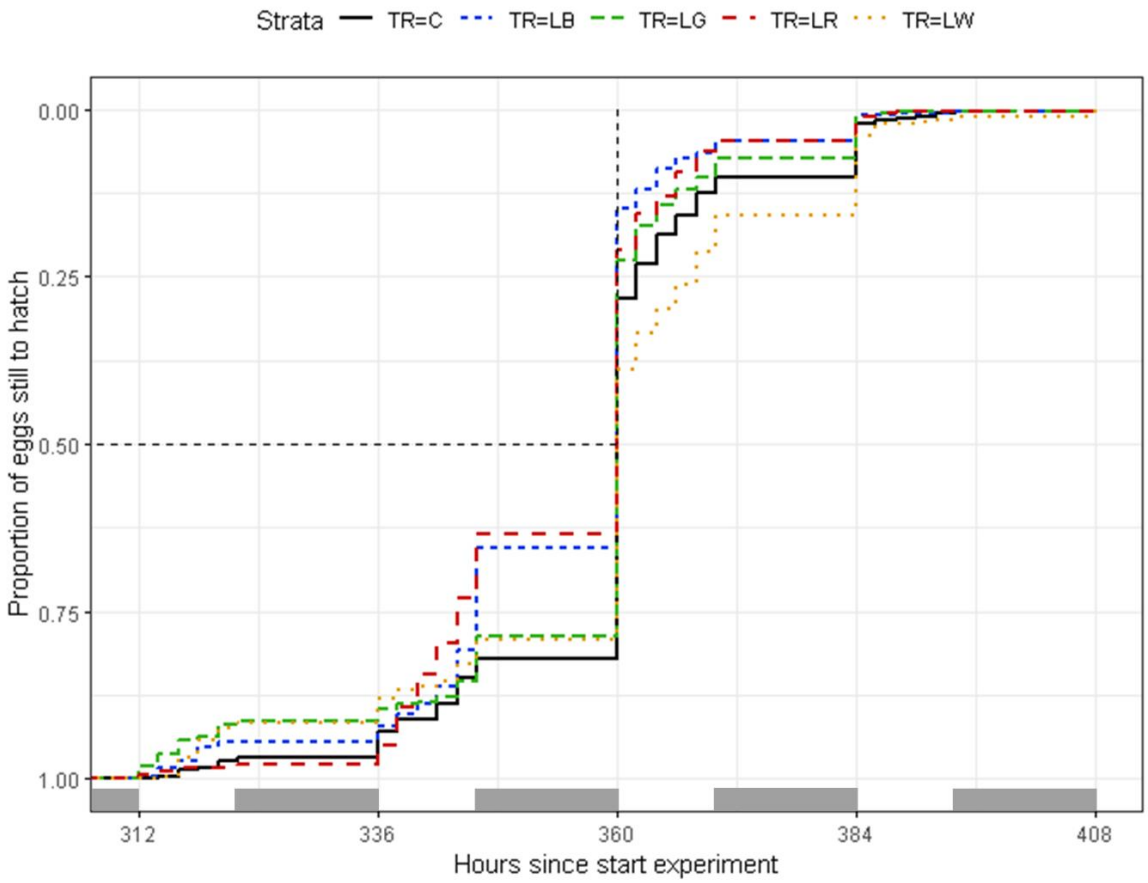
3.1 Hatching pattern survival analysis

In the survival analysis the mortality was left out, because in this case the subject of the analysis is hatching and not death. Mortality could also affect the pattern, but this effect would still be visible as eggs not hatching at the time where the pattern was affected by mortality.

In all treatments and the control there does not seem to be a daily hatching pattern (Figure 4). Although nothing can be said about a hatching pattern within the scotophase. The cox proportional hazard model showed that none of the ALAN treatments had a significantly different hatching pattern from the control. The hatching does seem to increase earlier in the blue and red treatment. These treatments were relatively close to being significantly different from the control with a p-value of 0.10 and 0.15 respectively.

Figure 4

The egg hatching pattern per treatment



Note. The number of eggs hatched goes up as the line goes up. The Y-axis is flipped in comparison with a usual survival plot as this is about hatching and not death. On the X-axis the hours since the start of the experiment are displayed. There is a tick every time the scotophase ends and the photophase starts again. Above the X-axis you the gray bars this represents when the scotophase or in case of the treatments the ALAN took place. The line goes up every time the hatchings were counted and there were caterpillars. The long horizontal parts of the lines above the gray bars come from only counting at the end of the scotophase.

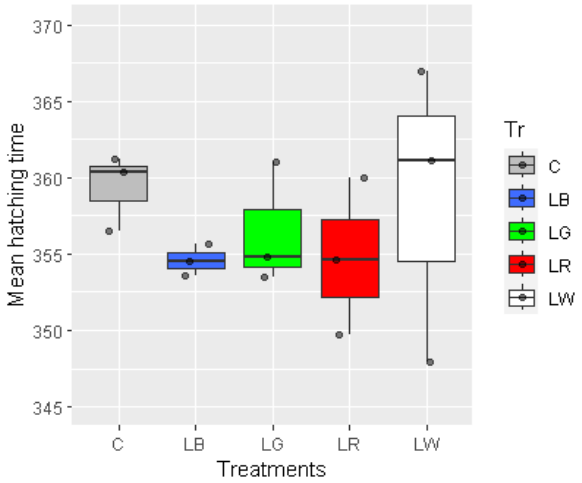
Before the petri dish was added as a random effect all treatments were significantly different from the control. The model was however significantly more predictive when the petri dish was added as a random effect. It was also significantly more predictive than a model with the petri dish as an explanatory factor and a model with ID as an explanatory factor with treatment as a random factor. This means that treatment is the main predictor, but there is also a lot of unexplained variation between petri dishes in the same treatment.

3.2 Mean hatching time and day

The mean hatching time of the control was 359 hours (15.0 days) after the start of the experiment (Figure 5). The blue, green, red and white ALAN treatments did not differ significantly from each other or the control (p=0.74). Their mean hatching time was 355, 356, 355, and 359 hours after the start of the experiment respectively. This is not surprising seeing how there were no significant differences between the hatching patterns either. Red and blue were not close to having a significantly earlier hatching time. This also means that there is no significant difference in the hatching day.

Figure 5

The mean hatching time per treatment



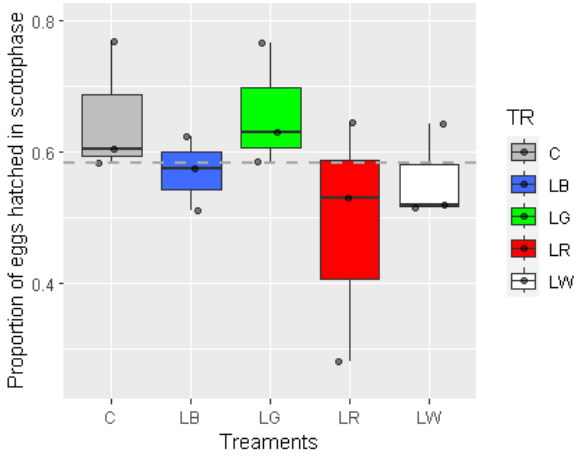
Note. This figure shows the mean hatching time per treatment. The X-axis shows the treatments, and the Y-axis shows the hours since the start of the experiment.

3.3 Hatching during the scotophase or photophase

There could also be a pattern where eggs are more likely to hatch during either the scotophase or the photophase. Whitin the treatments the proportion of hatching during the scotophase was significantly higher than the proportion of hatching during the photophase in the control (p=0.02), blue (p= 0.04), and green (p=0.01). The white treatment was close to being significant (p=0.12). The red treatment did not have more hatchings during the scotophase than the photophase (p=0.85). In this experiment the proportions of hatching during the scotophase is expected to be higher than the photophase because the scotophase was longer than the photophase. The scotophase took up 58% of the day. This means that if there was no preference for the photophase or scotophase the proportion of eggs hatched in the scotophase should be around 0.58. Neither the control or the treatments had a significantly higher or lower proportion of the hatching in the scotophase than 0.58 (control p= 0.36, LB p= 0.73, LG p= 0.29, LR p= 0.46, LW p= 0.61).

Figure 6

Proportion of eggs hatched during the scotophase or ALAN



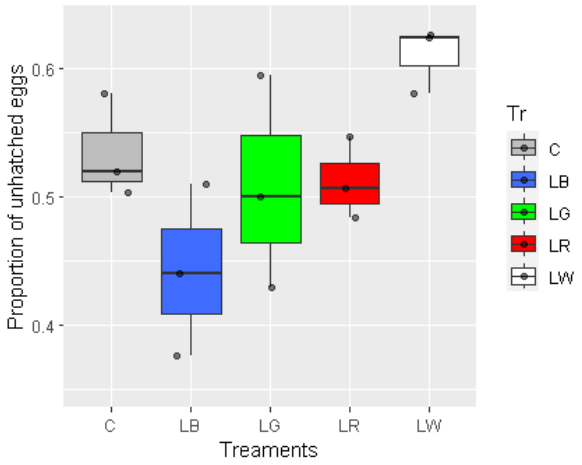
Note. This figure shows the proportion of eggs hatched during the scotophase of each treatment. The Y-axis shows the proportion of the hatched eggs that hatched in the scotophase. The X- axis shows the treatments. The gray dotted line is placed at a proportion of 0.58.

3.4 Mortality

The mean proportion of unhatched eggs in the control was 0.53. The mortality in the blue treatment was significantly lower with a proportion of 0.44 (p=0.012). The mortality in the white treatment was significantly higher than the mortality in the control with a proportion of 0.61 (p=0.036). The other treatments did not differ significantly from the control.

Figure 7

The mortality per treatment



Note. This figure shows the mortality. On the Y-axis you the proportion of unhatched eggs. On the X-axis you see the treatments.

4 Discussion

In this chapter results from the experiment and the analysis will be interpreted and discussed. The shortcomings and recommendations will also be discussed.

It is surprising that both the treatment with red ALAN and the treatment with blue ALAN start earlier than the other treatments and the control (Figure 4). Although the results were not significant, they were relatively close. With a larger sample size these results could have been significant. It would make most sense that only one of these treatments showed some kind of effect since the treatments have a big difference in wavelength. This is, however, not the case and the green and white treatments do not show such effects. This makes it more likely that there is no effect on the hatching pattern of the ALAN, but it still cannot be ruled out that ALAN does have an effect.

One factor making it hard to judge if there is an effect of ALAN on the egg hatching pattern is the variation between the petri dishes in the same treatment. This variation could stem from the parents. There were about five to ten clutches per petri dish. The number of clutches roughly reflects the amount of genetic variation as each clutch could be laid by a different female. Although in *Mamestra configurata* (Lepidoptera: Noctuidae) eggs are sometimes added on to already existing clutches (Ulmer et al., 2003). It is not known whether *M. brassicae* also does this. If this variation indeed has a genetic basis, this would mean that there are genes determining the time it takes until hatching.

The eggs were however also laid somewhere in the 36 to 6 hours before the experiment, so there is also variation in the age of the eggs before the experiment started. Which could also explain the variation. This would be very interesting to study. This could be done by taking clutches and separating them over different treatments. This is to see if the hatching time of eggs from the same clutch differ based on the ALAN treatment they received. Measures would need to be taken to make sure that clutches come from one female and that they all the eggs are laid at the same time.

Like the hatching pattern there were also no significant differences in the mean hatching time (Figure 5). There were also no treatments that were close to being significantly different. The real mean hatching times are likely lower than the mean hatching times found in this experiment. This is because the time used to calculate the means is the time that the hatchings were counted. Which was every two hours during the photophase and once at the end of the scotophase. There was no way to know when the eggs hatched in between counting. This means that eggs that hatched at the start of the scotophase would be reported as having the hatching time of the end of the scotophase. This means the hatching time gets pushed up and this results in higher means. Using manual counting there is no way to account for this. The data must always reflect what has been found and that is that the egg had hatched at the end of the scotophase.

In this experiment significantly more eggs hatched during the scotophase or ALAN than the photophase for the control, blue ALAN and green ALAN treatment (Figure 6). This is what would be expected of all groups if the hatching time was random because the scotophase and ALAN took up more of the day. The scotophase and ALAN took up 58% of the day. This means that if the embryos did not have a preference for hatching in the scotophase or photophase the proportion of eggs hatched in the scotophase should be 0.58. The proportion of eggs hatched during the scotophase was never significantly different from 0.58. This still does not mean that there is no preference for hatching in the scotophase. It only means that it was not proven or disproven with this experiment. An experiment where the length of the scotophases is different and possibly also where the photophase and scotophase are switched around would be able to answer this question. This also means that the hypothesis that hatching starts at the start of the photophase has not been proven.

The mortality in the blue ALAN treatment was significantly lower than the control and in the white ALAN treatment the mortality was significantly higher (Figure 7). This was surprising as in previous studies blue light or long wavelength light is associated with stronger negative effects (Boyes et al., 2021A). What is also surprising is that in all groups the mortality is high. This is surprising as a previous study on the survival of *M. brassicae* eggs found that all fertile eggs hatch in temperatures between 11 C^o and 18.5 C^o (Johansen, 1997). This could mean a lot of the eggs were unfertile, but that would not explain why the mortality in the blue treatment is lower and in the white treatment higher. It is possible that the mortality is caused by not receiving enough light. The blue ALAN treatment had the highest total irradiance. Total irradiance is different from LUX. The measurement unit LUX is based on visibility to humans, but to achieve 8 LUX of blue light more energy is needed than to achieve 8 lux of red light. This energy needed can be measured in total irradiance. Total irradiance is the surface area underneath the lines in Figure 2. This would not explain why the why the white ALAN treatment has a higher mortality as the total irradiance from the white ALAN is similar to those of the red and the green ALAN treatment and the control treatment has even less total irradiance.

It is also possible the intensity of the light during the photophase was too low to induce photoperiodism. It has been suggested that light intensity is also important for insect photoperiodism (Saunders, 2013). Although the light during the photophase was stronger than the light during the scotophase that the ALAN groups received, actual sunlight is a lot stronger. The difference between light and, dark and ALAN might not have been big enough. If this is the case, it explains why photoperiodism and a daily hatching pattern were not found in this study. A daily hatching pattern was found by Minis & Pittendrigh (1968). This was on a different species, but also with a stronger light intensity of 220 LUX in the photophase instead of 92 ± 1 LUX in our experiment. This could indicate that a stronger light level is indeed needed to induce photoperiodism. Although 220 LUX still does not come close to the strength of real sunlight. It is recommended to test the importance of irradiance in a follow up study. If it is found that total irradiance is important this experiment would need to be repeated with a higher irradiance.

The manual counting was not ideal, but infrared cameras that can film things as small as the eggs hatching are extremely expensive and were not within the budget. Other methods like having the eggs surrounded with sticky paper would not have sufficed as it would have relied on the caterpillars moving after they hatched. Pilots showed it could take the caterpillars several hours to move from the filter paper on to the sticky paper (Appendix D). This method was used by Minis & Pittendrigh (1968).

The flicking that was used to count the caterpillars when the hatching started to speed up is likely not a driver for the eggs to hatch. Although birds do eat eggs of members of the lepidoptera order. This usually happens in the winter (Cooper & Smith, 1995; Torgersen & Mason, 1987; Higashiura, 1989; Barbaro & Battisti, 2011). Eggs from *M. brassicae* are not present in the winter, but it cannot be ruled out an opportunistic bird will eat *M. brassicae* eggs when it stumbles upon them. The wind would also move the eggs in the wild which would not be a time-based event. This means it is also unlikely the embryo used the flicking as a time que. The same goes for the moving of the petri dishes to count and move them to their treatment.

Counting using the brush is a larger concern. The brushing could mimic a predatory insect. Eggs from *M. brassicae* are eaten by the larvae of one of the common green lacewing species (Klingen et al., 1996). There are also other insects that eat the eggs of members from the Noctuid family but are not confirmed to eat *M. brassicae eggs*. These include a member from the ladybug family, spiders from the harvestman family and damsel bugs (Pfannenstiel & Yeargan, 2002). Any fitness benefits from an

egg hatching pattern would not outweigh the fitness costs of getting eaten. The brushing could thus have caused the caterpillars to forgo their usual hatching pattern to escape the supposed predators. The brushing is however not likely to be used as a time que by the embryo as getting eaten would not happen on a given time in the wild.

The concerns above could be avoided had suitable infrared cameras been available within the budget. This would have also provided us with valuable information on the hatching during the scotophase. Whilst this experiment was running, we developed infrared cameras fitted with macro lenses that are able to film the egg hatching during the scotophase. It is recommended to repeat the experiment using the infrared cameras to get the data of the hatching during the scotophase.

Earlier research on moths always showed red light has the smallest effect in comparison to the dark control (Boyes et al., 2021A). All these studies were, however, on other life stages of moths. In these life stages moths have developed their eyes and nerve system. The nerves in the eyes are also the nerves that Agee (1973) tested and found was least reactive to red light. It is possible that moths react differently to different wavelengths when they are still an embryo and are still developing their nerve system and eyes. In this study red light was not significantly different from the control in any way. Although it might have an effect in the hatching pattern, but that result was not significant. The blue ALAN had reduced mortality compared to the control, but red ALAN did not differ significantly from the control. Since this study did not have any conclusive negative results, it is recommended to use red light as a way to mitigate the effect of ALAN on moths. This is because red light has been shown to reduce negative effect in other important aspects for moths as is discussed in detail in the introduction (Boyes et al., 2021A). However, more research is still needed on the effect of ALAN on the development of moths. This advice to use red light as mitigation is useful to governments and municipalities. They can preserve nature and mitigate the negative effect of light pollution by changing the light color of their streetlights. It is however important to also take in account other organisms that moths.

Further research on eggs should be performed using infrared cameras to monitor the hatching and possible genetic effects of the eggs should be taken into account by monitoring the clutches separately. A good follow up to this study would be to test whether a higher total irradiance does induce photoperiodism and reduce mortality in *M. brassicae* eggs.

5 Conclusion and recommendations

In this chapter the conclusions drawn from the research and the recommendations for research and mitigation of light pollution in relation to moths are summarized.

The aim of this study was to find any negative effect ALAN had on *M. brassicae* eggs. To serve as a resource on which could be relied for choices in relation to the mitigation of light pollution. To achieve this, eggs were experimentally exposed to blue, green, red and white ALAN at a level comparable to the light level at ground level under streetlighting during what would normally be the scotophase.

The results showed *M. brassicae* eggs have no daily hatching pattern during the photophase when their photophase consists of white light (414 nm – 659 nm, peaks 463 nm and 628 nm) with a strength of 92 ± 1 LUX. There were no significant differences in the hatching pattern between the treatments and the control. The blue and red ALAN treatment had an earlier start to hatching. This result was not significant, but it was close to being significant. With a larger sample size, it is possible these results become significant.

There was a lot of variation in the hatching pattern within treatments. This possibly has a genetic basis, but could also have been caused by eggs being laid at different times before the start of the experiment. It is recommended to study this further by repeating the experiment with clutches being divided over treatments and checking for differences within the clutch. Measures also need to be taken to make sure the eggs are laid at the same time.

There also no significant differences in the mean hatching time of different treatments. The small differences between treatments did also not come close to being significant.

More eggs hatched during the scotophase, but this was not significantly more than what would be expected given the longer length of the scotophase compared to the photophase. It cannot be ruled out there is no preference for either the scotophase or photophase. To find this out it is recommended to perform a study with different scotophase lengths.

The blue ALAN treatment had a significantly lower mortality than the control and the white ALAN treatment a significantly higher mortality. This could be due to total irradiance which was higher in the blue treatment compared to the other treatments, because more irradiance is needed to achieve the same LUX as the other treatments.

The total irradiance and the strength of the light during the photophase was low compared to the strength of real sunlight. It is possible that the light intensity was too low to achieve photoperiodism. It is recommended to test this hypothesis by doing an experiment with a range of light intensity of the same wavelength to test the effect of total irradiance. If light intensity is important this experiment would need to be repeated with higher light intensity during the photophase.

In this study red light was never significantly different from the control. In other studies, red light has smaller negative effects on other life stages of moths than longer wavelength light. Given this it is recommended to use red light to mitigate the effect of light pollution on moths. This is important information for governments and municipalities. However, it is important to also take into account the effect of ALAN on other organisms.

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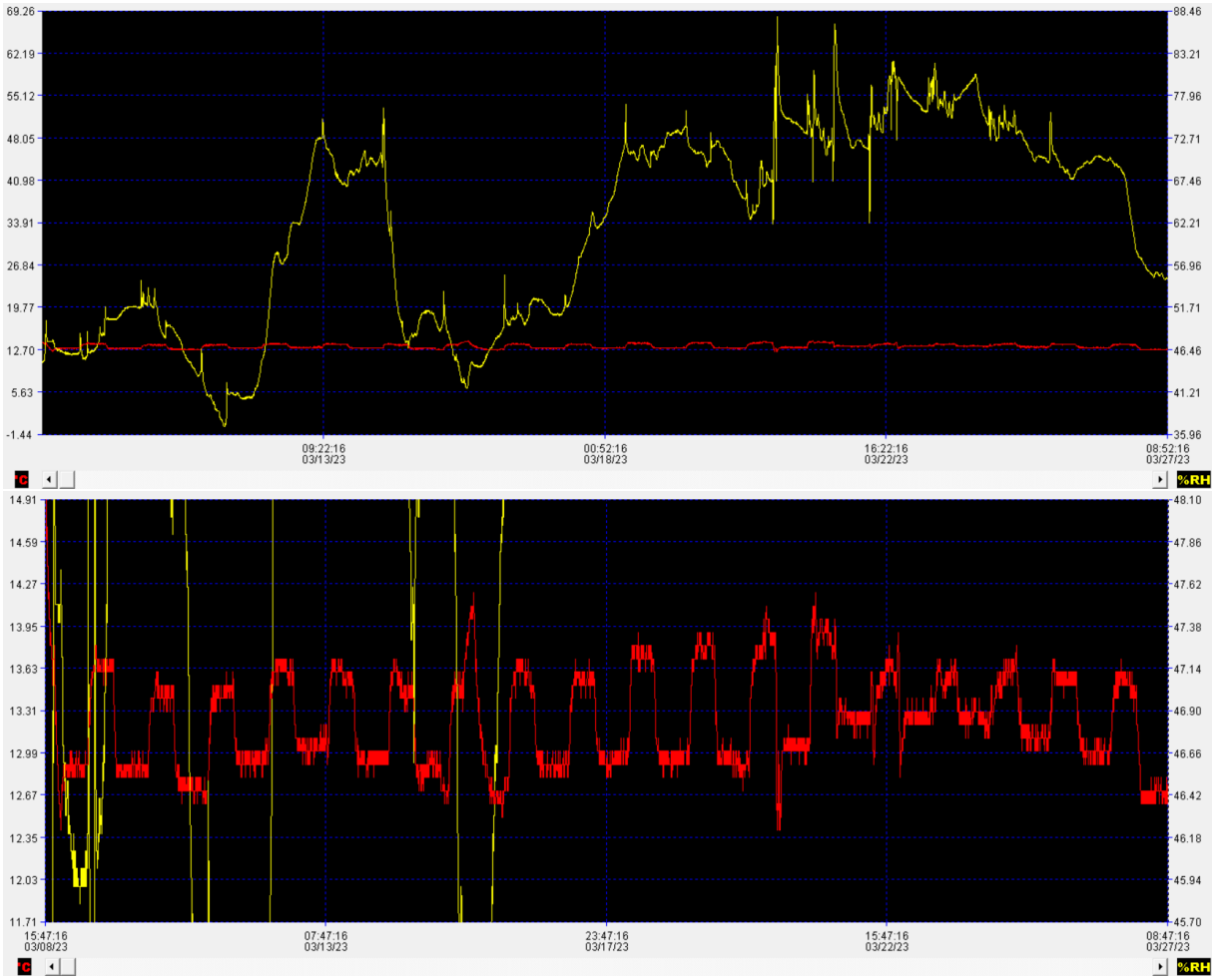
Appendices

Appendix A Temperature measurements

In the Figures A1, A2, A3 and A4 the humidity and the temperature are displayed. The logger used was the BL30 CLIMATE DATA LOGGER. One of the temperature and humidity loggers was formatted wrong and some of the treatments got mixed up. We are only sure which logger came out of the box where all petri dishes received their photophase. The other four loggers came out of the boxes where petri dishes received their ALAN treatment. The logger that was formatted wrong only recorded the first 45 hours of the experiment. The three loggers that logged the entire experiment are however very similar and since all treatments were at 8 LUX the temperature and humidity were likely the same in the last box with the logger that was formatted wrong.

Figure A1

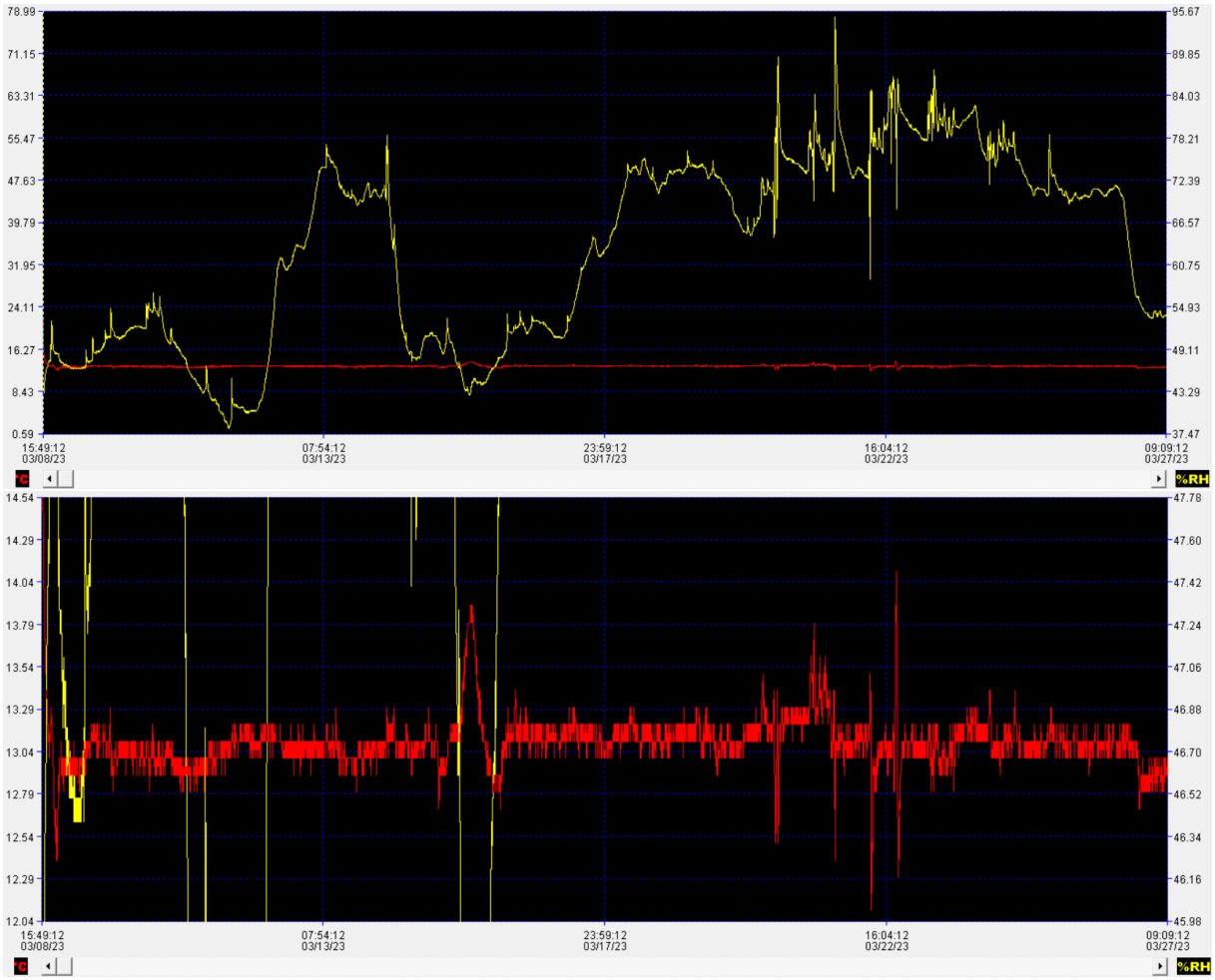
The temperature and humidity from the box where the control and the ALAN groups received their photophase



Note. The Y-axis on the left side shows the temperature in degrees Celsius. The red line is the temperature line. The Y-axis on the right side shows the humidity in relative humidity. The yellow line is the humidity line. The graph at the bottom is a zoom on the temperature. The X-axis at the bottom displays the time and date. The graph starts at the start of the experiment and ends when the experiment ended. The temperature starts high because the temperature logger itself needed to come down to the right temperature.

Figure A2

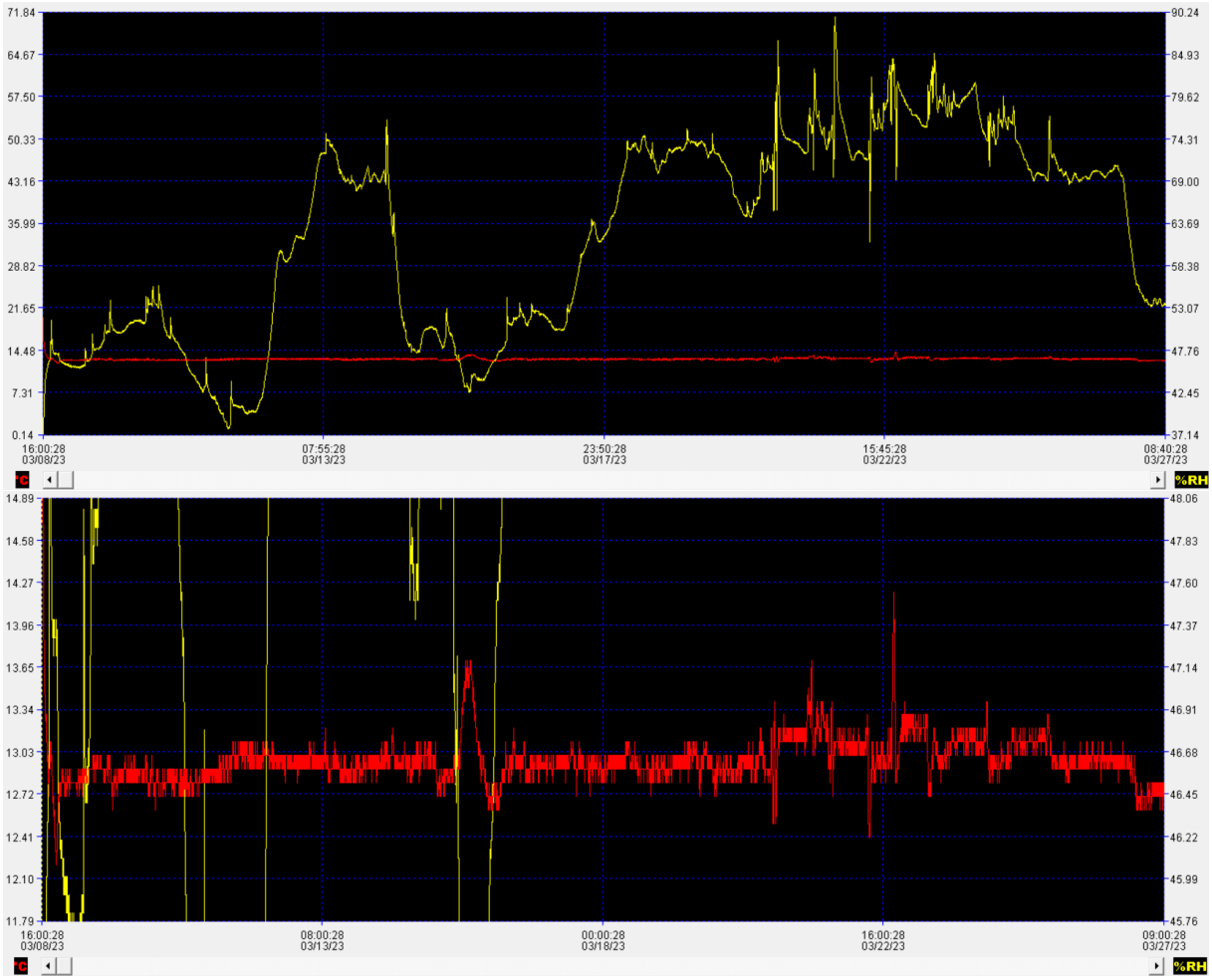
The temperature and humidity from one of the boxes where the ALAN groups received their ALAN



Note. The Y-axis on the left side shows the temperature in degrees Celsius. The red line is the temperature line. The Y-axis on the right side shows the humidity in relative humidity. The yellow line is the humidity line. The graph at the bottom is a zoom on the temperature. The X-axis at the bottom displays the time and date. The graph starts at the start of the experiment and ends when the experiment ended. The temperature starts high because the temperature logger itself needed to come down to the right temperature.

Figure A3

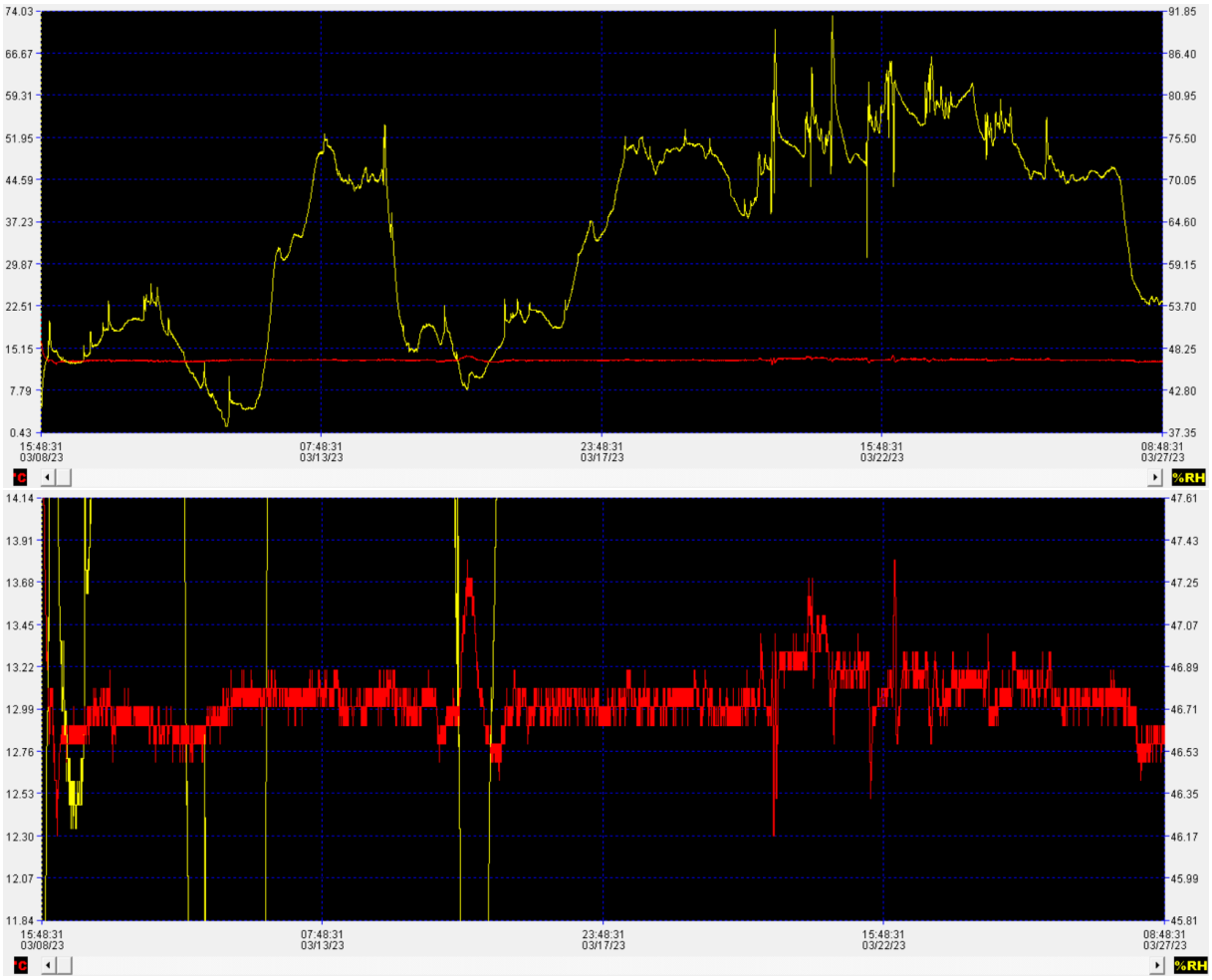
The temperature and humidity from another one of the boxes where the ALAN groups received their ALAN



Note. The Y-axis on the left side shows the temperature in degrees Celsius. The red line is the temperature line. The Y-axis on the right side shows the humidity in relative humidity. The yellow line is the humidity line. The graph at the bottom is a zoom on the temperature. The X-axis at the bottom displays the time and date. The graph starts at the start of the experiment and ends when the experiment ended. The temperature starts high because the temperature logger itself needed to come down to the right temperature.

Figure A4

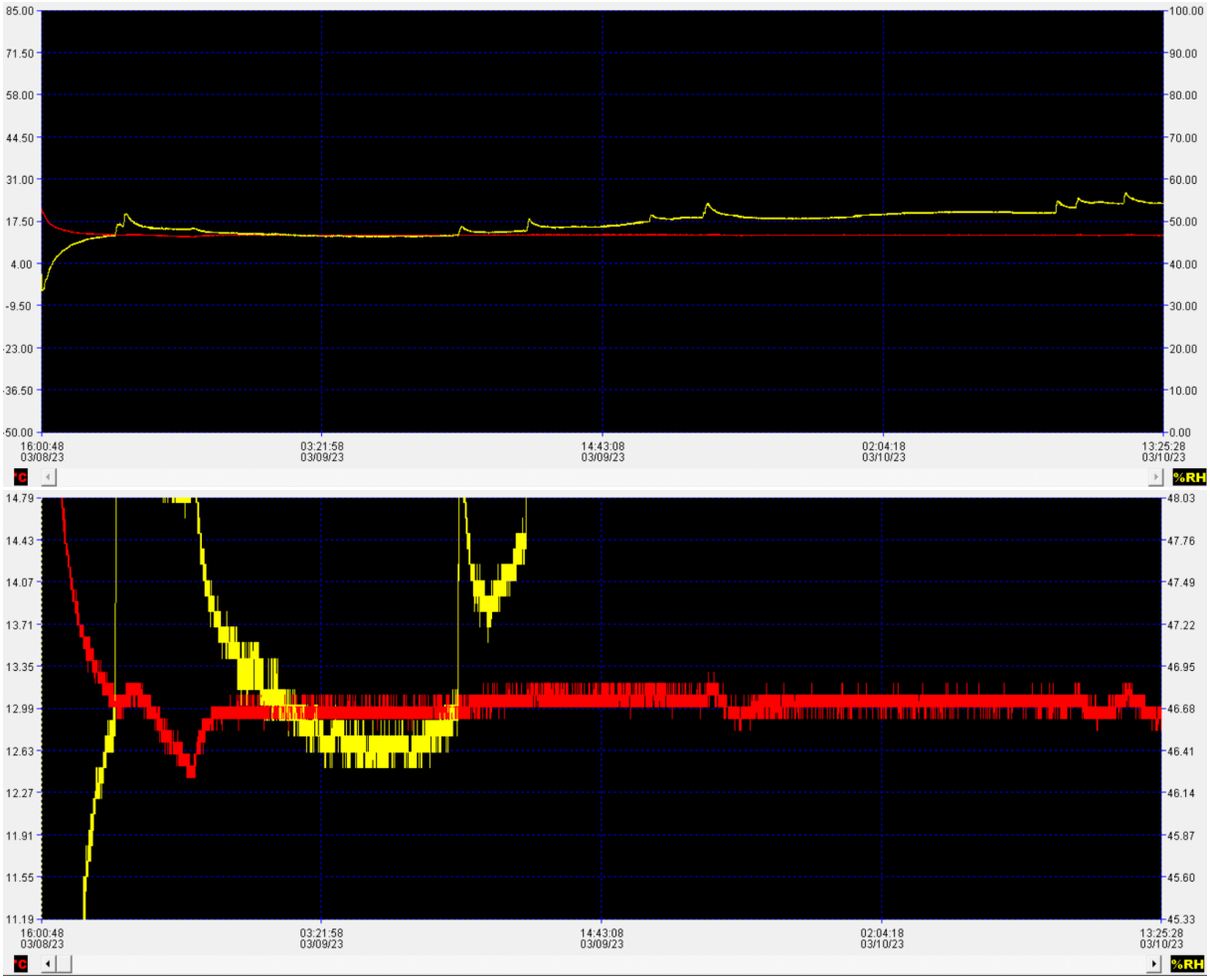
The temperature and humidity from a third one of the boxes where the ALAN groups received their ALAN



Note. The Y-axis on the left side shows the temperature in degrees Celsius. The red line is the temperature line. The Y-axis on the right side shows the humidity in relative humidity. The yellow line is the humidity line. The graph at the bottom is a zoom on the temperature. The X-axis at the bottom displays the time and date. The graph starts at the start of the experiment and ends when the experiment ended. The temperature starts high because the temperature logger itself needed to come down to the right temperature.

Figure A5

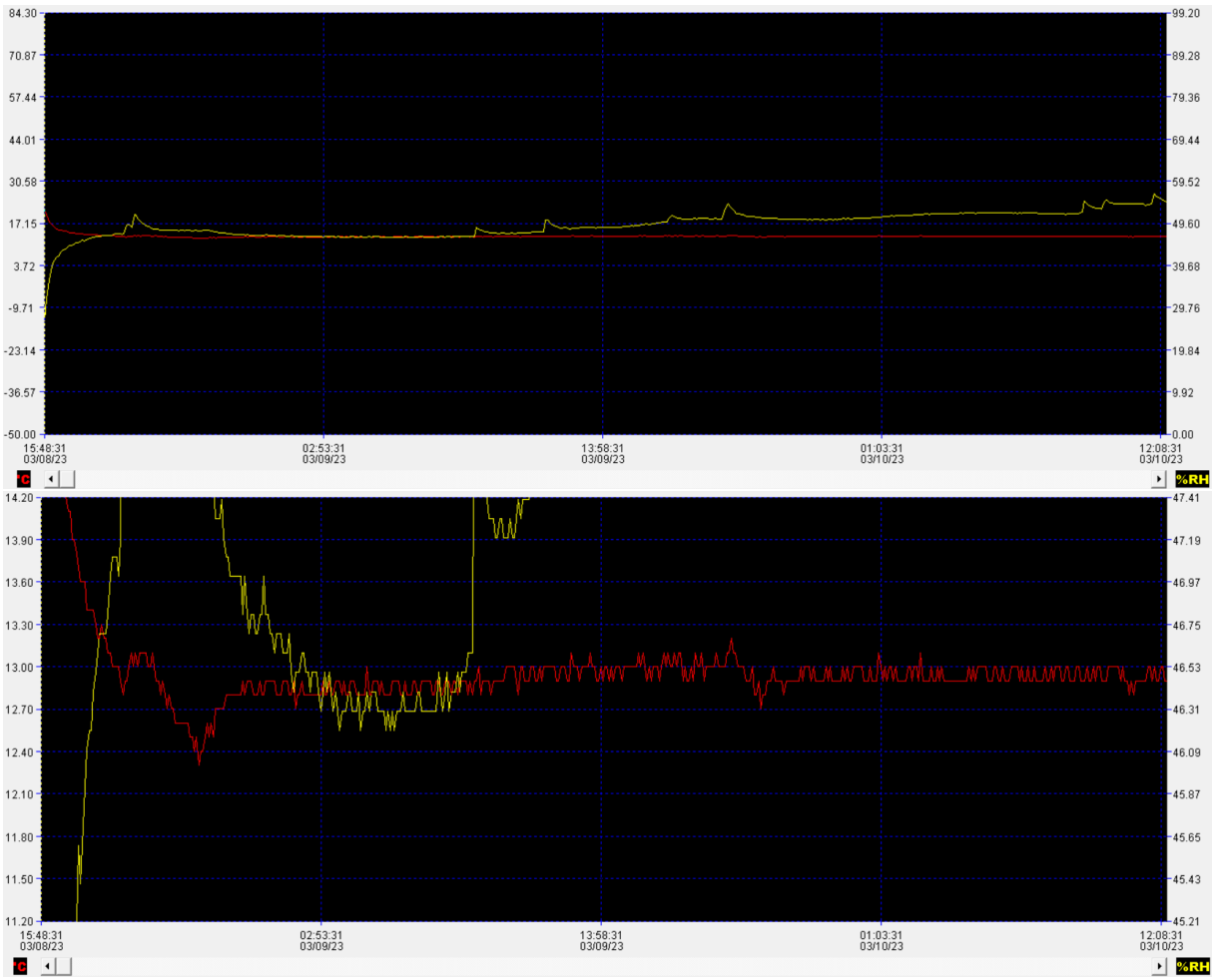
The temperature and humidity from the ALAN box which had the logger that was formatted wrong



Note. The Y-axis on the left side shows the temperature in degrees Celsius. The red line is the temperature line. The Y-axis on the right side shows the humidity in relative humidity. The yellow line is the humidity line. The graph at the bottom is a zoom on the temperature. The X-axis at the bottom displays the time and date. The graph starts at the start of the experiment and ends early because this logger was formatted wrong. The temperature starts high because the temperature logger itself needed to come down to the right temperature.

Figure A6

The temperature and humidity from one of the boxes where the ALAN groups received their ALAN zoomed in on the same time as the box that was formatted wrong for comparison



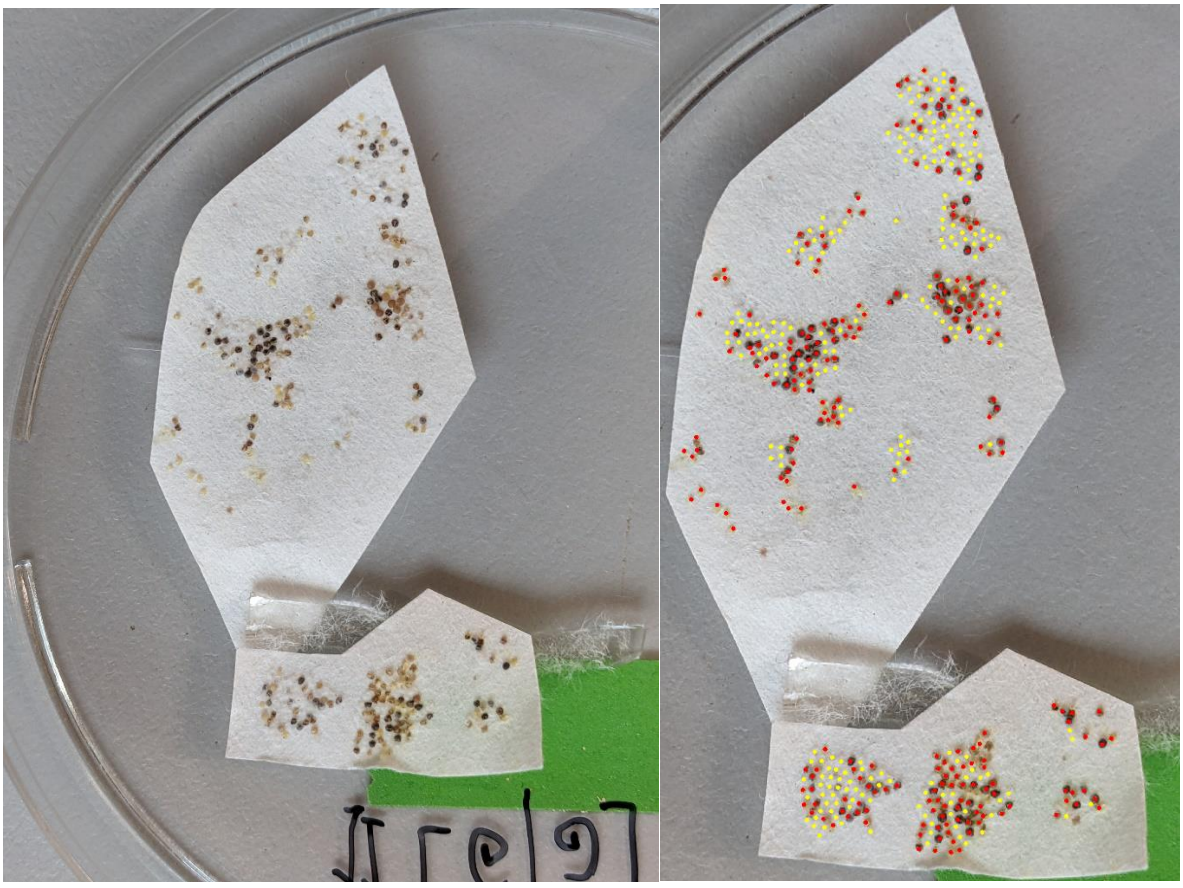
Note. The Y-axis on the left side shows the temperature in degrees Celsius. The red line is the temperature line. The Y-axis on the right side shows the humidity in relative humidity. The yellow line is the humidity line. The graph at the bottom is a zoom on the temperature. The X-axis at the bottom displays the time and date. The graph starts at the start of the experiment and ends early to compare it with the logger that was formatted wrong. The temperature starts high because the temperature logger itself needed to come down to the right temperature.

Appendix B Egg counting

DotDotGoose was used to mark and count eggs (Ersts, 2023). This program allows you to place a dot on the eggs and counts the dots automatically. This makes the counting of the number of eggs more precise, and it is impossible to lose count. For the mortality assessment this also made it possible to take the time to open eggs to see if there was still an embryo inside, making the mortality data more precise. Below is an example of the markings of one of the repetitions of a group receiving the green ALAN treatment (Figure B1). Hatched eggs are not very visible on the picture as only the translucent eggshells were left on the filter paper. They were visible through the stereoscope but hard to photograph (Figure B2). The mortality data for each petri dish can be found in Table B1. The pictures of the mortality in the petri dishes can be found in the Figures B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13, B14, B15, B16 and B17. Not the entire petri dish is in the picture and some of the photos are stitched together, but all of the eggs are visible on the picture.

Figure 1B

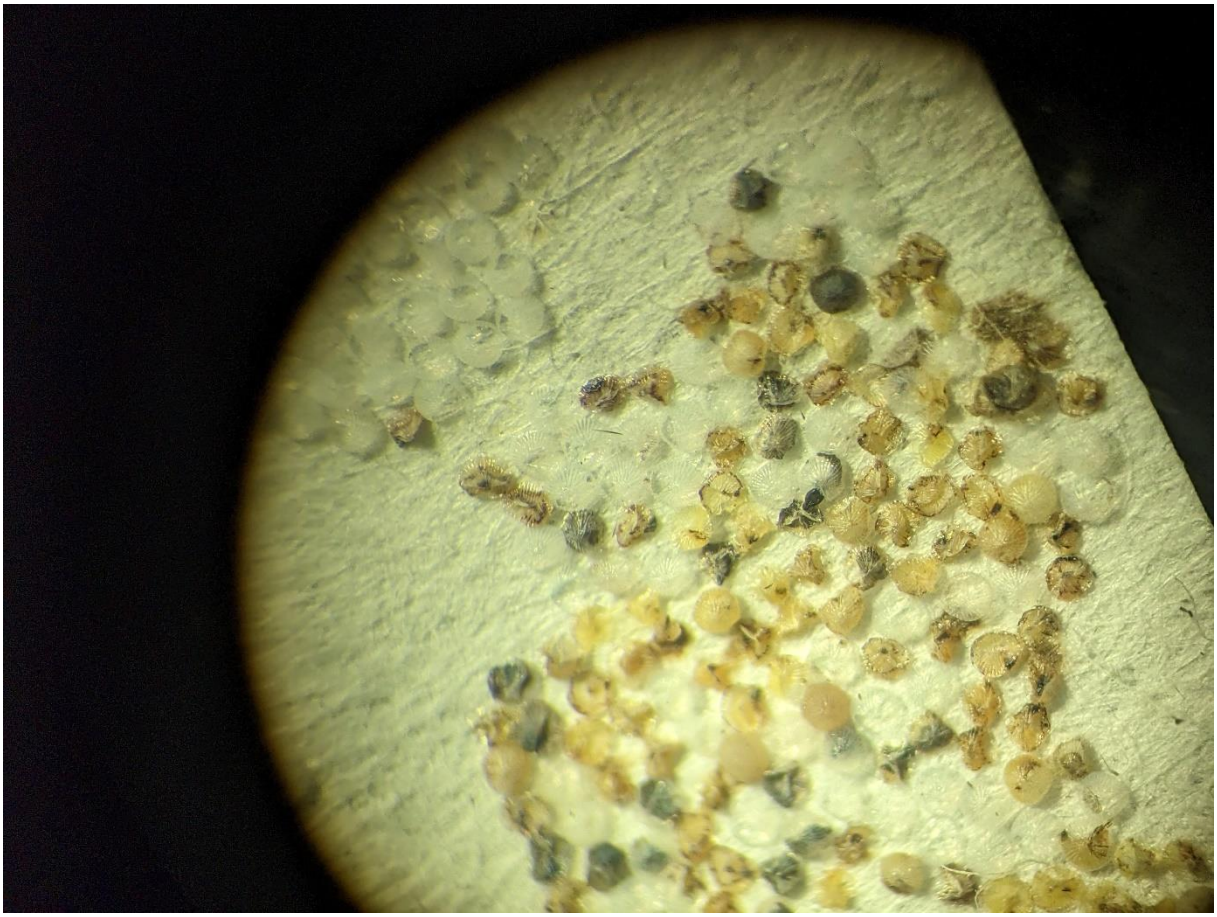
An example of the eggs in a petri dsih before and after counting the mortality



Note. On the left the picture before using DotDotGoose. On the right the picture after marking and counting with DotDotGoose. The red dots are unhatched eggs and the yellow dots are hatched eggs.

Figure B2

The eggs through the microscope with the eggshells clearly visible



Note. The eggshells in the top of the picture that the microscope was focused on were clearly visible.

Table B1

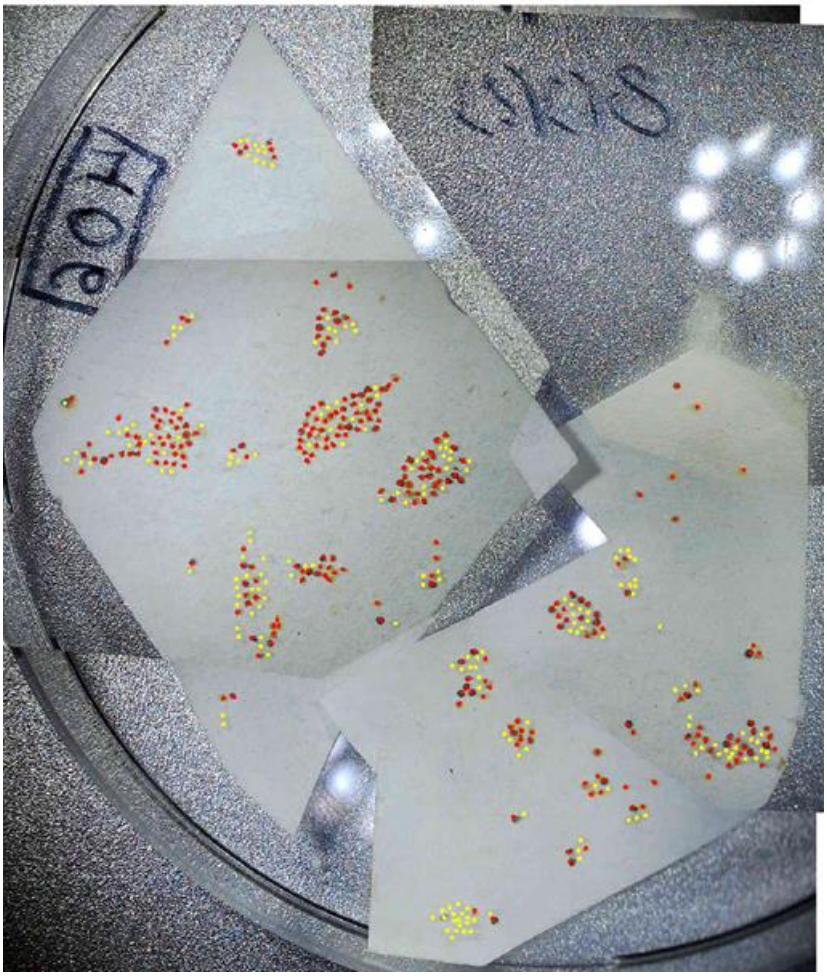
The mortality data

ID	Tr	Total_egg	Hatched_Eggs	Unhatched_eggs
1	C	460	193	267
2	C	435	216	219
3	C	410	197	213
4	LW	394	148	246
5	LW	457	171	286
6	LW	572	240	332
7	LR	384	174	210
8	LR	275	142	133
9	LR	413	204	209
10	LG	406	232	174
11	LG	486	243	243
12	LG	343	139	204
13	LB	383	188	195
14	LB	523	293	230
15	LB	367	229	138

Note. All the data on mortality gathered using the methods descriped earlier in this apendix.

Figure B3

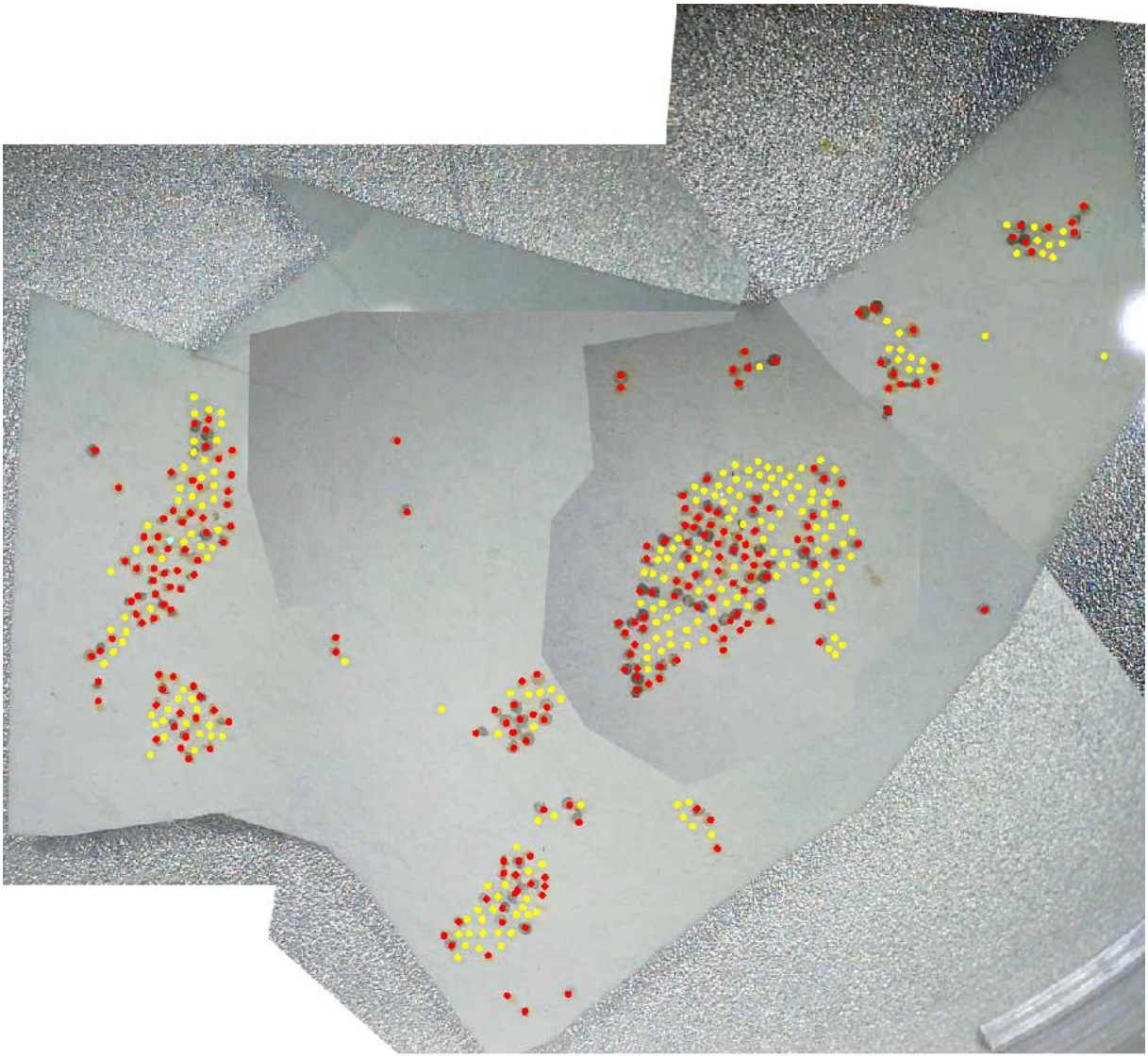
The mortality for the first repetition of the control group 1C



Note. The mortality for the first repetition of the control group 1C. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B4

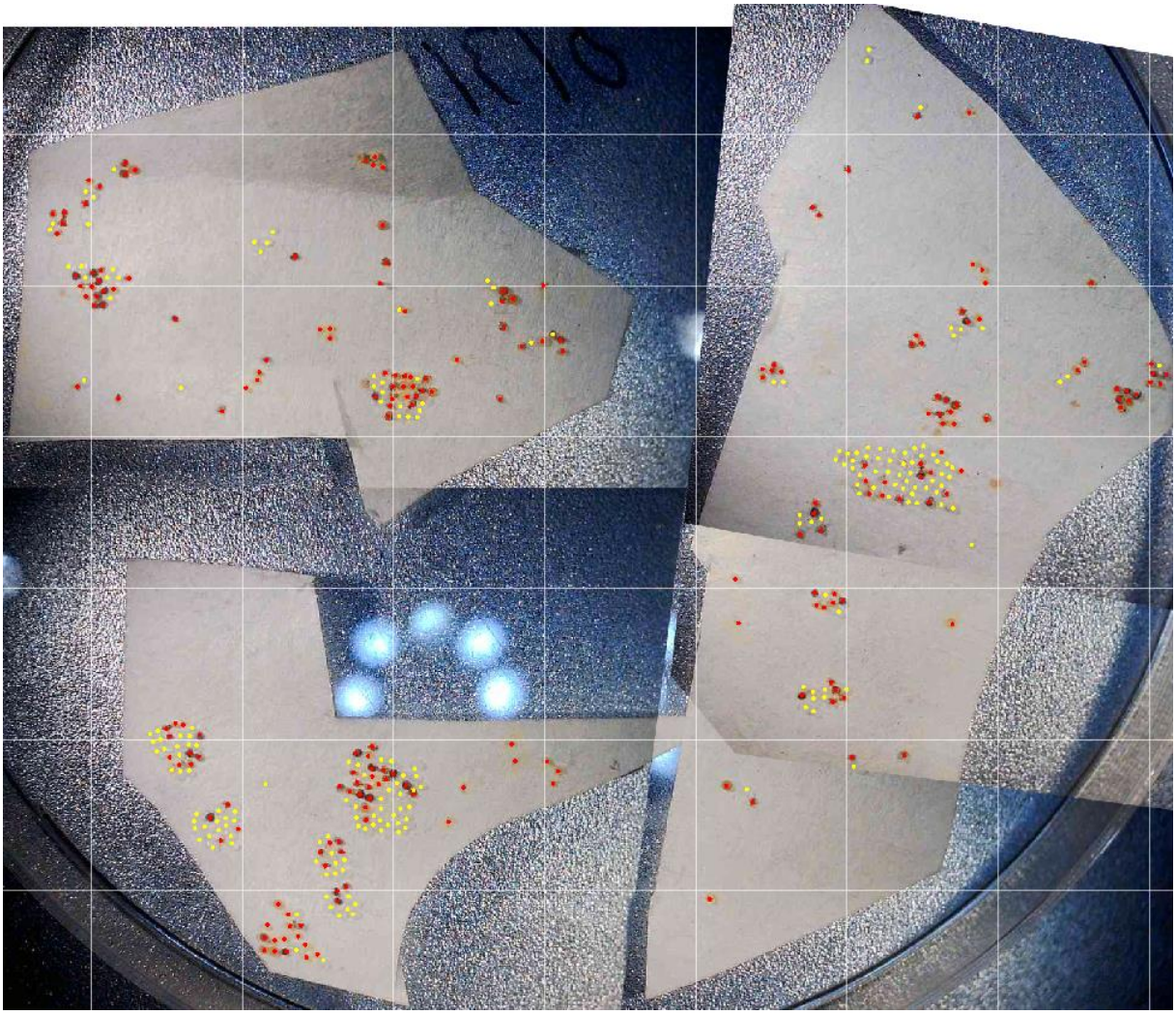
The mortality for the second repetition of the control group 2C



Note. The mortality for the second repetition of the control group 2C. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture. The blue dot in this picture should be ignored.

Figure B5

The mortality for the third repetition of the control group 3C



Note. The mortality for the third repetition of the control group 3C. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

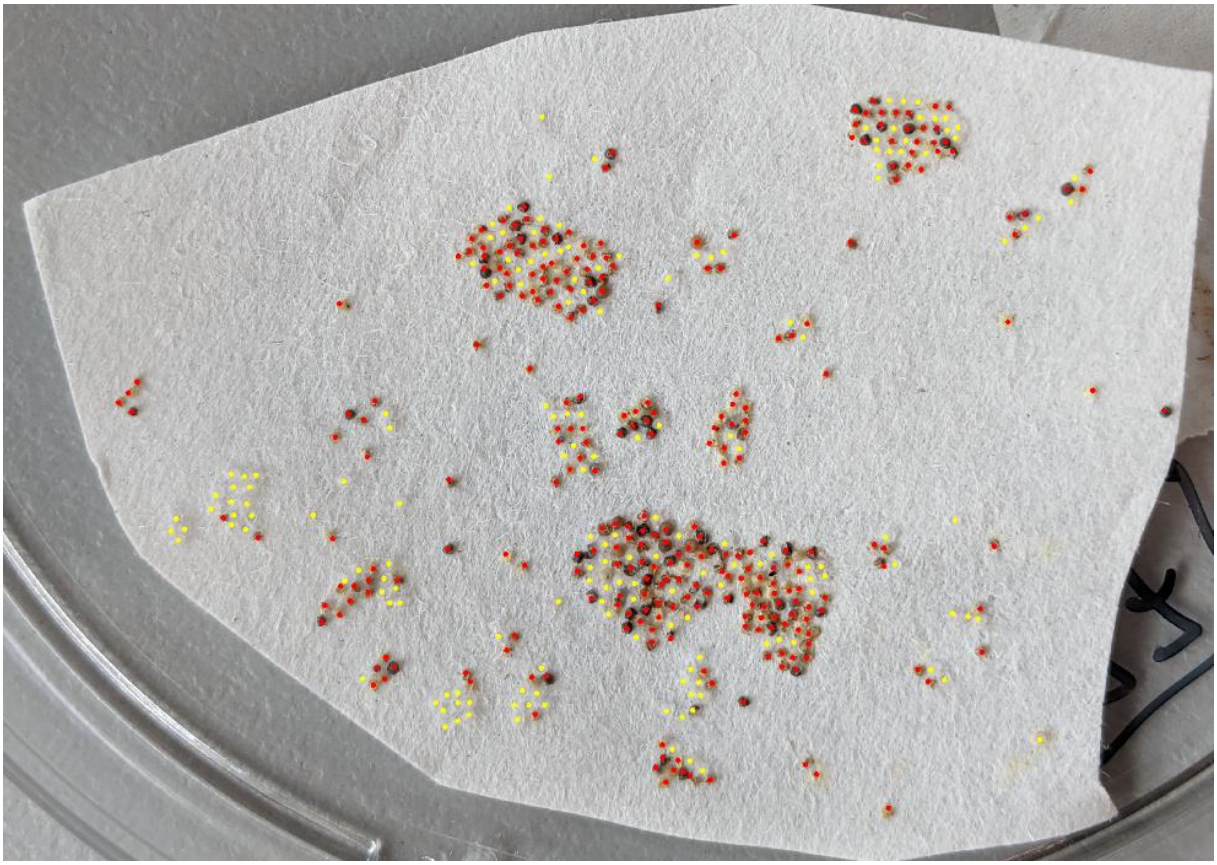
Figure B6

The mortality for the first repetition of the white ALAN treatment group 4LW

Note. The mortality for the first repetition of the white ALAN treatment group 4LW. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B7

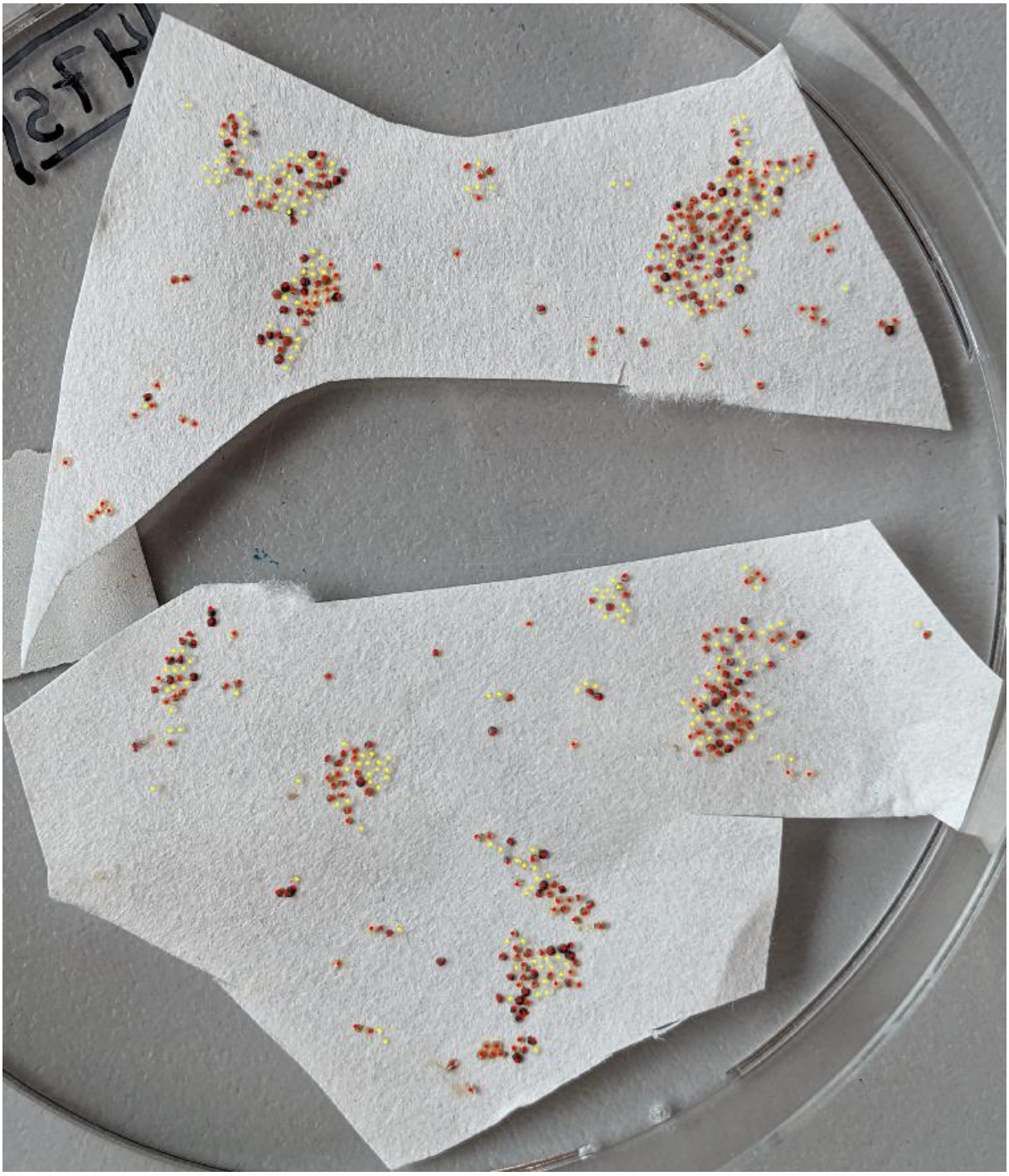
The mortality for the second repetition of the white ALAN treatment group 5LW



Note. The mortality for the second repetition of the white ALAN treatment group 5LW. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B8

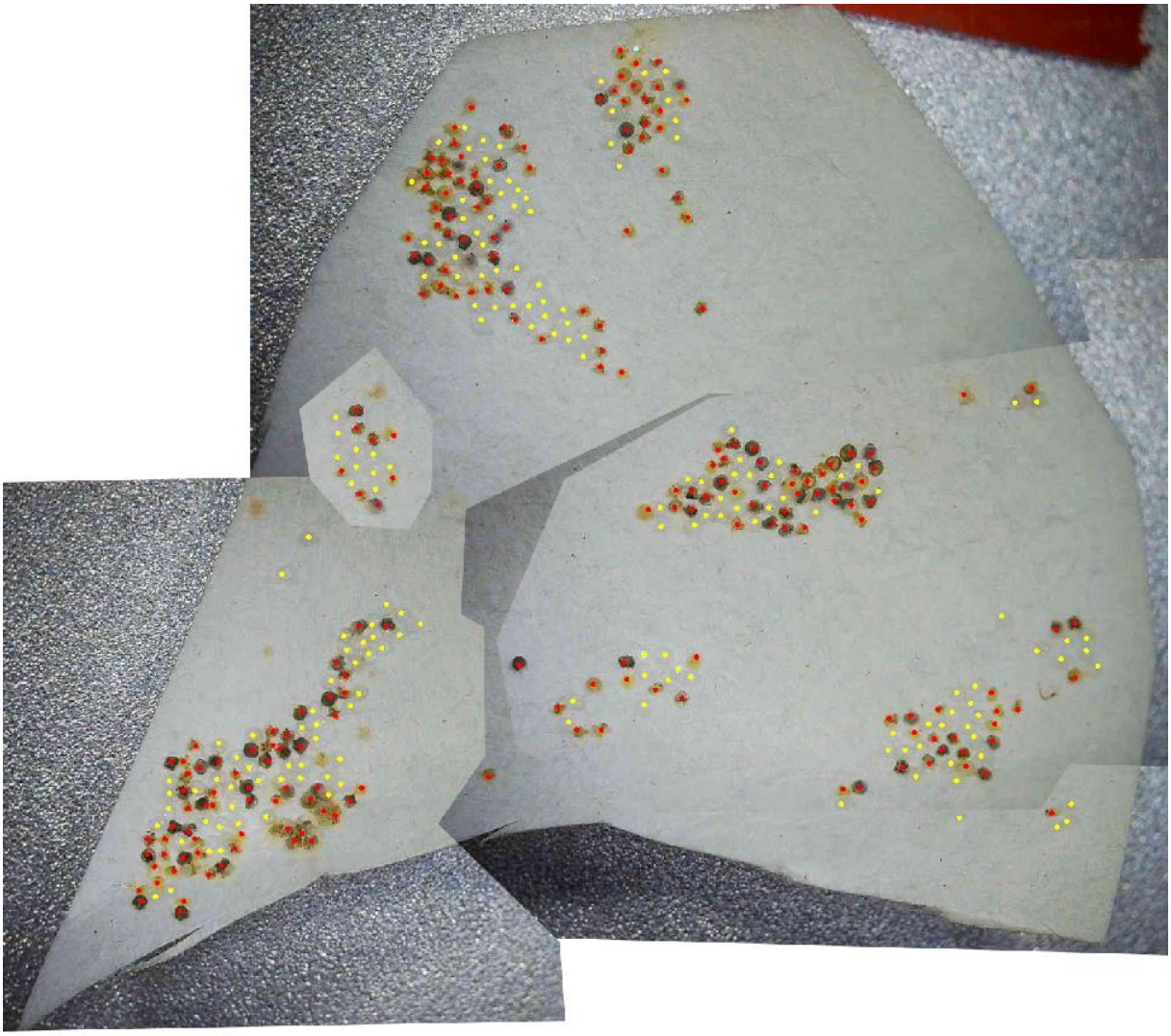
The mortality for the third repetition of the white ALAN treatment group 6LW



Note. The mortality for the third repetition of the white ALAN treatment group 6LW. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B9

The mortality for the first repetition of the red ALAN treatment group 7LR



Note. The mortality for the first repetition of the red ALAN treatment group 7LR. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

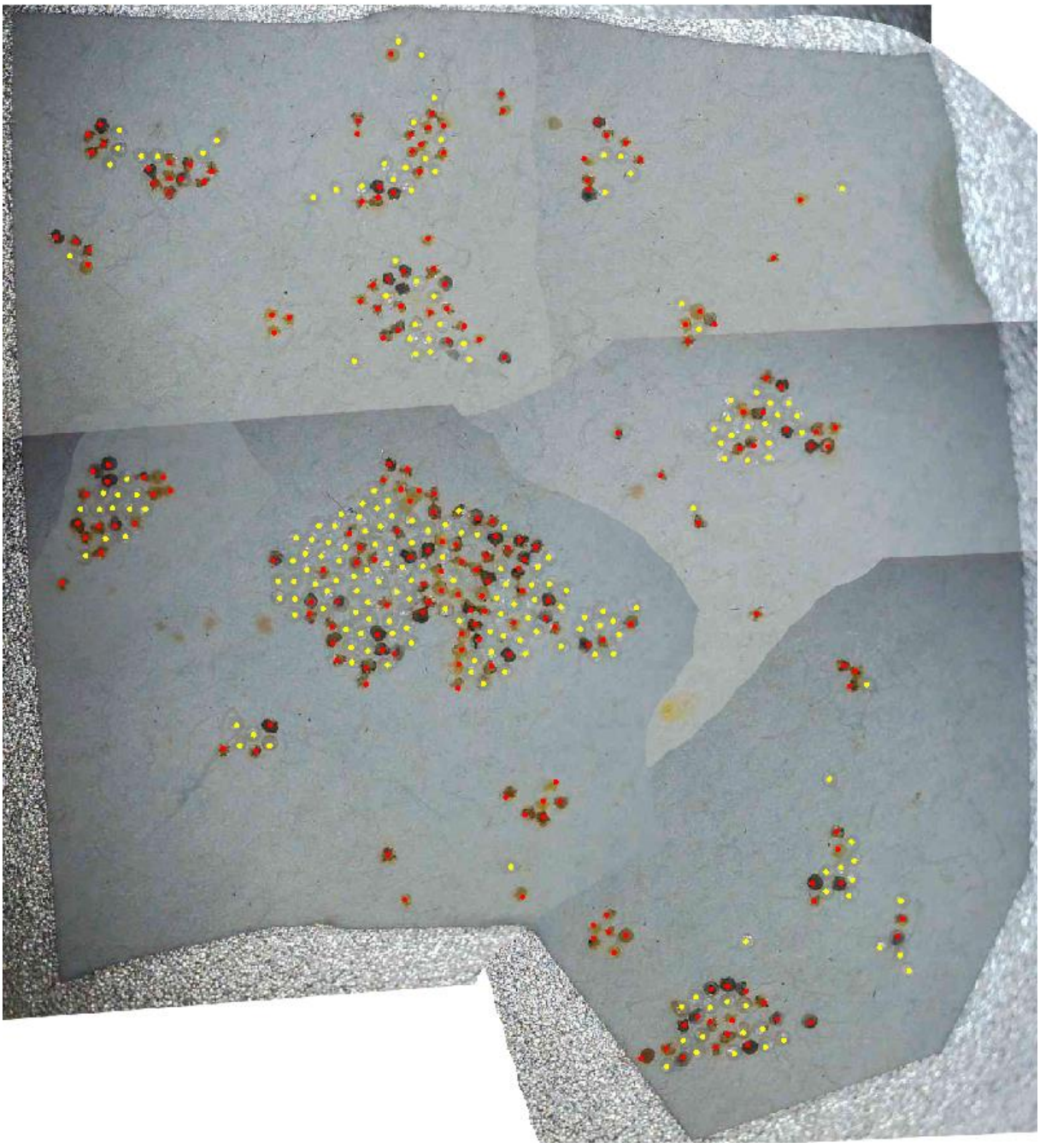
Figure B10

The mortality for the second repetition of the red ALAN treatment group 8LR

Note. The mortality for the second repetition of the red ALAN treatment group 8LR. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B11

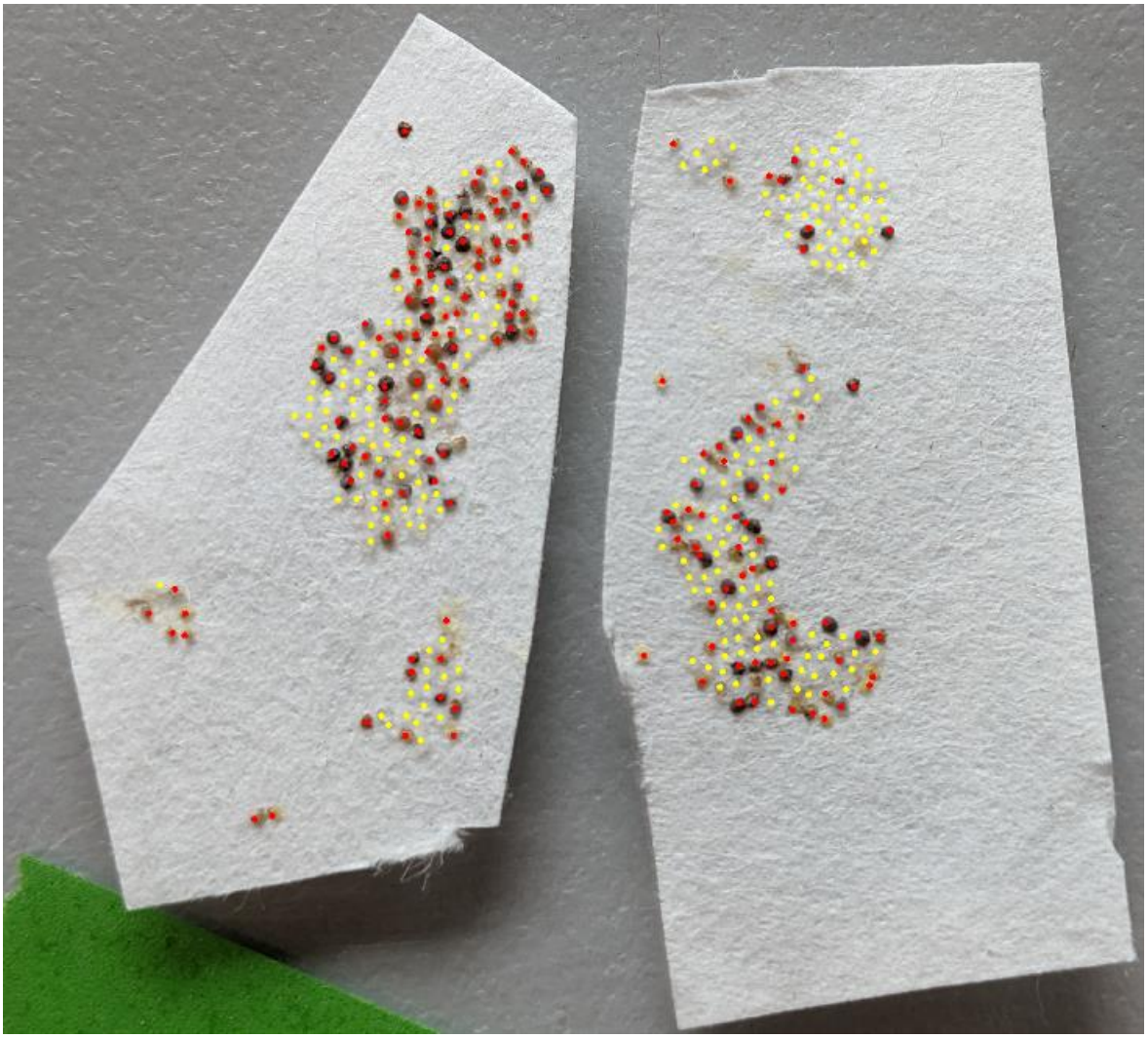
The mortality for the third repetition of the red ALAN treatment group 9LR



Note. The mortality for the third repetition of the red ALAN treatment group 9LR. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B12

The mortality for the first repetition of the green ALAN treatment group 10LG



Note. The mortality for the first repetition of the green ALAN treatment group 10LG. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

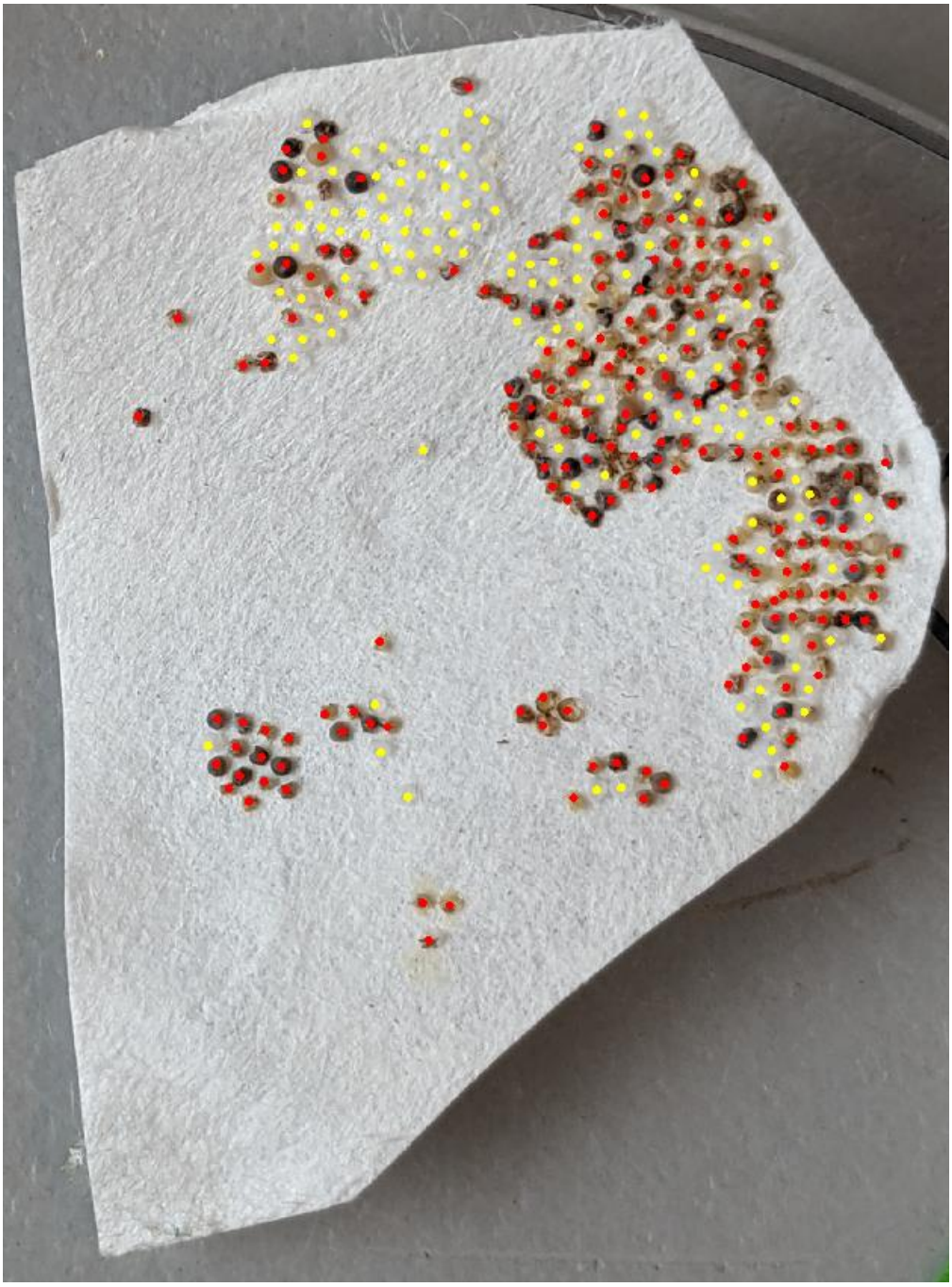
Figure B13

The mortality for the second repetition of the green ALAN treatment group 11LG

Note. The mortality for the second repetition of the green ALAN treatment group 11LG. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B14

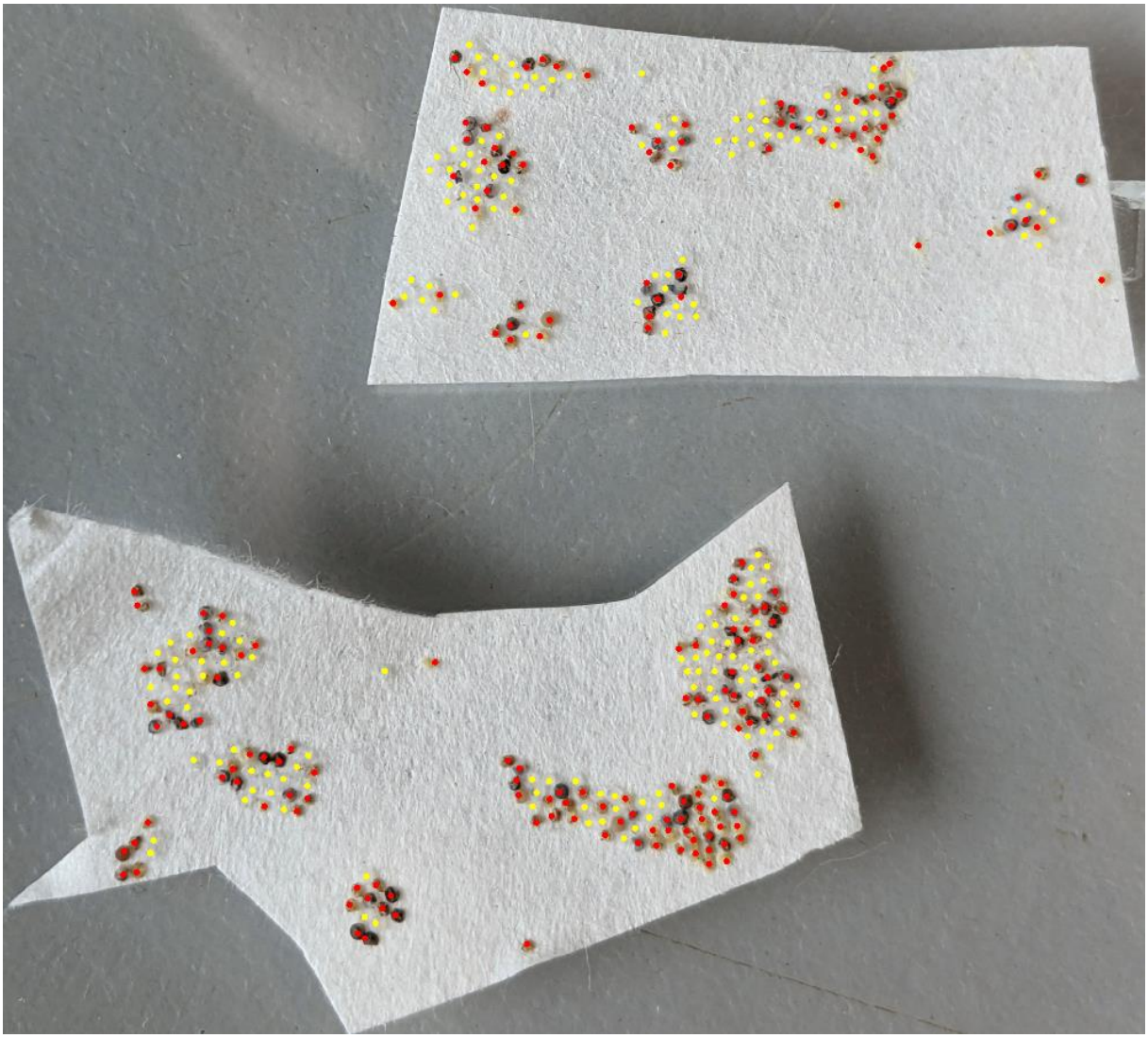
The mortality for the third repetition of the green ALAN treatment group 12LG



Note. The mortality for the third repetition of the green ALAN treatment group 12LG. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B15

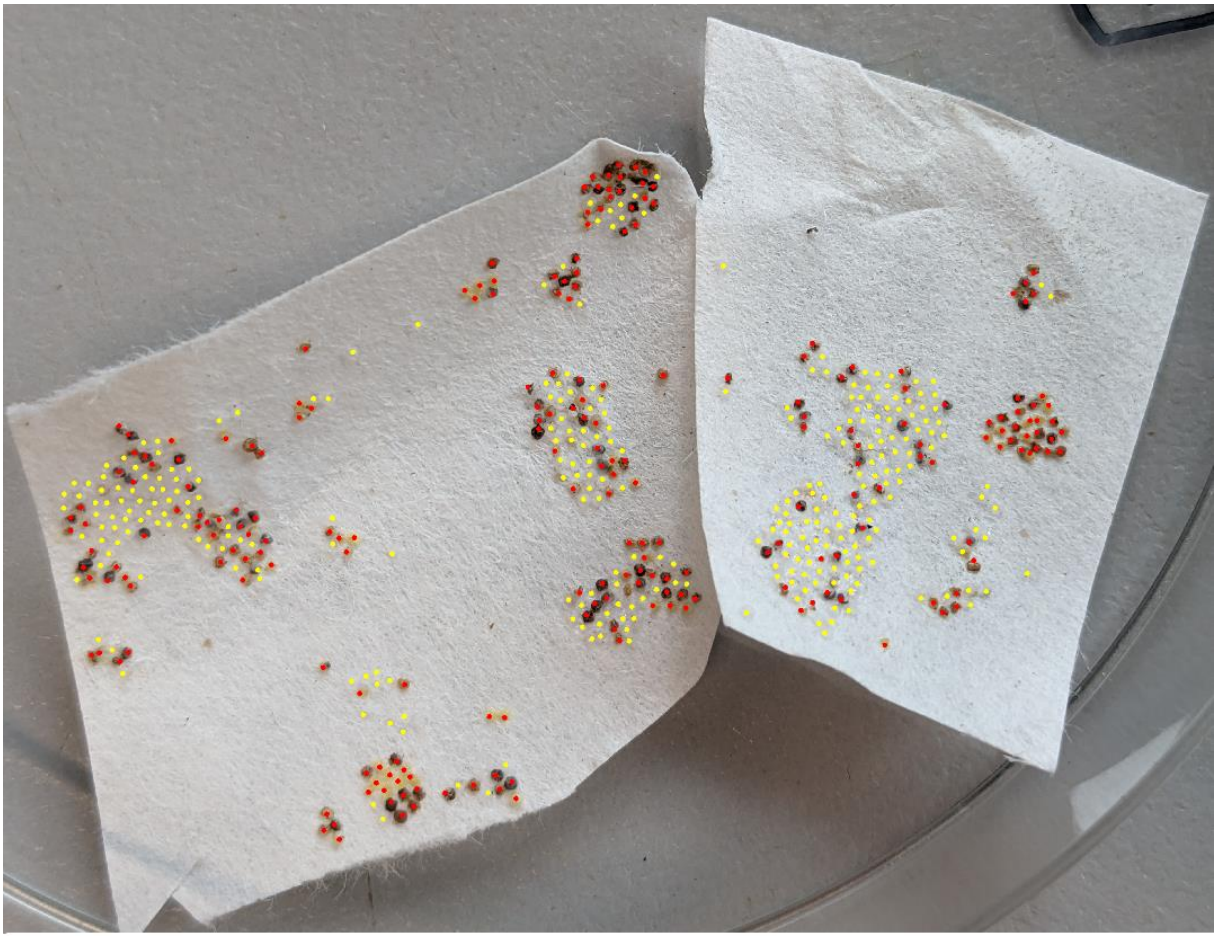
The mortality for the first repetition of the blue ALAN treatment group 13LB



Note. The mortality for the first repetition of the blue ALAN treatment group 13LB. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B16

The mortality for the second repetition of the blue ALAN treatment group 14LB



Note. The mortality for the second repetition of the blue ALAN treatment group 14LB. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Figure B17

The mortality for the third repetition of the blue ALAN treatment group 15LB

Note. The mortality for the third repetition of the blue ALAN treatment group 15LB. The red dots are unhatched eggs and the yellow dots are hatched eggs. All the eggs from this repetition are in the picture.

Appendix C Statistical analysis R-script

Below you can find the R-script used to do the statistical analysis of the results.

```
#################################################################

## What is the effect of ALAN on Mamestra brassicae eggs

## Pelle van Hilst - pelle.v.hilst@gmail.com - June 2023

#################################################################

rm(list=ls())

##############################

# 0 - Load libraries

##############################

#install.packages("fitdistrplus")

#install.packages("ggpubr")

#install.packages("ggplot2")

#install.packages("dplyr")

#install.packages("tidyverse")

##install.packages("survival")

#install.packages("survminer")

#install.packages("readxl")

#install.packages("glmmTMB")

#install.packages("brms", type="source")

#install.packages("nlme")

#install.packages("lme4")

#install.packages("gridExtra")

#install.packages("coxme")

#install.packages("coxphw")


library(fitdistrplus)

library(ggpubr)

library(ggplot2)

library(dplyr)

library(tidyverse)
```

```
library(betareg)
library("survival")
library("survminer")
library(readxl)
library(glmmTMB)
library(brms)
library(nlme)
library(lme4)
library(gridExtra)
library(coxme)
library(coxphw)
##############################
# 1 - Source files
##############################
setwd("C:/Users/pelle/Downloads/Mamestra_brassica_eggs_ALAN/Mamestra_brassicae_eggs_ALAN
")
#file with hatching times
pre_hatching_data <- read_excel("Hours_eggs_Mamestra_brassicae_2.0.xlsx",
                    sheet=1,col_names = TRUE)
head(pre_hatching_data)

#file with mortality
mortality_data <- read_excel("Mamestra_brassicae_ALAN_mortality.xlsx",
                      sheet=1,col_names = TRUE)
head(mortality_data)

##############################
# 2 - creating data frame hatching times
##############################
#Adding a column with hatching day based on hatching hour
```

```
# Define the ranges of hours within a day
hours_day <- c(289, 313, 337, 361, 385, 409)
days <- c("12", "13", "14", "15", "16")

# Add a new column based on range conditions
pre_hatching_data$hatching_day <- cut(pre_hatching_data$Time, breaks = hours_day,
                                      labels = days, include.lowest = TRUE)
print(pre_hatching_data)

# Giving each individual hatched egg their own row with hatching time and an
#individual egg ID

hatching_data<-pre_hatching_data %>%
  slice(rep(row_number(), times = Caterpillars)) %>%
  select(-Caterpillars) %>%
  mutate(Egg_ID = row_number())

print(hatching_data)

##############################
# 3 - Mortality
##############################

# Calculate the proportion of mortality
mortality_data$proportion_unhatched <- mortality_data$Unhatched_eggs /
mortality_data$Total_egg
print(mortality_data)

# ID needs to be character
mortality_data$ID <- as.character(mortality_data$ID)
```

```
#plot the proportions of unhatched eggs per treatment
##Assigns colors to treatment's##
Tr_colors<-data.frame(treatment=c("C", "LB", "LG", "LR", "LW"),
                      color=c("grey", "#3f6cff", "green", "red", "white"))

##create a box plot with ggplot2, with colors##
ggplot(mortality_data, aes(x=Tr, y=proportion_unhatched, fill=Tr))+geom_boxplot()+
  geom_jitter(width=0.2, height=0, alpha=0.5)+
  scale_fill_manual(values=Tr_colors$color)+
  labs(title="Mortality per treatment", x="Treaments", y="Proportion of unhatched eggs")+
  ylim(0.35, 0.65)

#Test for beta distribution

# Perform Shapiro-Wilk test
shapiro.test(mortality_data$proportion_unhatched)

# Perform Kolmogorov-Smirnov test for each category
ks_mortality_data <- ks.test(mortality_data$proportion_unhatched, "pnorm",
                             mean(mortality_data$proportion_unhatched), sd(mortality_data$proportion_unhatched))
print(ks_mortality_data)

## Step (1) Plot
descdist( data = mortality_data$proportion_unhatched , discrete = FALSE)
descdist(data = mortality_data$proportion_unhatched, discrete = FALSE, boot=1000)

## Step (2) Fit

fitdist(mortality_data$proportion_unhatched,"beta")
max(mortality_data$proportion_unhatched)
```

```
#   values must be in [0-1] to fit a beta distribution
beta_mortality_data = fitdist(mortality_data$proportion_unhatched, "beta")
normal_mortality_data = fitdist(mortality_data$proportion_unhatched, "norm")
weibull_mortality_data = fitdist(mortality_data$proportion_unhatched, "weibull")
gamma_mortality_data = fitdist(mortality_data$proportion_unhatched, "gamma")

plot(beta_mortality_data)
plot(normal_mortality_data)
plot(weibull_mortality_data)
plot(gamma_mortality_data)

## Step (3) Estimate parameters

print(beta_mortality_data)
print(normal_mortality_data)
print(weibull_mortality_data)
print(gamma_mortality_data)

summary(beta_mortality_data)
summary(normal_mortality_data)
summary(weibull_mortality_data)
summary(gamma_mortality_data)



#test with a beta distribution
# Fit a beta regression model with a random effect using glmmTMB
# Define a custom control object
custom_control <- glmmTMBControl(optimizer = optim, optArgs = list(method="BFGS"))

# Fit  model TR ID with the specified optimizer
model_TMB <- glmmTMB(proportion_unhatched ~ Tr + (1 | ID), data = mortality_data,
```

```
                family = beta_family("logit"),
                control = custom_control)

print(model_TMB)
summary(model_TMB)
diagnose(model_TMB)

model_TMB2 <- glmmTMB(proportion_unhatched ~ (1 | ID), data = mortality_data,
                 family = beta_family("logit"))
summary(model_TMB2)

model_TMB3 <- glmmTMB(proportion_unhatched ~ Tr , data = mortality_data,
                 family = beta_family("logit"))
summary(model_TMB3)
anova(model_TMB, model_TMB3)

mortality_data_treatment<- aggregate(proportion_unhatched~Tr,
                            data=mortality_data, FUN=mean)
AIC(model_TMB3)
AIC(model_TMB)
print(mortality_data_treatment)

anova(model_TMB, model_TMB3)

################################
# 4 - Survival model
################################

# Fit first Cox proportional hazards model with only treatment
cox_model1_TR <- coxph(Surv(Time, status) ~ TR, data = hatching_data)
summary(cox_model1_TR)
```

```
cox_model6_ID <- coxph(Surv(Time, status) ~ ID, data = hatching_data)
summary(cox_model6_ID)

#testing linearity
plot(predict(cox_model1_TR), residuals(cox_model1_TR, ype= "martingale"),
    ylab= "Fitted values", xlab= "Martingale residuals",
    main= "Residuals plot model0", las=1)
abline(h=0)
lines(smooth.spline(predict(cox_model1_TR), residuals(cox_model1_TR,
                                    type= "martingale")), col="red")

plot(predict(cox_model1_TR), residuals(cox_model1_TR, type= "deviance"),
    ylab= "Fitted values", xlab= "Deviance residuals",
    main= "Residuals plot model0", las=1)
abline(h=0)
lines(smooth.spline(predict(cox_model1_TR), residuals(cox_model1_TR,
                                    type= "deviance")), col="red")
# Generate survival curves using the survfit function
kepler_model <- survfit(Surv(Time, status) ~ TR, data = hatching_data)

# Visualize survival curves ----------------------------------------------
kepler_plot <- ggsurvplot(kepler_model,
                pval = TRUE,
                conf.int = FALSE,
                risk.table = FALSE,
                risk.table.col = "strata",
                linetype = "strata",
                surv.median.line = "hv",
                ggtheme = theme_bw(),
                palette = c("black", "#0036e6", "#30b119", "#ca0000", "#e19b00"),
```

```
                xlim = c(312, 408),
                xlab = "Hours since start experiment",
                ylab = "Proportion of eggs still to hatch",
                break.x.by = 24)

# Flip the y-axis
kepler_plot$plot <- kepler_plot$plot + scale_y_reverse()

# Modify the plot title
kepler_plot$plot <- kepler_plot$plot +
  ggtitle("Egg hatching pattern")

# Display the modified plot
kepler_plot

# Generate survival curves using the survfit function
kepler_model_ID <- survfit(Surv(Time, status) ~ ID, data = hatching_data)

# Visualize survival curves ----------------------------------------------
kepler_plot_ID <- ggsurvplot(kepler_model_ID,
                pval = TRUE,
                conf.int = FALSE,
                risk.table = FALSE,
                risk.table.col = "strata",
                linetype = "strata",
                surv.median.line = "hv",
                ggtheme = theme_bw(),
                palette = c("black", "black", "black", "blue", "blue", "#0036e6", "#30b119",  "green",
"green", "#ca0000", "red", "red",
                        "orange", "orange",  "#e19b00"),
                xlim = c(312, 408),
```

```
                xlab = "Hours since start experiment",
                ylab = "Proportion of eggs still to hatch",
                break.x.by = 24)

# Flip the y-axis
kepler_plot_ID$plot <- kepler_plot_ID$plot + scale_y_reverse()

# Modify the plot title
kepler_plot_ID$plot <- kepler_plot_ID$plot +
  ggtitle("Egg hatching pattern")

# Display the modified plot
kepler_plot_ID

#Shoenfeld test for ph, test to see if data is proportional
cox.zph(cox_model1_TR)
par(mfrow=c(1,1))
plot(cox.zph(cox_model1_TR)[1])
abline(h=0, col="red")

#second plot
plot(cox.zph(cox_model1_TR)[2])
abline(h=0, col="red")

#Cox model with random effect with coxme package TR and ID as a random effect
coxme_model3_TR_ID <- coxme(Surv(Time, status) ~ TR + (1|ID), data = hatching_data)
print(coxme_model3_TR_ID)

#Cox model with random effect with coxme package TR and ID as a random effect
coxme_model5_ID_TR <- coxme(Surv(Time, status) ~ ID + (1|TR), data = hatching_data)
print(coxme_model5_ID_TR)
```

```
#Shoenfeld test for ph, test to see if data is proportional
cox.zph(coxme_model3_TR_ID)
par(mfrow=c(1,1))
plot(cox.zph(coxme_model3)[1])
abline(h=0, col="red")

#second plot
plot(cox.zph(coxme_model3_TR_ID)[2])
abline(h=0, col="red")

#AIC
AIC(cox_model1_TR)
AIC(coxme_model3_TR_ID)
AIC(coxme_model5_ID_TR)
AIC(cox_model6_ID)
anova(cox_model1_TR, coxme_model3_TR_ID, test="LRT")
anova(cox_model1_TR, coxme_model3_TR_ID, test="LRT")
anova(coxme_model3_TR_ID, coxme_model5_ID_TR, test="LRT")
anova(coxme_model3_TR_ID, cox_model6_ID, test="LRT")
#best model is model with TR and ID as random factor


##############################
# 5 - Mean hatching date
##############################

#create data frame
# Calculate means using aggregate()
pre_mean_hatching_day <- aggregate(as.numeric(as.character(hatching_data$hatching_day)),
                                   by = list(hatching_data$ID),
```

```
                    FUN = mean, na.rm = TRUE)
print(pre_mean_hatching_day)

# Rename the columns in the means data frame
colnames(pre_mean_hatching_day) <- c("ID", "mean_hatching_day")

# Merge means with df2 based on Repetitions
mortality_data_mean_hatching_day <- merge(mortality_data, pre_mean_hatching_day, by = "ID",
all.x = TRUE)
print(mortality_data_mean_hatching_day)

#Test for normal distribution
# Perform Shapiro-Wilk test
shapiro.test(mortality_data_mean_hatching_day$mean_hatching_day)

# Perform Kolmogorov-Smirnov test for each category
ks_mortality_data_mean_hatching_day <-
ks.test(mortality_data_mean_hatching_day$mean_hatching_day,
        "pnorm", mean(mortality_data_mean_hatching_day$mean_hatching_day),
        sd(mortality_data_mean_hatching_day$mean_hatching_day))
print(ks_mortality_data_mean_hatching_day)
## Step (1) Plot
descdist( data = mortality_data_mean_hatching_day$mean_hatching_day , discrete = FALSE)
descdist(data = mortality_data_mean_hatching_day$mean_hatching_day, discrete = FALSE,
boot=1000)

## Step (2) Fit

max(mortality_data_mean_hatching_day$mean_hatching_day)

#   values must be in [0-1] to fit a beta distribution
```

```
normal_mortality_data_mean_hatching_day =
fitdist(mortality_data_mean_hatching_day$mean_hatching_day,
                                                "norm")
weibull_mortality_data_mean_hatching_day =
fitdist(mortality_data_mean_hatching_day$mean_hatching_day,
                                                "weibull")
gamma_mortality_data_mean_hatching_day  =
fitdist(mortality_data_mean_hatching_day$mean_hatching_day,
                                                "gamma")


plot(normal_mortality_data_mean_hatching_day)
plot(weibull_mortality_data_mean_hatching_day)
plot(gamma_mortality_data_mean_hatching_day)


## Step (3) Estimate parameters


print(normal_mortality_data_mean_hatching_day)
print(weibull_mortality_data_mean_hatching_day)
print(gamma_mortality_data_mean_hatching_day)


summary(normal_mortality_data_mean_hatching_day)
summary(weibull_mortality_data_mean_hatching_day)
summary(gamma_mortality_data_mean_hatching_day)



# Run a one-way ANOVA
hatching_day_model1 <- aov(mean_hatching_day ~ Tr, data = mortality_data_mean_hatching_day)
summary(hatching_day_model1)


# Conduct pairwise comparisons with Bonferroni correction
pairwise.t.test(mortality_data_mean_hatching_day$mean_hatching_day,
mortality_data_mean_hatching_day$Tr, p.adjust.method = "bonferroni")
```

```
# Apply Tukey's HSD test
tukey_result <- TukeyHSD(hatching_day_model1)
print(tukey_result)

##Assigns colors to treament's##
Tr_colors<-data.frame(treatment=c("C", "LB", "LG", "LR", "LW"),
                      color=c("grey", "#3f6cff", "green", "red", "white"))

## Longer y axis
ggplot(mortality_data_mean_hatching_day, aes(x = Tr, y = mean_hatching_day, fill = Tr)) +
  geom_boxplot() +
  geom_jitter(width = 0.2, height = 0, alpha = 0.5) +
  scale_fill_manual(values = Tr_colors$color) +
  labs(title = "Mean hatching day per treatment", x = "Treatments", y = "Mean hatching day") +
  ylim(13.8, 14.6)

mean_hatching_day_treatment<- aggregate(mean_hatching_day~Tr,
                        data=mortality_data_mean_hatching_day, FUN=mean)

print(mean_hatching_day_treatment)

##############################
# 6 - Scotophase photophase
##############################

# create data frame

# Group the original data frame by "ID" and calculate the proportion of "dark" and "light"
scotophase_data <- hatching_data %>%
  group_by(ID) %>%
```

```
  summarise(dark_proportion = sum(LD == "Dark") / n(),
            light_proportion = sum(LD == "Light") / n(),
            TR = first(TR))  # Retain the first value of TR within each group

# View the new data frame
print(scotophase_data)



#Test for normal distribution
# Perform Shapiro-Wilk test
shapiro.test(scotophase_data$dark_proportion)

# Perform Kolmogorov-Smirnov test for each category
ks_scotophase_data <- ks.test(scotophase_data$dark_proportion, "pnorm",
                              mean(scotophase_data$dark_proportion), sd(scotophase_data$dark_proportion))
print(ks_scotophase_data)
## Step (1) Plot
descdist( data = scotophase_data$dark_proportion , discrete = FALSE)
descdist(data = scotophase_data$dark_proportion, discrete = FALSE, boot=1000)

## Step (2) Fit

fitdist(scotophase_data$dark_proportion,"beta")
max(scotophase_data$dark_proportion)

#   values must be in [0-1] to fit a beta distribution
normal_scotophase_data = fitdist(scotophase_data$dark_proportion,
                                 "norm")
weibull_scotophase_data = fitdist(scotophase_data$dark_proportion,
                                  "weibull")
gamma_scotophase_data  = fitdist(scotophase_data$dark_proportion,
```

```
                                "gamma")

plot(normal_scotophase_data)
plot(weibull_scotophase_data)
plot(gamma_scotophase_data)

## Step (3) Estimate parameters

print(normal_scotophase_data)
print(weibull_scotophase_data)
print(gamma_scotophase_data)

summary(normal_scotophase_data)
summary(weibull_scotophase_data)
summary(gamma_scotophase_data)

#run anova
scotophase_data_aov <- aov(dark_proportion ~ TR, data=scotophase_data)
summary(scotophase_data_aov)

# Run a glm
scotophase_data_model0 <- glm(dark_proportion ~ TR, data = scotophase_data)
summary(scotophase_data_model0)

# Conduct pairwise comparisons with Bonferroni correction
pairwise.t.test(scotophase_data$dark_proportion, scotophase_data$TR, p.adjust.method =
"bonferroni")

##create a box plot with ggplot2, with colors and line for scotophase length##
ggplot(scotophase_data, aes(x=TR, y=dark_proportion, fill=TR))+geom_boxplot()+
  geom_jitter(width=0.2, height=0, alpha=0.5)+
```

```
  scale_fill_manual(values=Tr_colors$color)+
  labs(title="Proportion of caterpillars hatched in the
  scotophase or during the ALAN", x="Treaments", y="Proportion of eggs hatched in scotophase")+
  geom_hline(yintercept=0.5833333333, linetype="dashed", color="darkgrey", linewidth = 1)+
  ylim(0.25,0.8)

##create a box plot with ggplot2, with colors and line for scotophase length##
ggplot(scotophase_data, aes(x=TR, y=light_proportion, fill=TR))+geom_boxplot()+
  geom_jitter(width=0.2, height=0, alpha=0.5)+
  scale_fill_manual(values=Tr_colors$color)+
  labs(title="Proportion of caterpillars hatched in the
  photophase", x="Treaments", y="Proportion of eggs hatched in photophase")+
  geom_hline(yintercept=0.5833333333, linetype="dashed", color="darkgrey", linewidth = 1)+
  ylim(0,1)



# Split the data frame into subsets based on TR groups
grouped_light_dark <- split(scotophase_data, scotophase_data$TR)

# Perform a t-test for each group
ttest_scotophase_data <- lapply(grouped_light_dark, function(subset) {
  dark_proportion <- subset$dark_proportion
  light_proportion <- subset$light_proportion
  t_test <- t.test(dark_proportion, light_proportion)
  return(t_test$p.value)
})

# View the p-values for each group
print(ttest_scotophase_data)

# Specify the desired value to compare against
```

```
scotophase_length<- 0.5833333333  # Replace with your desired value



# Subset the data for each category and perform t-tests
for (category in unique(scotophase_data$TR)) {
  scotophase_subset_data <- scotophase_data[scotophase_data$TR == category, "dark_proportion"]
  print(scotophase_subset_data)
  # Perform t-test comparing the mean to the desired value
  t_test <- t.test(scotophase_subset_data, mu = scotophase_length)

  # Print the results
  cat("Category:", category, "\n")
  cat("Test statistic:", t_test$statistic, "\n")
  cat("p-value:", t_test$p.value, "\n")
  cat("\n")
}

##############################
# 7 - Mean hatching time
##############################
#create data frame
# Calculate means using aggregate()
pre_mean_hatching_time <- aggregate(as.numeric(as.character(hatching_data$Time)),
                          by = list(hatching_data$ID),
                          FUN = mean, na.rm = TRUE)
print(pre_mean_hatching_time)

# Rename the columns in the means data frame
colnames(pre_mean_hatching_time) <- c("ID", "mean_hatching_time")
print(pre_mean_hatching_time)
```

```
# Merge means with df2 based on Repetitions
mean_hatching_time <- merge(mortality_data, pre_mean_hatching_time, by = "ID", all.x = TRUE)
print(mean_hatching_time)

#Test for normal distribution
# Perform Shapiro-Wilk test
shapiro.test(mean_hatching_time$mean_hatching_time)

# Perform Kolmogorov-Smirnov test for each category
ks_mean_hatching_time <- ks.test(mean_hatching_time$mean_hatching_time,
                                 "pnorm", mean(mean_hatching_time$mean_hatching_time),
                                 sd(mean_hatching_time$mean_hatching_time))
print(ks_mean_hatching_time)
## Step (1) Plot
descdist( data = mean_hatching_time$mean_hatching_time, discrete = FALSE)
descdist(data = mean_hatching_time$mean_hatching_time, discrete = FALSE, boot=1000)

## Step (2) Fit

max(mean_hatching_time$mean_hatching_time)

#   values must be in [0-1] to fit a beta distribution
normal_mean_hatching_time = fitdist(mean_hatching_time$mean_hatching_time,
                                    "norm")
weibull_mean_hatching_time = fitdist(mean_hatching_time$mean_hatching_time,
                                     "weibull")
gamma_mean_hatching_time  = fitdist(mean_hatching_time$mean_hatching_time,
                                    "gamma")

plot(normal_mean_hatching_time)
plot(weibull_mean_hatching_time)
```

```
plot(gamma_mean_hatching_time)

## Step (3) Estimate parameters

print(normal_mean_hatching_time)
print(weibull_mean_hatching_time)
print(gamma_mean_hatching_time)

summary(normal_mean_hatching_time)
summary(weibull_mean_hatching_time)
summary(gamma_mean_hatching_time)



# Run a one-way ANOVA
hatching_time_model1 <- aov(mean_hatching_time ~ Tr, data = mean_hatching_time)
summary(hatching_time_model1)

# Conduct pairwise comparisons with Bonferroni correction
pairwise.t.test(mean_hatching_time$mean_hatching_time, mean_hatching_time$Tr,
p.adjust.method = "bonferroni")

# Apply Tukey's HSD test
tukey_result <- TukeyHSD(hatching_time_model1)
print(tukey_result)

##Assigns colors to treament's##
Tr_colors<-data.frame(treatment=c("C", "LB", "LG", "LR", "LW"),
                      color=c("grey", "#3f6cff", "green", "red", "white"))

## Longer y axis
ggplot(mean_hatching_time, aes(x = Tr, y = mean_hatching_time, fill = Tr)) +
```

```
  geom_boxplot() +
  geom_jitter(width = 0.2, height = 0, alpha = 0.5) +
  scale_fill_manual(values = Tr_colors$color) +
  labs(title = "Mean hatching time per treatment", x = "Treatments", y = "Mean hatching time") +
  ylim(345, 370)

mean_hatching_time_treatment<- aggregate(mean_hatching_time~Tr,
                                         data=mean_hatching_time, FUN=mean)

print(mean_hatching_time_treatment)

##############################
# Trash
##############################

##Treatment and petri dish (ID)
#model_beta <- betareg(proportion_unhatched ~ Tr + factor(ID), data = mortality_data)
#summary(model_beta)

##Treatment
#model_beta2 <- betareg(proportion_unhatched ~ Tr, data = mortality_data)
#summary(model_beta2)

##petri dish (ID)
#model_beta3 <- betareg(proportion_unhatched ~ (1|ID), data = mortality_data)
#summary(model_beta3)

#AIC(model_beta)
#AIC(model_beta2)
#AIC(model_beta3)
```

```
#Test with different R package


#Test with different R package brms
#formula_TRID_beta <- bf(proportion_unhatched ~ Tr + (1 | ID))
#family <- brmsfamily("beta", link = "logit")
#model_brms_TRID <- brm(formula_TRID_beta, data = mortality_data, family = family)
#summary(model_brms_TRID)


# Specify the desired value to compare against
#desired_value <- 0.5  # Replace with your desired value


# Subset the data for each category and perform t-tests
#for (category in unique(hatchingtime$Tr)) {
 # subset_data <- hatchingtime[hatchingtime$Tr == category, "proportion_scotophase"]
  #print(subset_data)
  # Perform t-test comparing the mean to the desired value
  #t_test <- t.test(subset_data, mu = desired_value)


  # Print the results
#  cat("Category:", category, "\n")
 # cat("Test statistic:", t_test$statistic, "\n")
  #cat("p-value:", t_test$p.value, "\n")
  #cat("\n")
#}


# Compute survival curves kepler ------------------------------------------------


kepler_model <- survfit(Surv(Time, status) ~ TR + (1|ID), data = hatching_data)
summary(kepler_model)
summary(kepler_model)$table
```

```
#Simple plot
plot(kepler_model, conf.int=F, xlim = c(312, 408),
    xlab = "Hours since start experiment",
    ylab = "Proportion of eggs still to hatch", main= "Egg hatching pattern", las= 1)
abline(h=0.5, col="red")
# Visualize survival curves ----------------------------------------------
kepler_plot <- ggsurvplot(kepler_model,
                          pval = TRUE,
                          conf.int = FALSE,
                          risk.table = FALSE,
                          risk.table.col = "strata",
                          linetype = "strata",
                          surv.median.line = "hv",
                          ggtheme = theme_bw(),
                          palette = c("black", "#0036e6", "#30b119", "#ca0000", "#e19b00"),
                          xlim = c(312, 408),
                          xlab = "Hours since start experiment",
                          ylab = "Proportion of eggs still to hatch",
                          break.x.by = 24)

# Flip the y-axis
kepler_plot$plot <- kepler_plot$plot + scale_y_reverse()

# Modify the plot title
kepler_plot$plot <- kepler_plot$plot +
  ggtitle("Egg hatching pattern")

# Display the modified plot
kepler_plot

# Perform log-rank test TR
```

```
#kepler_logrank <- survdiff(Surv(Time, status) ~ TR, data = hatching_data)
#print(kepler_logrank)

# Perform log-rank test petri dish
#kepler_logrank2 <- survdiff(Surv(Time, status) ~ (1|ID), data = hatching_data)
#print(kepler_logrank2)

# Perform log-rank test petri dish
#kepler_logrank3 <- survdiff(Surv(Time, status) ~ TR, (1|ID), data = hatching_data)
#print(kepler_logrank3)

#log rank test with only two treatments each time

# Specify the column and values to remove
#column_TR <- "TR"
#left_over_LRLG <- c("LW", "C", "LB")

# Remove rows with specified values in the specified column
#LRLG_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LRLG), ]

# Perform log-rank test LRLG
#kepler_logrank_LRLG <- survdiff(Surv(Time, status) ~ TR, data = LRLG_hatching_data)
#print(kepler_logrank_LRLG)


# Perform log-rank test LRLG
#left_over_LRLB <- c("LW", "C", "LG")
#LRLB_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LRLB), ]
#kepler_logrank_LRLB <- survdiff(Surv(Time, status) ~ TR, data = LRLB_hatching_data)
#print(kepler_logrank_LRLB)
```

```
# Perform log-rank test LRLW
#left_over_LRLW <- c("LB", "C", "LG")
#LRLW_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LRLW), ]
#kepler_logrank_LRLW <- survdiff(Surv(Time, status) ~ TR, data = LRLW_hatching_data)
#print(kepler_logrank_LRLW)

# Perform log-rank test LRC
#left_over_LRC <- c("LB", "LW", "LG")
#LRC_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LRC), ]
#kepler_logrank_LRC <- survdiff(Surv(Time, status) ~ TR, data = LRC_hatching_data)
#print(kepler_logrank_LRC)

# Perform log-rank test LWC
#left_over_LWC <- c("LB", "LR", "LG")
#LWC_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LWC), ]
#kepler_logrank_LWC <- survdiff(Surv(Time, status) ~ TR, data = LWC_hatching_data)
#print(kepler_logrank_LWC)

# Perform log-rank test LWC
#left_over_LWLB <- c("C", "LR", "LG")
#LWLB_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LWLB), ]
#kepler_logrank_LWLB <- survdiff(Surv(Time, status) ~ TR, data = LWLB_hatching_data)
#print(kepler_logrank_LWLB)

# Perform log-rank test LWC
#left_over_LWLG <- c("LB", "LR", "C")
#LWLG_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LWLG), ]
#kepler_logrank_LWLG <- survdiff(Surv(Time, status) ~ TR, data = LWLG_hatching_data)
#print(kepler_logrank_LWLG)

# Perform log-rank test LWC
```

```
#left_over_CLG <- c("LB", "LR", "LW")
#CLG_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_CLG), ]
#kepler_logrank_CLG <- survdiff(Surv(Time, status) ~ TR, data = CLG_hatching_data)
#print(kepler_logrank_CLG)

# Perform log-rank test LWC
#left_over_LBLG <- c("C", "LR", "LW")
#LBLG_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_LBLG), ]
#kepler_logrank_LBLG <- survdiff(Surv(Time, status) ~ TR, data = LBLG_hatching_data)
#print(kepler_logrank_LBLG)

# Perform log-rank test LWC
#left_over_CLB <- c("LG", "LR", "LW")
#CLB_hatching_data <- hatching_data[!(hatching_data[[column_TR]] %in% left_over_CLB), ]
#kepler_logrank_CLB <- survdiff(Surv(Time, status) ~ TR, data = CLB_hatching_data)
#print(kepler_logrank_CLB)
```

Appendix D Pilot clock design

Several different methods for monitoring the caterpillars were considered. One of them was using sticky paper on a clock with eggs on the hour dial. The idea was that the caterpillars would jump off on the clock face when they hatched in search of food. They would fall on the current time since they were on the hour dial and stick in place. This way we could see when the caterpillars hatched. The caterpillars would however take several hours to jump off the dial. They could also jump off on both sides which would make a large difference in the time it would look like they hatched. What this looked like can be seen in the Figures D1, D2 and D3. The jumping could have been the reason caterpillar stayed on the dial for several hours, however more testing also showed that the caterpillars did not walk directly on to sticky paper when it was placed next to them without the need to jump.

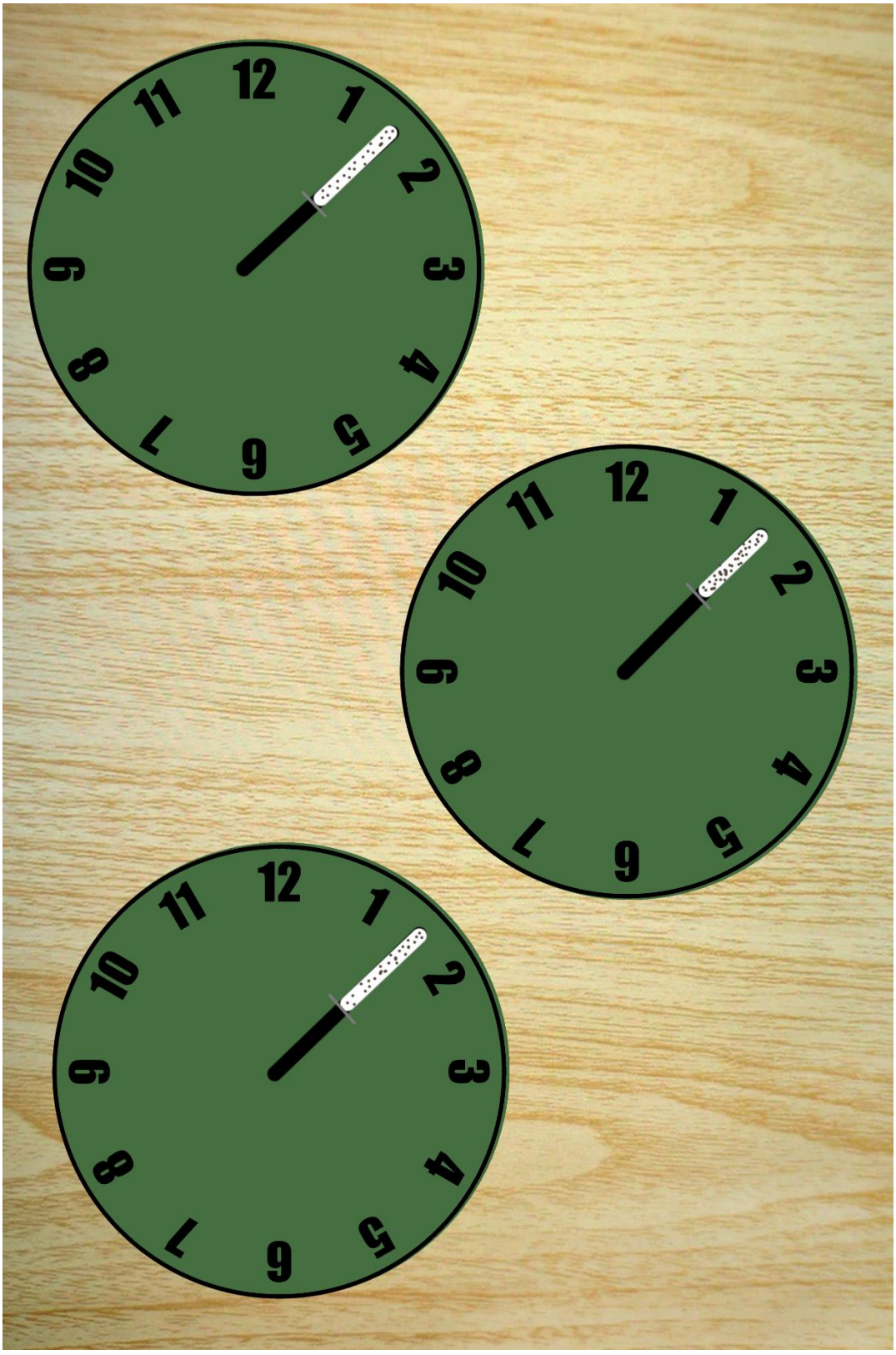
Figure D1

One of the clock designs to monitor the caterpillar hatching

Note. The caterpillars would be on the hour dial. The brow lines would represent the caterpillars that fell of and got stuck on the time where they jumped off. There would also be bariers preventing the caterpillars from walking up and down the dial that are not in this figure. This design was with a 24 hour clock. A 12 hour clock was also considered and would be more accurate.

Figure D2

The design of the layout the clocks would have had in the box



Note. Three clock would have fit inside the boxes used fort he experiment. This design also shows the barier on the clocks.

Figure D3

One of the pilots testing the clock design

Note. It seems to work relatively well, but the hatching started several hours earlier than where the first caterpillars were stuck on the paper.