SUSINCHAIN
SUSTAINABLE INSECT CHAIN

Report on storage and transport conditions of insect eggs

Deliverable D15

INAGRO

September 2020

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Introduction

The Task 2.5 Demonstration of transport and storage possibilities of insect eggs, small larvae and pupae) and led by INAGRO aims to demonstrate preservation possibilities for the eggs such that they do not hatch, but without affecting the hatching ratio. This knowledge allows for the storage and transportation of eggs, and, therefore, allows further (decentralised) expansion of the industrial insect sector.

Based on lab scale tests with eggs and pupae, in which the ideal transport conditions are determined, actual transports are organised. During these transports, the environmental condition is closely monitored with temperature, oxygen and humidity sensors. This will allow the necessary transport containers for this purpose to be determined.

The activities and results developed in this task are presented in this Deliverable which consists of the following reports:

- Report: Transport experiments on House Cricket (*Acheta domesticus*)
- Report: Transport experiments on Black Soldier Fly (*Hermetia Illucens*)
- Report: Transport experiments on *Tenebrio molitor*

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Report: Transport experiments

House Cricket (*Acheta domesticus*)

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Chapter 1 – Introduction

Eggs are sensitive to temperature fluctuations and dehydration, which currently causes yield loss. Furthermore, eggs can hatch during transportation.

The aim of this task is to mimic conditions during transportation by package delivery services and demonstrate the effects on two life stages (nymphs and eggs). This knowledge will improve methods for storage and transportation of crickets and therefore allow further (decentralized) expansion of the industrial insect sector. Based on lab scale tests with eggs and nymphs, in which the ideal transport conditions are determined, actual transports will be organized. In broad terms, the experiments aim to clarify several points related to transport of crickets.

To ensure the optimal number of cricket nymphs in the container during transportation, a density experiment will be conducted. This initial experiment will assess the survival percentage of the nymphs living at different densities in similar sized transportation containers.

We will monitor how variation in transport conditions (i.e. the total transport time, the external temperature during transport and whether it takes place in a high- or low-humidity environment) affects indirect environmental determinants of cricket health, such as:

- Humidity within the transport boxes (expected to increase over time)
- CO_2 levels (expected to increase with crickets respiration)

The direct effect of the above parameters on cricket health will be assessed by determining:

- Survival depending on transportation time
- Increase in biomass during transport.

We will furthermore determine the effect of transport conditions (primarily temperature) on cricket eggs by testing whether certain conditions will affect survival and relative hatch-rate of eggs. By comparing these data to our data on

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nymphs transported under similar conditions, we will assess whether transport of eggs or nymphs will be most effective.

Due to delays in the work-package, experiments are yet to be started. While the overall goals and the below outline of the experiments will not be changed, details in the design and execution of the experiments may be modified if deemed more appropriate.

Overall, we believe that insight into the parameters described above will greatly inform on the optimal transport condition for crickets in the future.

Chapter 2 – Material and methods

1. Experimental goals

Our experiments can broadly be divided into three groups:

1. Initial experiment on optimal density
2. Experiments on nymph growth and survival depending on transport conditions
3. Experiments on the relative effect of extreme transport conditions on egg hatch-rate.

2. Density experiments

The experimental work described in this section will be performed prior to the transportation experiments (described below) and will be conducted at Bugging Denmark's climate controlled cricket production container in Copenhagen. The overall purpose of the experiments is to determine the optimal density of cricket nymphs in the transportation boxes.

Cricket nymphs will be 4th instar nymphs. A visual observation of size will ensure approximately similar instar of all nymphs.

The crickets will be placed in transport containers currently being used for transporting crickets and other insects. The boxes measure 10 cm x 10 cm x 6 cm. Within the boxes a piece of egg carton (9 cm x 9 cm x 5cm) will increase the surface area and provide

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shelter for the crickets. Water and food will be provided as water agar solution and grinded chicken feed respectively. Four different densities will be tested in triplicates n=80, n=120, n=160, n=200. The designated number of crickets will be stored in darkness at 25°C. After 6 days survival rates will be determined by counting surviving nymphs. The optimal density for the purpose of the down-stream experiments is defined as the density with the greatest survival percentage.

Figure 1 – Four diferente densities of 4th instar crickets will be placed in containers with egg carton (grey triangels) and kept in a dark environment for 6 days to mimic transport conditions. After six days, the survival rate depending on density will be determined.

3. Effect of transport conditions on nymph growth and survival

The experiments will primarily be performed in the climate-controlled container unit funded by SUSINCHAIN to perform cricket-related experiments at University of Copenhagen. For the more extreme environmental conditions (e.g. to achieve 5°C), it may be necessary to use other equipment.

While external environmental conditions (i.e. the climate outside the transportation boxes) will remain constant throughout the experiments, separate sensors registering RH and CO2-levels will be placed inside selected transportation boxes to inform on the environmental changes within the micro-environment in the boxes as transport progresses.

Temperature will be regulated inside the climate-controlled container unit to 4 different temperatures (5°C, 15°C, 25°C, 35°C). To create high- and low-humidity external conditions, the transportation boxes will be placed in larger containers, some of which will contain wet kitchen-towel to increase relative humidity (RH). This is a frequently used approach when performing experiments on crickets. To continuously monitor the changes in humidity and CO_2-levels in the micro-environment inside the transportation boxes, a sensor will be placed in the set of boxes that are only to be opened after 6 days and thus represent the longest studied transportation time.

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The crickets will be placed in the transport boxes together with above mentioned sensors and egg carton. Water will be provided by an agar solution and food as grinded chicken feed. The crickets will be stored in darkness until quantification.

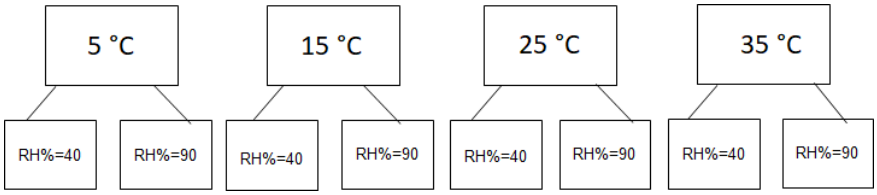

Figure 2 – A visual representation of the experimental set-up. Each box represents an experimental temperature that will be tested under two different humidities. Experiments will be done in triplicate.

For each of the four temperatures, 24 boxes (representing 2 RH-conditions x 4 time points x 3 replicates) are populated with crickets.

The transportation containers are placed in larger containers, which are stored for the designated amount of time: 1, 2, 3 or 6 days.

At each time-point the following will actions will be taken:

- Registering RH% in the boxes
- Registering CO_2-levels in the boxes
- Counting the number of live crickets in the box
- Measuring the biomass of all live crickets in the box

The above data will inform on the effect of transport conditions on the micro-environment inside transportation boxes and how this environment in turn affects cricket survival and growth. The results will therefore allow a better understanding of what goes on during cricket transport, which will help optimize cricket survival during future transports.

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4. Relative effect of transport on eggs

To gain insight into the advantages/disadvantages of transporting eggs rather than 4th instar hatchlings, the relative effect of transport conditions on egg hatch-rate will be studied.

These experiments will focus on the relative influence of transport conditions rather than absolute hatch-rate.

Adult crickets will be allowed to lay eggs in coconut coir for 24 hours and are subsequently removed. The coconut coir will be mixed to ensure equal distribution of eggs (similar to what can be expected during transport) and divided into 9 equally large parts. Each part will be placed inside a transportation box. Three of the boxes will be stored at 5°C, three at 15°C, while the remaining three will be stored at the ideal hatching temperature of 33°C.

The coir is stored at designated temperatures for 6 days, mimicking a prolonged transportation time. After the simulated transportation time, the eggs will be allowed to hatch over a 14 day period at 33°C. The number of new hatchlings will be determined after 1, 3, 5, 9 and 14 days either by manual counting or by determining the average weight of 100 individuals and the total weight of the hatchling population.

The results will inform on the overall delay and reduction in egg hatching due to transport taking place under sub-optimal conditions.

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Report: Transport experiments

Black Soldier Fly (*Hermetia Illucens)*

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Chapter 1 – Introduction

During the last years, the industry dedicated to the mass production of beneficial insects and especially *Hermetia illucens* has increased significantly. This is basically due to the global need for other nutritional sources in the field of human and animal consumption.

It is for this reason that it is necessary to jointly establish the basic parameters necessary for the breeding and storage of the different stages of these insects, and thus be able to maintain the established standards.

In *Hermetia illucens*, as in most insects, temperature and humidity directly affect the growth and development of the different stages of the biological cycle (Gullan & Cranston, 2000).

The cuticle of the exoskeleton is known to consist of a superficial lipid layer that is impermeable to water. The transpiration rate through the cuticle in species adapted to humid climates is higher than in drier environments (Wigglesworth, 1984). For this reason, it is important to know the different mechanisms used by insects for this purpose. Females have been shown to possess various strategies during oviposition, such as mass grouping of eggs and selection of the oviposition site. This is the case of BSF females since, to avoid egg dissection and starvation of newly emerged larvae, they usually lay eggs in dry crevices near a humid resource (Booth & Sheppard, 1984). In different laboratory studies with *H. illucens* it was determined that the optimal range of humidity for the development of the species is between 50 to 99% (Furman et al., 1959; Tingle et al., 1975; Bradley & Sheppard, 1983; Booth & Sheppard, 1984). A maximum viability of eggs has been shown at 30 °C (Chia et al. 2018), and the development time was decreased as the incubation temperature increased.To develop an egg transport protocol, it is important to understand and verify the right egg incubation conditions and the effect of incubation conditions on the survival and eclosion time of eggs.

Studies on different BSF strains and in transport scenarios where “incubation conditions” are applied for 1 day (express transport) or 3 days (average transport) are needed to build the full picture. Therefore, the purpose of the following study is to evaluate the optimal relative humidity and temperature for the incubation of *Hermetia Illucens* (BSF) eggs.

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Chapter 2 – Humidity Experiment / Material and methods

1. Experimental design

The study will be conducted in a climate chamber located in HiProMine S.A. Robakowo - Poland. We follow the following experimental design:

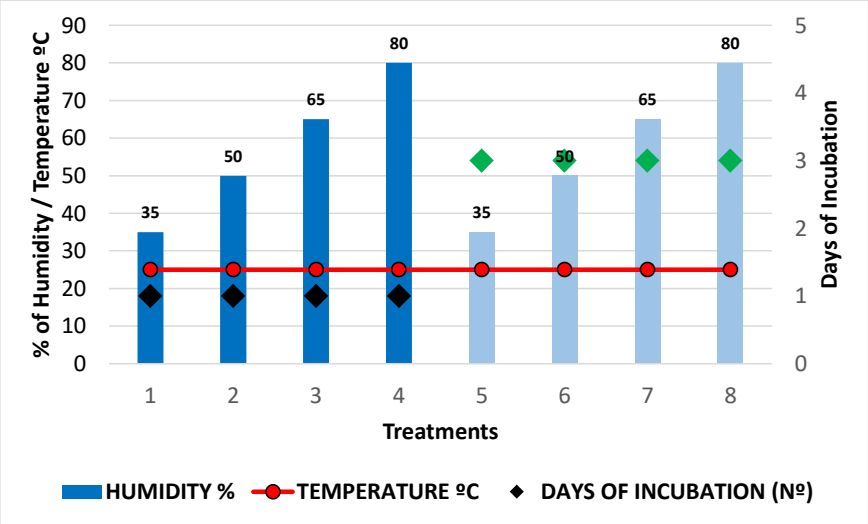

Figure 1: Graph showing 8 different treatments (tested humidity (%): 35, 50, 65 and 80; days of incubation: 1 day and 3 days; temperature tested of all treatments: 30 ºC). Each treatment has 3 replicates.

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2. Experimental workflow

To start the experiment, 3 g of eggs (24 hours old) will be placed on a mesh covering a 250 ml plastic box (Figure 2), after that the box with eggs will be put it inside a climate chamber and divided in the different treatments.

After the incubation of the different treatments (1 day or 3 days of incubation inside climate chamber) the eggs of 3 replicates of each treatment will be placed into a new 250 ml box, this step will be repeated daily during 4 times, in this way we will ensure that all the eggs hatch. All the boxes with eggs will kept it in laboratory conditions (30 ºC – 65 %RH). The neonates, which hatch into the box each day, will be quantified.

Figure 2: Photo showing the placement of the fresh eggs on the mesh.
©HiProMine S.A. Robakowo - Poland

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Quantification Method used:

1. Measure the square meters of the 250 ml box,
2. Randomly take four photos of the selected box (Figure 3),
3. We measure the area of the photo,
4. We quantify the neonates of each photo,
5. Finally, we carry out all the necessary calculations to determine the number of hatchlings.
6. We repeat steps 1 to 4, as many times as necessary.

Figure 3: Photo showing the small newly emerged larvae that will be counted. © HiProMine S.A. Robakowo – Poland

Applying this method will allow the measurement of the following parameters:

- Start day of experiment (date)
- Number of hatchlings during incubation (when taken out of the incubator if neonates were observed)
- Number of hatchlings after day 1
- Number of hatchlings after day 2
- Number of hatchlings after day 3

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- Number of hatchlings after day 4
- % Mortality

The eggs from the remaining 3 replicates of each treatment, will be placed into a new bigger box (1000 ml) with chicken feed for the hatch and kept under laboratory conditions (30 ºC – 65 %RH). The box with feed will be replaced daily (for 4 days), in this way we make sure to separate the hatchlings of each treatment per age. The neonates, which hatch onto the feed will be fed if necessary. After two days, the small larvae will be quantified (**IMPORTANT:** this step will be repeated three times. if necessary, the neonates will be changed to a bigger box and feed will be added).

Applying this method will allow the measurement of the Survival Rate during three days of each treatment.

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Chapter 3 – Temperature Experiment / Material and methods

1. Experimental design

The study will be conducted in a bioreactor located in Insektentechnologiecenter Hermetia Baruth in Berlin / Germany and a climate chamber Bioflytech S.L. – Spain. We follow the following experimental design:

Hermetia Baruth

Treatment ID	Temperature (ºC)	Incubation time (days)	Relative humidity (%)
1	23	1	60
2	27	1	60
3	31	1	60
4	35	1	60
5	23	3	60
6	27	3	60
7	31	3	60
8	37	3	60

Table 1: Table showing 8 different treatments (tested temperature (ºC): 23, 27, 31 and 35; days of incubation: 1 day and 3 days; humidity of all treatments: 60 %). Each treatment has 6 replicates.

Bioflytech S.L.

Treatment ID	Temperature (ºC)	Incubation time (days)	Relative humidity (%)
1	20	1	60
2	25	1	60
3	30	1	60
4	20	3	60
5	25	3	60
6	30	3	60

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Table 2: Table showing 6 different treatments (tested temperature (ºC): 20, 25 and 30; days of incubation: 1 day and 3 days; humidity tested of all treatments: 60 %). Each treatment has 3 replicates.

2. Experimental workflow

Hermetia Baruth

To start the experiment, 3g of eggs (24 hours old) will be placed on a mesh (far enough apart that they do not form clumps and die) covering a 500 ml plastic box (Figure 1), after that the box with eggs will be put it inside a climate chamber and divided in the different treatments.

Bioflytech S.L.

To start the experiment, 3 g of eggs (24 hours old) will be placed on a mesh covering a 250 ml plastic box (Figure 2), after that the box with eggs will be put it inside a climate chamber and divided in the different treatments.

Figure 1. Photo showing the placement of eggs on the mesh cover prior to incubating them. ©Hermetia Baruth

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Figure 2. Setup boxes. Top box: with small holes at the top to allow ventilation and a mesh in the bottom to place the eggs. Bottom box: used to transfer the neonates. © Bioflytech S.L. – Spain

Hermetia Baruth

After the incubation phase (1 day or 3 days of incubation inside the bioreactor), the eggs of 3 replicates of each treatment will be placed into a new 500 ml box, this step will be repeated daily until all the eggs are hatched. All the boxes with eggs will be kept under laboratory conditions (30 ºC – 60 %RH). The neonates, which hatch into the box each day, will be quantified daily following R&D Center **HiProMine S.A. Robakowo's protocol** (image analysis).

Bioflytech S.L.

After the incubation phase, (1 day or 3 days of incubation inside the climate chamber), the eggs of 3 replicates of each treatment will be placed into a new 250 ml box, this step will be repeated every day until all the eggs are hatched. All the boxes with eggs will be kept under laboratory conditions (30 ºC – 60 %RH). The neonates that hatch will be quantified daily following R&D Center **HiProMine S.A. Robakowo's protocol** (image analysis).

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Applying this method will allow the measurement of the following parameters:

- Start day of experiment (date)
- Number of hatchlings during incubation (when taken out of the incubator if neonates were observed)
- Number of hatchlings
- % Mortality

Hermetia Baruth

The eggs from the remaining 3 replicates of each treatment, will be placed into a new bigger box (1000 ml) with chicken feed for the hatch and kept under laboratory conditions (30 ºC – 65 %RH). The box with feed will be replaced daily (for 4 days), in this way we make sure to separate the hatchlings of each treatment per age. The neonates, which hatch onto the feed will be fed if necessary. After two days, the small larvae will be quantified (**IMPORTANT:** this step will be repeated three times. if necessary, the neonates will be changed to a bigger box and feed will be added).

Applying this method will allow the measurement of the Survival Rate during three days of each treatment.

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Chapter 4 – Future experiments

HiProMine S.A. Robakowo – Poland

Effect of Relative Humidity Percentage on *Hermetia illucens* egg incubation at 25 ºC

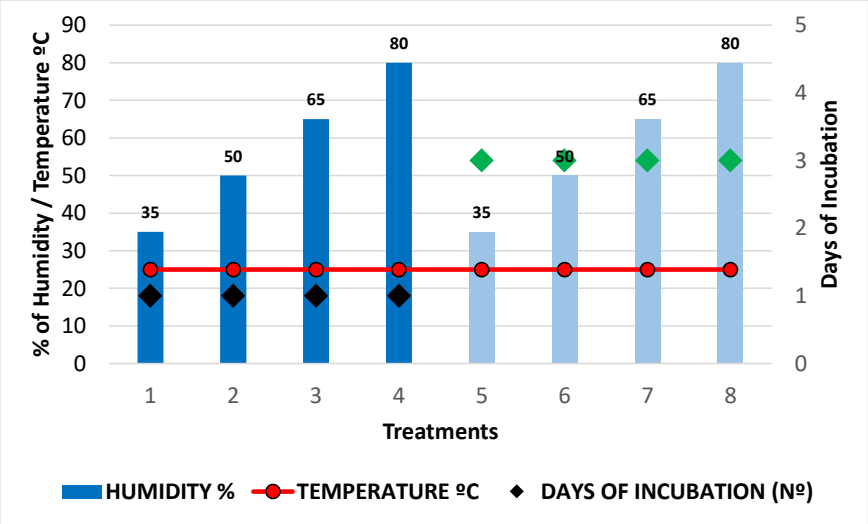

Figure 1: Graph showing 8 different treatments (tested humidity (%): 35, 50, 65 and 80; days of incubation: 1 day and 3 days; temperature tested of all treatments: 25 ºC). Each treatment has 3 replicates.

Hermetia Baruth

When the optimal temperature is identified, a new experiment with a gradient of relative humidity level can be designed.

Base Transport Method

A series of egg transport events will be organised between two partners. A partner will send the eggs “Egg Donor” and a partner will receive the eggs and record the data “recipient”. Depending on the parameter tested, different containers (treatments) will be prepared by the donor and sent to a partner, while two controls should be done:

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- Control 1: Eggs from the same pool will be placed on substrate (Gainesville diet) and let to hatch on the feed. The larvae are to be quantified.
- Control 2: Eggs from the same pool will be put in the same container used in the transport, and under the same settings, but will stay at the donors facility under controlled conditions and will be opened and put on feed at the time of shipment delivery.

The shipment delivered to the recipient will be assessed using the method as the controls.

The container can be a bag, a bottle, or a tube that is supplied with a structural element that prevents the shaking of eggs. The amount of eggs and the setup is to be agreed on between the donor and the recipient. An amount of 10 g of eggs recommended.

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Chapter 6 – Conclusion

From an industrial point of view, the production of black soldier fly (BSF) involves two different but complementary sub-processes. The first process deals with producing eggs in cages (or egg production units) while the second involves the rearing of larvae on defined substrates to either treat wastes or to produce proteins and fats. Maintaining high egg yield might be a big challenge for business models that focus on waste management. Specialised business models that focus on producing and selling eggs can play major roles in the development of the BSF industry by supplying waste management facilities with eggs and/or supply eggs to other facilities specialised in larvae rearing.

For the development of such businesses, and to enhance the cooperation between the BSF industry and the academic partners, it's important to develop egg transport protocols where the materials can be prepared fast while maintaining minimum mortality under transport conditions.

Available literature on the incubation of eggs (Details can be seen in the file: Literature review on the transportation of Black Soldier Fly (Hermetia illucens)) can provide a basis for the egg transport investigations. In addition to the available literature, our in-house investigations have confirmed that factors such as temperature, storage humidity, and CO2 concentrations play a major role the development time and hatching rate of eggs. Nevertheless, most of our in-house investigation/experiences on egg transfer were done in a trial-and-error approach rather than a scientific method, and data obtained with better methods is needed.

This preliminary report aims to report the protocols we developed in order to initiate a chain of transport trials followed by data collection with the aim of assessing and evaluating different "shipment mentioned transport parameters. The goal of these trails is to develop a transport protocol which can be done at minimum technical requirements and benefits all institutions and companies working with BSF.

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Report: Transport experiments

Tenebrio molitor

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Chapter 1 – Introduction

It is expected that the insect industry will grow rapidly the coming years. Due to this growth the number of animal transports will increase and is likely to evolve in a similar way as other animal farms. The latter would mean that there would be specialized breeders and fatteners. With frequent transportation of early life stages between them.

The current knowledge is very limited (see Literature review on the transport conditions of *Tenebrio molitor*) and frequently the data is inadequate to predict the survival during transport as the long exposure period is not representative.

In this study we focussed on the transportation of eggs and young larvae (hereafter neonates) as these are expected to compromise the bulk of transports between breeders and fatteners. It is assumed, based on partner experience, that the pupae are to fragile for transport. The animals were exposed to different climatic conditions between 1 and 6 days to mimic real world transportation conditions:

1 day:	express national transport
2 days:	express international transport
3 days:	regular transport
6 days:	delayed transport

With a large temperature range between 5 and 40 °C and humidity between 20 and 80 %.

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Chapter 2 – Material and methods

3. Colony information

The mealworms used in this study have been bred at the Inagro Insect Research Centre since 2013. They are kept in 60x40 cm plastic crates (inner surface area of 2000 cm²) at a temperature of 27 °C ± 1 °C SD, 60 % ± 3 % SD relative humidity and in the dark except during feeding. The animals are fed ad libitum with INSECTUS Mealworm Grow (Mijten nv, Belgium) and chopped chicory roots. The CO_2 concentration is monitored and kept below 1500 ppm. In order to gather eggs for the experiments, 8x250 grams of beetles were allowed to lay eggs for 24 h for 4 consecutive days in flour. After oviposition the eggs and substrate were collected and sieved on a 0.5 mm sieve. These clean eggs were used in the experiments.

4. Egg transport part one

Fifteen times two grams of freshly harvested eggs were placed in between 20 grams of wheat bran in a plastic crate of 10 by 8 by 3 (l*w*h). Three crates were placed in standard rearing conditions in our climate room (27 °C 60 % RV) to assess the baseline hatchability (hereafter our control). The other 12 crates were placed in a climate room with varying climate conditions. After 1 day 3 random replicates were removed and placed in the standard rearing conditions. This was repeated for 2, 3 and 6 days. The logic behind the exposure temperatures was:

The climate conditions varied between 5°C and 40°C (in 5°C increments) and 20 and 80 % relative humidity (in 20 % increments). The goal was to assess all combinations, but this was in practice impossible due to the limitations of our equipment especially cold/dry and hot/humid combinations.

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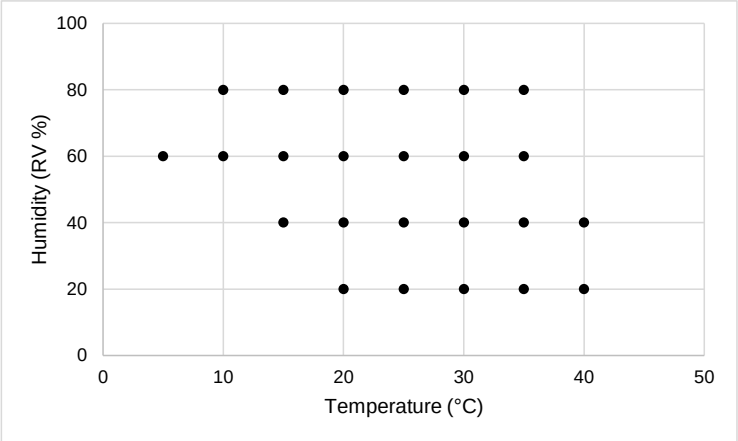

The crates were checked every workday to assess whether or not (some) eggs have hatched and if the hatching was delayed. Two weeks after the first larvae are observed the number of neonates was estimated via subsampling and compared to the number of neonates in the control. Comparing the number of neonates to the number of eggs is near impossible because counting the eggs is difficult with a high risk of damaging them and the weight of the eggs is not always equal as they accumulate dirt on the shell.

5. Egg transport part two

Based on the results of part one, extreme scenario's will be selected where the eggs in the current set-up had no or a limited survival. Several options will be assessed to optimize the crate in a cost efficient way to increase the survival in these extreme scenario's and ensure economic viable transportation is possible. Amongst others this may include, dampening the wheat bran before transport and the use of heat/cool packs.

6. Neonate transport

Based on the results of part one, extreme scenario's will be selected where the had no or a limited survival. These conditions will be replicated with 4 weeks old

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larvae (± 5 mg). Literature indicates that they may be less prone to, short term, exposure to these extremes. This will give us an indication at which conditions it is better to send larvae compared to eggs.

7. Pupae transport

Because the pupae are fairly resistant to temperature and humidity extremes, no climate conditions will be assessed. However, the pupae are more fragile and even small wound can kill the pupae. Therefore it is expected that the pupae are more likely to be harmed by the transportation handling and road conditions than the climate conditions. We are currently looking for a scientific way to assess how much they endure before adverse effects occur. One possible way would be to measure acceleration/deceleration during transport and compare this with the maximum G forces a pupae can endure.

8. Real world transportation

Finally, some real world transportation will be performed to assess the real world temperature and humidity variation during transport.

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Chapter 3 – Results

1. Egg transport part one

At 11-09-2020, 74 conditions were assessed successfully, 2/3 of the projected total, resulting in 222 measurements or more than 30 000 larvae counted. A full statistical analysis will be performed to assess the optimal conditions and extremes when the full data set is available.

The first preliminary results indicate that 35 °C is the upper temperature limit even at a humidity of 60 % with an approximate 50 % decrease in hatchability after 1 day and no survivors after 6 days. At 30 °C and a low humidity, there is still a significant negative effect, but this effect is no longer present at higher humidity. The lower temperature limit is 10 °C with adverse effects starting at 6 days or longer irrespective of the humidity. At 5 °C there is a significant drop after only 48 hours. Interestingly, there might be a beneficial effect at 15 °C with a slightly higher hatchability compared to the control. However these results need to be verified.

These very preliminary results indicate that the optimal envelope is between 15 and 25 °C, even if the transport is delayed (6 days). Express transport can occur in a slightly larger envelope of 10 to 30 °C (with a high humidity).

2. Egg transport part two

For the second part we will further assess transport at 10 and 35 °C to assess if it is possible to mitigate the adverse effects by increasing the humidity even higher, change the substrate or change the substrate/egg ratio.

3. Neonate transport

Since very little eggs survived at 5 °C and 40 °C, we will assess the survival of neonates at these temperatures.

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Chapter 4 – Discussion

A lot of work has already been performed in for this work package, each temperature and humidity combination is 1 week work and currently 15 have been performed. When all possible combinations are assessed, it will be the most elaborate dataset on hatchability in different temperatures/humidity's to date. It will provide evidence on what is possible during realistic transport conditions and what is not. Based on the current preliminary data we would advise not to go below 15°c or above 30 °C (in a dry environment). If necessary to transport eggs when it is not possible to stay within those limits, express transport should be arranged to ensure the eggs arrive on site in 1 day or less.

Chapter 5 – Future experiments

In the coming months we will continue with the first phase of the experiment to ensure all combinations, that are possible with our equipment, are performed and verified if needed. Thereafter the other egg and neonate experiments will follow soon.

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