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# The effect of humidity on water loss rates in the brassy leaf beetle (*Phratora vitellinae*)

Master's thesis in Biology: Ecology, Behavior, Evolution and  
Biosystematics

Supervisor: Cameron Ghalambor

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## ABSTRACT

There are growing reports of global insect declines. Many factors seem to contribute to observed declines, and especially climate change. Previous studies have primarily focused on the impact of rising temperatures on desiccation resistance, especially in species from arid environments. Given the role that climate change plays in impacting insect populations, and the potential for an increase in frequency and intensity of extreme weather events, a greater understanding of how environmental factors related to changes in precipitation such as relative humidity affect individuals and species will be important in predicting consequences of future change. This study compared rates of mass lost as a proxy for water loss at low (~10%), medium (~35%) and high (~65%) humidity levels in the brassy leaf beetle (*Phratora vitellinae*) and investigated if a prior acclimation to high humidity impacted the rates of mass lost compared to non-acclimated individuals. We found that when moved in to low and medium desiccation treatments, beetles from a high humidity pre-treatment lost water at the same rate as those who did not experience acclimation. Mass loss was greater in low than in medium and high humidity. In addition, non-acclimated individuals who were transferred from low and medium humidity treatments into a high humidity treatment experienced the same rate of mass gain, supporting the view that prior acclimation does not impact subsequent rates of water loss, and that acute humidity is an important factor driving water loss rates in these beetles. These findings may suggest minimal desiccation resistance in these species or that these levels and durations of lowered humidity do not pose stressful enough conditions to induce a strong desiccation resistant response. However, given their wide geographic range, studies that incorporate other factors such as a combination of temperatures and humidity as well as behavior are needed to predict future changes in population dynamics of this species.

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## 1. INTRODUCTION

There is a growing recognition of global insect declines (e.g. Baranov et al., 2020; Fox, 2013; Hallmann et al., 2017; Harris et al., 2019; for review see: Wagner, 2020). Insects are under threat from many factors such as habitat destruction, nitrification, climate change and agricultural intensification (Wagner et al., 2021). However, much is still not known to what extent each of these stressors contribute to these observed declines, especially how they will act on different temporal and spatial scales (Boggs, 2016; Wagner et al., 2021). Insects play vital roles in ecosystems such as pollination, decomposition, and seed dispersal. They also make up the primary diet of other important taxa such as insectivorous birds, who have seen parallel declines in abundance (Møller, 2019). In addition, they are responsible for the majority of biotic pollination of wild plants as well as the world's food crops (Allsopp et al., 2008; Klein et al., 2007; Prather et al., 2013). Given their global importance and the vital roles they play in supporting ecosystems, close attention and understanding of the mechanisms behind these declines are essential in safeguarding the biodiversity all living things depend on (Harvey et al., 2023; Wagner et al., 2021).

On a global scale, climate change seems to play a significant, if not leading role in insect declines. Multiple long-term studies have consistently shown significant declines in insect populations even within protected areas, providing compelling evidence that climate change plays a leading role (Baranov et al., 2020; Hallmann et al., 2017; Halsch et al., 2021). For example, Harris et al. (2019) found an alarming 83% decline in Coleoptera abundance in a protected U.S. forest over four decades, likely driven by increased temperatures and reduced winter snowfall. Not all impacts of climate change will be negative, however, some insect communities may see an increase in abundances due to a changing climate (Fox, 2013), and changes in abundance and distribution of insects that carry vector borne diseases may have severe consequences for human health (reviewed in Gage et al., 2008). Such patterns suggest the positive and negative responses to climate change may not be easy to predict as they reflect multiple environmental stressors and differences among species in their physiology.



Rising temperatures represent one aspect of climate change that could be driving declines in insect abundance. Elevated temperatures can impact insect populations either through direct or indirect effects. Projections of how climate warming will impact insects often focus on the direct effects of temperature exceeding the critical maximum thermal tolerances (e.g. Deutsch et al., 2008; Sunday et al., 2010, 2012). Yet, the negative impacts of thermal stress are often due to indirect impacts related to how temperature impacts different physiological functions. For example, in ectotherms elevated temperatures cause increased metabolic rates, which in turn increases oxygen demands and energy requirements (e.g. Deutsch et al., 2015; Huey & Kingsolver, 2019; Pörtner, 2002). One understudied indirect impact of temperature is its impact on water loss and desiccation. Another important abiotic factor related to global climate change that can have important direct and indirect impacts on insect abundance, distribution, and desiccation, is changes in precipitation (Chown et al., 2011). Despite its recognized importance in shaping species distributions, it has been given less attention than the impacts of rising temperatures. Yet, changes in water availability can have profound impacts on insect populations including impacting the thermal performance curve and desiccation, and therefore effects of changes in precipitation and related factors such as humidity, warrant an increased focus (Chown et al., 2011). Due to their large surface area to volume ratio, terrestrial insects are especially susceptible to desiccation (Chown & Nicolson, 2004; Harrison et al., 2012).

There are three main ways that insects can lose water: through the cuticle, respiration, and excretion (Chown & Nicolson, 2004). Moreover, insects can gain water from food, drink, and water vapor absorption. In some species, such as sap and blood feeders, an excess of water may pose a challenge, while in others, reducing water loss is the primary goal. To survive insects have adapted various ways to prevent desiccation and balance water gains and losses through these avenues (O'Donnell, 2022). For example, sap or blood feeders may produce enormous amounts of urine while feeding to counteract the water gains, while other species can survive with water poor food sources (O'Donnell, 2022).

Cuticular water loss is accepted as the main source of water loss in the majority of insects (Hadley, 1994 as cited in Harrison et al., 2012). Epicuticular lipids provide a waterproofing barrier and their composition has been found to play an important role in the amount of

desiccation they confer. For instance, it has been found that waterproofing properties increase with an increase in chain length, decrease in branching structure, and increase in amount of cuticular hydrocarbons (CHCs) (Gibbs, 2002; Hadley, 1977). In addition, various findings show that there is phenotypic plasticity in the production of the composition of CHCs in response to desiccation and various other related environmental factors such as temperature stress or cold shock (e.g. Leeson et al., 2020; Menzel et al., 2018), and such plasticity in CHC production may allow for adaptation to changing climactic conditions (Leeson et al., 2020).

Most studies find respiratory water loss to account for a much smaller proportion of water loss as compared to cuticular water loss in resting insects. A comprehensive review found that respiratory water loss makes up 20% or less of total water loss (Chown, 2002). However, this amount can be significantly higher in xeric species or when insects are involved in energetically demanding processes such as flight (Chown, 2002; Harrison et al., 2012; Zachariassen, 1996). Respiratory water losses can be reduced through modifications in the opening and closing of the spiracles. For example, there is evidence that some small flying insects make fine tune adjustments to the opening of their spiracles (Lehmann, 2001), and other species make use of discontinuous gas exchange in order to maintain water balance (though see Chown, 2002, for an in depth discussion).

Excretion is another main aspect of the water balance in insects. The Malpighian tubules and the hindgut are the main structures in the excretory system that regulate water balance (O'Donnell, 2022). Studies have shown that insects can change the concentration of their urine, and amount of food consumed and excreted based on hydration status (Harrison et al., 2012). Recent research has begun to detect the complex role of the neuroendocrine system in regulating water absorption and secretion from the Malpighian tubules and hindgut (Harrison et al., 2012). The significance of excretion's contribution to water balance largely depends on the environment the insects occupy and their source of food (O'Donnell, 2022). For instance, large differences exist between species that feed on sap or blood versus those that do not (O'Donnell, 2022).

Acclimation can affect how water is lost through the cuticle, respiratory, and excretory systems, and as such can play a major role in desiccation resistance. There are a handful of studies that

have looked at the effect of temperatures and humidity acclimation on desiccation resistance, but these studies provide mixed results. For the most part prior exposures to stressful temperatures or humidity levels seem to increase the capacity to tolerate desiccation, through a reduction in the subsequent rate of water loss. For instance, Fischer and Kirste (2018) found that butterflies (*Bicyclus anynana*) held at higher temperatures and at lower humidity levels had higher subsequent desiccation resistance as compared to those that were acclimated in low temperature or high humidity conditions providing support for adaptive phenotypic plasticity in response to high temperatures and/or low humidity conditions. Similar adaptive phenotypic responses following acclimation to low humidity, have been found in fruit flies (*Drosophila melanogaster*) (Bazinet et al., 2010; Hoffmann, 1990), and Bazinet et al. (2010) speculated this was done through a reduction in cuticular water loss. However, some studies show differing mechanisms such as an increase in desiccation tolerance rather than a reduction in the rate of water lost (Parkash et al., 2014; Terblanche et al., 2005). Regardless of the mechanism, these studies have all shown acclimation to occur over very short periods of time ranging from 2 hours (Hoffmann, 1990) to 24 hours (Fischer & Kirste, 2018), with the strength of the response sometimes increased by the duration of pre-exposure (Hoffmann, 1990). However, other studies have found the effects of acclimation on water loss to be less conclusive. For example, Kleynhans and Terblanche (2011) found that the impact of temperature and humidity levels on water loss rates varied in direction and magnitude across species and did not show clear patterns across ecotypes of tsetse flies (*Glossina* spp). Studies on *Drosophila leontia* found that acclimation during development can show similar patterns with humidity, though not temperature, impacting subsequent rates of water loss (Parkash & Ranga, 2014). Given that these findings are inconclusive, with some studies showing acclimation to be a significant factor affecting subsequent water loss rates (Bazinet et al., 2010; Fischer & Kirste, 2018) and others finding it to be insignificant (Terblanche et al., 2005), further research is warranted to better understand the mechanisms underlying these responses and their role in desiccation resistance, particularly with regard to humidity levels.

In this study, I focus on how changes in relative humidity affect the rate of water loss in the brassy leaf beetle (*Phratora vitellinae*) and how acclimation potentially impacts patterns of water loss. I hypothesized that if beetles can sense differences in humidity and are able to control their

rate of water loss, then beetles should lose less water under low humidity conditions. Alternatively, if water loss is merely a reflection of the evaporation rate, then water loss rates should be higher at lower humidity levels. Additionally, I asked if acclimation influenced subsequent rate of water loss. I hypothesized that if water loss simply reflects a passive response to humidity conditions, then all individuals regardless of their acclimation history will lose water at the same rate, such that under drier conditions water is lost at a faster rate. Alternatively, if water loss is under active control, we might observe beetles to alter their rates of water loss depending on their acclimation history.

## 2. METHODS

### **Study Species**

*Phratora vitellinae* are leaf beetles that feed on species of willow (*Salix* spp) and poplar (*Populus* spp). They are amongst the most important pest species of willow in Europe, and widespread throughout Fennoscandia (Hellén et al., 1939 as cited in Urban, 2006). The larvae produce a defensive secretion derived from the salicin chemicals of its host plants (Pasteels et al., 1983). Adults overwinter near host plants and emerge in springtime at which time they feed on host plants and lay their eggs there. Larvae feed on host plants for several weeks and pupate in the soil beneath them. New adults emerge in late summer and feed on the host plants until it's time to overwinter (Urban, 2006). Given the importance of *Salix* as a foundation species in North temperate ecosystems (Canty et al., 2016; Cronk et al., 2015), understanding the physiological tolerance of one of its insect herbivores is of general interest.

### **Experimental Design**

Foliage beating was used to collect individual beetles from *Salix* spp. trees in Trondheim, Norway (63°24'04.9"N 10°27'26.8"E) during September 2022. All individuals (n=40 total) were brought back to the lab within one hour of collection and placed in pre-tared 30ml glass vials with unique ID numbers. Vials were open at the top, and fine mesh was secured over openings with rubber bands to allow for airflow into the vial, while preventing escape. Half of these individuals (n=20) were randomly assigned to a 24-hour high humidity acclimation treatment (described below) that they were subjected to before beginning the desiccation trials, while the other half (n=20) started with the 36 hours desiccation (low and medium humidity) treatments immediately. Two mortalities occurred before the desiccation trials began, therefore data from these individuals was excluded and analyses for the non-acclimated group were made from n=18 individuals. Initial insect mass was measured to the nearest .0001 gram for all individuals. To decrease measurement error, two measurements were taken for each individual at each time, and the average of those two measurements was recorded. Subsequent measurements of mass loss are estimates of “total water loss” and are assumed not to reflect significant changes in fat or protein because of the relatively short duration of the study.

### *Acclimation Treatment*

To test the effect of acclimation to a high level of humidity, we assigned beetles to a moderately high humidity treatment and measured if this altered water loss rates under subsequent lower humidity relative to individuals that did not receive this acclimation treatment. We used  $n=20$  freshly captured individuals and randomly assigned them to receive the 24-hour period of acclimation before placing them in their respective desiccation treatments. The acclimation chamber was set to 65% humidity and 17°C. During the acclimation period the insects were given no food or water. After 24 hours in the acclimation chamber, all individuals were weighed again twice and the average of the two weights was recorded again. These beetles were then transferred in to the low and medium humidity treatments (described below) and followed over the next 36 hours. The rates at which they lost water in the low and medium humidity treatments were directly compared to another set of individuals ( $n=18$ ) that did not experience this acclimation and were directly placed under low and medium humidity conditions after being collected from the field. In addition, we compared the average change in mass over 24 hours between the beetles that were placed from the field in the high humidity acclimation treatment and the beetles that were placed immediately in the low and medium humidity desiccation treatments (see flow chart in **Figure 1**).

### *Low and Medium Humidity Treatments (The Desiccation Treatments)*

To test if the high humidity acclimation condition altered subsequent water loss relative to non-acclimated individuals, and to test whether differences in relative humidity affected the rate at which water was lost in general, we compared water loss of the acclimated and non-acclimated beetles under low and medium humidity levels for 36 hours. For the non-acclimated beetles,  $n=18$  wild caught individuals were placed in the low and medium desiccation treatments immediately. Specifically, we randomly assigned half of the individuals  $n=9$  to a low humidity treatment, and the other half  $n=9$  to a medium humidity treatment. For the low humidity treatment, a desiccation chamber was filled with 150ml of desiccant to reduce the relative humidity to ~10%. For the medium humidity treatment, individuals were left outside the desiccator at ambient humidity which remained around 35% throughout the experiment. Temperature was approximately 21°C for both groups. All beetles were weighed two times to the nearest .0001 gram at eight time intervals over 2 days, and the average of the two measurements

was recorded. Time was recorded in hours as: 0,4,8,12,24,28,32,36 accordingly. All vials were handled in a similar way for similar amounts of time, and bugs were weighed in a random order, as quickly as possible to minimize the amount of time spent outside the desiccation chamber.

Additionally, to examine the consequences of an increase in humidity on water loss, at the end of the 36 hours in the low and medium humidity treatments, the beetles that did not undergo acclimation initially, were transferred to the 65% acclimation chamber for 24 hours to assess the impact on water loss rate.

### **Statistical Methods**

All analyses were conducted in R (R Core Team 2022). Because repeated measures were taken for each individual over time, individual ID was included as a random effect in all models to account for the non-independence of measurements for each individual. Mass (in grams) was the response variable in all models. A visual inspection of residuals showed that mass followed a normal distribution.

We first tested the effects of humidity on mass loss in the first 24 hours between individuals exposed to the three different humidity treatments (low 10%, medium 35%, and high humidity/acclimation group 65%). A linear mixed effect model with time and humidity and their interaction as fixed effects and individual ID as a random effect were used. Mass between the three groups was compared at time 0 and 24 hours (see **Table 1; Appendix 1** for summary outputs). Mass measurements for individuals in the acclimation group were time shifted forward 24 hours, so that their mass measurements could be directly compared to the insects in the low and medium humidity treatments once they were placed in these treatments after acclimation.

Next, to test the effects of acclimation and humidity on mass loss during desiccation, we first used a model that tested the effects of initial mass, acclimation, and humidity on mass loss over the first 36 hours. The full model included as fixed effects: time, humidity, acclimation, and their interactions, as well as initial mass and the interaction between initial mass and time (**Table 2**). Individual ID was included as a random effect. A subset of the data from time > 0 was used for this model to account for the fact that mass at time 0 was equivalent to initial mass and therefore

had to be removed from the response variable to avoid a direct correlation between the response and predictor variables. We found that there was no significant effect of initial mass on the rate at which water was lost, therefore the model was simplified to remove this effect, and individual ID as a random effect was deemed sufficient to account for the variation in starting mass of each individual. The subsequent simplified model looked at humidity and acclimation, using the full dataset with mass measurements from time 0 (**Table 3**). Humidity, acclimation, time, and their interactions were used as fixed effects, and individual ID as a random effect (refer to **Table 3**). Because the effect of acclimation was not significant (see results below), the model was further simplified by removing acclimation, and the final parsimonious model tested the effect of humidity level alone. Thus, in the final model, time and humidity and their interaction were used as fixed effects and individual ID was used as a random effect (**Table 4**).

Lastly, for the subset of individuals that did not experience the high humidity acclimation treatment, we tested the effects of moving these beetles from low and medium desiccation treatments into the high humidity treatment from time 36 to 60. A linear mixed effect model with time and humidity and their interaction as fixed effects and individual ID as a random effect were used. Mass between the two groups (originating from low or medium desiccation treatments) was compared at time 36 and 60 hours (see **Table 5; Figure 5**).



### 3. RESULTS

#### **Differences in Mass Lost Between Low, Medium and High (Acclimation) Humidity Treatments Over the First 24 Hours**

Individual ID did not explain any significant variation in mass loss during the first 24 hours (variance=1.606e-06, residual=1.527e-07; **Table 1**). At time 0, beetles in the low and medium humidity treatments did not differ significantly in average mass from the high humidity acclimation chamber (see **Table 1; Figure 2**). After 24 hours the beetles in the acclimation chamber (high humidity) had not lost any significant mass from time 0 ( $p=0.659$ ), and the average mass of individuals in the low and medium humidity treatments was significantly lower than the average mass of beetles kept at high humidity ( $p=2.44e-05$  and  $p=0.000146$  respectively, see **Table 1; Figure 2**) The beetles in the low and medium humidity treatments did not vary significantly from each other after 24 hours (see **Appendix 1; Appendix 2**).

#### **Testing Effects of Initial Mass, Acclimation, and Humidity on Mass Over 36 Hours**

In the initial model (refer to **Table 2**), individual ID again did not explain any significant variation in mass (variance=1.854e-07, residual=4.567e-08). The results of this initial full model showed that there was no effect of initial mass on the rate of mass lost, as the interaction effect between time and initial mass was not significant ( $p=0.491$ ; **Table 2**). Significant effects of acclimation were found in this first model ( $p=0.000614$ ).

When the model was simplified without the effect of initial mass, acclimation was not significant ( $p=0.288$ ; **Table 3; Figure 3**). Again, individual ID did not explain any significant variation in the variation of mass seen in the model (variance=1.587e-06, residual=8.109e-08). There was a significant effect of time on mass ( $p<2e-16$ ; **Table 3**). There was also a significant interaction between humidity and time ( $p=0.0291$ ; **Table 3**), indicating that rate of mass loss was different between low and medium humidity. Beetles in the low humidity treatment lost on average  $4.382e-05$  grams of mass per hour, while beetles in medium humidity treatment lost on average  $3.569e-05$  grams per hour. In other words, beetles in low humidity treatment lost mass on average at a rate 18.55% faster than those in medium humidity. In addition, rate of mass lost

between the acclimation and non-acclimation groups was not significantly different ( $p=0.2877$ ; **Table 3; Figure 3**).

Because acclimation was not seen to have a significant effect on the variation in mass lost, it was removed from the model to increase the power to test how humidity alone affected mass loss (water loss) over time (see **Table 4**). Again, we found that individual ID did not contribute to any significant change in mass (see **Table 4**). The final model showed a significant effect of humidity ( $p=0.000794$ ). Beetles in the low humidity treatment lost on average  $4.179e-05$  grams of mass per hour, while beetles in the medium humidity lost on average  $3.3027e-05$  grams per hour (**Table 4; Figure 4**). In other words, beetles in the low humidity treatment lost mass at a rate 20.75% faster than those in the medium humidity.

#### **Effects on Water Loss of Moving Non-Acclimated Beetles from Low and Medium Humidity to High Humidity, at the End of the 36 Hours (Time 36-60 Hours)**

In the model testing the average change in mass over the last 36-60 hours of the non-acclimated beetles, who were transferred from the low and medium humidity desiccating treatments into the high humidity chamber, individual ID did not explain any significant variation in mass seen in the model (variance= $1.965e-06$ , residual= $5.931e-08$ ; **Table 5**). At time 36, the average mass of beetles from both the low and medium humidity treatment did not differ significantly from each other ( $p=0.7943$ ; **Table 5; Figure 5**). After 24 hours in high humidity (at time 60), beetles from both groups had gained a significant amount of mass from time 36 and again were not significantly different from each other ( $p=0.2358$ ; **Table 5; Figure 5**).

#### 4. DISCUSSION

Concern about the effects of climate change on insects has largely focused on the impacts of temperature warming (Chown et al., 2011). However, less attention has been given to the potential impacts of prolonged droughts and the risk of desiccation (Chown et al., 2011). In this study, we examined how water loss rates changed in response to different levels of humidity in the brassy leaf beetle (*Phratora vitellinae*). We tested if acclimation to a high humidity affected subsequent rates of water loss in low and medium desiccating conditions. We also tested the hypothesis that lowered humidity levels would increase the rate of water loss in these beetles if they passively respond to elevated evaporation rates, versus the alternative hypothesis that the beetles should exhibit reduced water loss if they have the ability to actively control water loss. Our results show that acclimation to a high humidity does not impact subsequent rates of water loss under reduced humidity conditions. In addition, we found that individuals lose mass faster under drier conditions, suggesting that water loss in these beetles may strictly be a response to the environmental humidity. Alternatively, given that the relative humidity in the low treatment was 74% lower than that of the medium treatment, yet the rate of water loss increased by only 21%, it is possible that the lack of a proportional decrease in mass lost indicates some level of desiccation resistance under low humidity conditions. We discuss these results in more detail below.

##### **Mass Lost Over 24 Hours in High (Acclimation), Medium and Low Humidity**

When observing mass lost in the first 24 hours in the low, medium and high (acclimation) humidity treatments, only individuals placed in the desiccating treatments (~35% relative humidity and ~10% humidity) lost a significant amount of mass (**Table 1; Figure 2**). Beetles in the low and medium humidity treatment both lost on average 10-11% of their initial mass. Beetles that were in the acclimation chamber, at 65% humidity, did not lose any significant mass, indicating that they maintained their water content (**Table 1; Figure 2**). Such humidity levels are common in Trondheim during the summer months, suggesting these conditions may reflect optimal humidity levels for these beetles, which allows them to resist desiccation. In addition, after undergoing 36 hours in the desiccating treatments, individuals transferred to this high

humidity (acclimation) chamber actually gained mass (**Table 5; Figure 5**) further supporting the idea that these conditions represent optimal, hydrating conditions (see below).

### **Mass Lost Over 36 Hours at Low and Medium Humidity (Desiccating Trials)**

#### *Impact of Acclimation*

When observing mass (water) lost in the desiccation treatments over 36 hours, acclimation was found to have no significant impact (**Table 3**). Interestingly, this contrasts with many previous findings on acclimation to different humidity levels. Rapid acclimation responses have been observed in insects in response to environmental changes such as prior exposures to low humidity levels (Hoffmann, 1990; Parkash & Ranga, 2014). Other studies have shown evidence of rapid acclimation responses to pre-treatments of low and high humidity and temperature levels (Bazinet et al., 2010; Fischer & Kirste, 2018; Parkash et al., 2014; Terblanche et al., 2005). Therefore, we had expected that there may be a difference in water loss rates following the 65% humidity acclimation treatment. Specifically, if insects had acclimated to a higher relative humidity, we may have expected them to fare worse in subsequent dry conditions. For instance, (Fischer & Kirste, 2018) found that butterflies acclimated to 90% relative humidity vs 50% relative humidity fared worse in subsequent desiccating environments, losing more mass and living shorter. It is possible that the pretreatment conditions were not long enough for acclimation to occur, however acclimation has been found to occur within a matter of hours in other species (e.g. Fischer & Kirste, 2018; Hoffmann, 1990). Perhaps other environmental variables such as temperature or food availability play a larger role in creating an acclimation response in this species. Exposure to certain environmental triggers, do not always elicit an acclimation response. For example, Terblanche et al. (2005) found that thermal acclimation had no impact on the desiccation rate. Future follow up studies could examine if a pre-treatment of acclimation to low and high humidity and low and high temperatures would make the effect of acclimation clearer. Although a significant effect of acclimation was found in the first model, which included initial mass (**Table 2**), this was likely due to the confounding effect of initial mass being a significant predictor on its own.

### *Impact of Humidity*

When observing mass lost in the desiccation treatments over 36 hours, the humidity level was found to have a significant impact on the rate of water lost. We found that individuals in the low ~10% humidity treatment lost mass on average 21% faster than those in the medium ~35% humidity treatment (**Table 4; Figure 4**), suggesting the impact of relative humidity alone drives water loss. Because mass loss was greater in the lower humidity treatment, we were unable to reject the hypothesis that mass lost was merely an outcome of passive evaporation, which suggests there is no active resistance to water loss, and it is just a function of the humidity level. But, considering that the mass lost between treatments was not proportional to the decrease in humidity levels between them, this could suggest that there is some sort of desiccation resistance happening.

Changes in the cuticle is one possible mechanism that could explain the less than proportional reduction of water loss rate. Cuticular water loss is accepted as the major source of water loss in most insects at rest (Chown & Nicolson, 2004; Harrison et al., 2012), and because our study species did not originate in an arid environment (Trondheim), we can assume that the majority of water loss seen during the experiment was due to cuticular losses. Therefore, if the beetles did have desiccation resistance it would have likely happened through a reduction of water loss from this route. This could have been accomplished by an increase in chain length, decrease in branching, or increase in overall saturation of cuticular hydrocarbons (CHCs), as these properties of cuticular lipids have been found to provide greater waterproofing abilities (Gibbs, 2002; Hadley, 1977). For instance, phenotypic plasticity in CHC production, characterized by an increase in saturation, has been shown to enhance desiccation resistance in translocated populations of dung beetles in Australia (*Onthophagus taurus*) (Leeson et al., 2020). There are various other examples of phenotypic plasticity in the production of CHCs in both terrestrial and aquatic insects (e.g. Botella-Cruz et al., 2021; Otte et al., 2018; Parkash & Ranga, 2014). It is possible that after 24 hours in the desiccating environment, insects began to change the structure of the cuticle by increasing production of CHCs or modifying the composition of epicuticular lipids in other ways. This could explain why we saw no difference in average mass lost between individuals in the low and medium humidity treatments when looking at only the first 24 hours (both groups lost on average 10-11% of their initial mass) (**Appendix 1; Figure 2**), yet we did

see a significant difference in the rate of water loss between low and medium humidity levels, with beetles in the low humidity treatment losing mass 20% faster than those in the medium treatment, when followed for the full 36 hour period of desiccation (**Table 4; Figure 4**). A repeat study could investigate the composition of the cuticular lipids before and after the experiment to see if the structure of the cuticle changed in insects exposed to drier conditions.

Another factor that could have contributed to desiccation resistance is a reduction of metabolic rate, either through reduced activity or a change in gas exchange mechanisms. Some insects can adjust their method of gas exchange from continuous to discontinuous, which can result in a lower rate of water loss (Williams et al., 2010). In addition, some species have the ability to finetune the opening of their spiracles to compensate for water losses (Lehmann, 2001). However, since the temperature was the same in both the low and medium humidity treatments, and there were no observed differences in activity levels, we assumed that metabolic rate was similar in both chambers and did not play a large role in the seen differences in water loss rates.

#### *Initial Mass (Body Size)*

When investigating the impact of initial mass on mass lost over the 36 hour desiccation trials, we found no significant impact. Like other organisms, when comparing across species, insects have been found to have resting mass specific metabolic rates that scale inversely with their mass (Chown et al., 2007), though the scaling of metabolic rate can vary within and amongst life stages (Greenlee & Harrison, 2005). In addition, various studies have found body size to play a role in inter- and intraspecific desiccation resistance, with larger sizes conferring greater desiccation resistance (Le Lagadec et al., 1998; Lighton et al., 1994; Nervo, Roggero, Chamberlain, Caprio, et al., 2021; Nervo, Roggero, Chamberlain, Rolando, et al., 2021; Renault & Coray, 2013). We therefore expected that a higher initial mass would have resulted in a slower water loss rate due to larger individuals having either higher water stores, lower metabolic rates, smaller surface area to volume ratios or a combination of these. However, in contrast, our study showed that initial mass had no effect on the rate of water being lost (**Table 1**). Although surprising, a similar finding was found on a study of four species of water beetles (*Enochrus*), where contrary to expectations, body size did not have a significant effect on the rate of water lost both between and amongst species (Pallarés et al., 2016). Follow up studies could be done to

confirm the effect of body size on mass lost in this species. For instance, future studies could examine if differences in body mass amongst sex play a role, which has been seen in other studies (Nervo, Roggero, Chamberlain, Rolando, et al., 2021), as sex could be a confounding factor or investigating if body mass plays a larger role seasonally or within different life stages (Greenlee & Harrison, 2005). In our case, because we collected individuals towards the end of the season, near overwintering times, they may have undergone other physiological changes such as in the composition of their cuticle (e.g. Kárpáti et al., 2023) or hemolymph (e.g. Crosthwaite et al., 2011) to prepare for overwintering, giving them a greater tolerance to desiccation, and undermining the possible role body mass could play at other times. For example, glycerol levels have been found to increase from summer to winter as beetles prepare for overwintering (van der Laak, 1982), which has been found to increase desiccation resistance (Yoder et al., 2006). If individuals in our study had undergone physiological changes such as these, this could have led to a decrease in the magnitude of effect of lowered humidity levels on the rate at which they lost water. It would be interesting to carry out this experiment throughout the field season, including different life stages, to see if the effect of humidity varied seasonally or between developmental stages.

### **Final 24 Hours in High Humidity (Acclimation Chamber) of Non-Acclimated Beetles**

When examining the change in mass over the last 36-60 hours of the non-acclimated beetles, who were transferred from the low and medium humidity desiccating treatments into the high humidity chamber, we found that all beetles gained the same amount of mass once put in the high humidity chamber; all beetles gained roughly 4% mass (**Table 5; Figure 5**). This supports the conclusion that it is not the previous history of humidity conditions experienced, but rather the current or acute level of humidity being experienced that drives the rate of water loss in these beetles. Regardless of whether individuals had experienced a prior history of 36 hours in a low or medium humidity treatment, once moved in to 65% relative humidity all responded similarly, in this case, gaining the same amount of mass. Given the observed gain in mass at 65% humidity rather than a maintenance of mass (as compared to the acclimation beetles who maintained a constant mass in the 65% humidity in the first 24 hours), this could mean that a 65% humidity level is not simply benign, but rather hydrating for these insects. Perhaps the acclimation beetles were already maximally hydrated and fed at the time they entered the 65% acclimation chamber

(at time 0) as they came directly from the field, there was no need to increase water consumption. Whereas in contrast, when the non-acclimation individuals went in to the 65% high humidity chamber, after spending the first 36 hours in the laboratory desiccating, without access to food and water, they were able to rehydrate. Previous studies have shown that some species of insects can absorb water vapor directly from humid air (reviewed in O'Donnell, 2022) through their mouths or rectum (e.g. Bernotat-Danielowski & Knülle, 1986), which is the likely mechanism in the absence of food or water. In dehydrated flea larvae (*Xenopsylla cheopis*) the critical equilibrium humidity for water vapor rectal absorption has been found to occur around 65% relative humidity where water balance could be maintained or increased above this level (Knülle, 1967).



## 5. CONCLUSION

Previous studies have primarily focused on the impact of temperatures on desiccation resistance, especially in xeric species (Addo-Bediako et al., 2001; Chown & Nicolson, 2004). Given the role that climate change plays in impacting insect populations, a greater understanding of how environmental factors related to changes in precipitation such as relative humidity, affects individuals and species will be important in predicting consequences of future change (Chown et al., 2011). This study's focus on humidity levels alone in a population originating in a wet environment provides a first step in understanding how insects might respond to future changing precipitation patterns. In addition, the use of a widespread focal insect species makes future comparative studies across environmental gradients possible. Although it remains unclear if or how much desiccation resistance the beetles had, further investigations can investigate this. The current study indicates that this species is sensitive to acute changes in humidity levels as seen by differences in the rate of mass gained or lost depending on the level of humidity, and therefore may be more vulnerable to levels of lowered humidity in the areas in its range where it occupies more xeric conditions. Because the components of desiccation resistance can vary in surprising ways (Kleynhans & Terblanche, 2011), follow up studies that look at a range of humidity levels and temperatures may be useful in predicting responses to climatic changes in this species. In addition, species of certain functional groups may be more severely impacted by changes in climate due to potential differences in their ability to tolerate water loss and this can have cascading effects on ecosystem services and functions (Nervo, Roggero, Chamberlain, Caprio, et al., 2021). Therefore, studies including different functional groups in the same geographic area may be valuable for predicting changes to the local ecosystem structure. Other factors not focused on in this study, that can affect desiccation traits include habitat type (Chown, 1993; Le Lagadec et al., 1998), nesting behavior (Nervo, Roggero, Chamberlain, Caprio, et al., 2021), and phylogenetic relatedness (Nervo, Roggero, Chamberlain, Caprio, et al., 2021). Follow up studies which include other species and functional groups occupying an assortment of habitat types in Trondheim would provide insight into how these factors impact insect physiology and population dynamics locally.

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## 7. TABLES

**Table 1.** Summary table for linear mixed effect model comparing average mass for low, medium, and high (acclimation chamber) treatments at time 0 and 24. Mass (in grams) is the response variable and time (as a factor in hours) and humidity level (low, medium, or high) were fixed effects with individual ID as a random effect.

| <b>Fixed Effects</b>  | <b>Estimate</b> | <b>Std. Error</b> | <b>P-value</b> |
|-----------------------|-----------------|-------------------|----------------|
| (Intercept)           | 0.0094150       | 0.0002966         | < 2e-16 ***    |
| Time24                | -0.0000550      | 0.0001236         | 0.659006       |
| HumidityL             | -0.0001261      | 0.0005323         | 0.814000       |
| HumidityM             | -0.0005150      | 0.0005323         | 0.339418       |
| Time24:HumidityL      | -0.0010783      | 0.0002218         | 2.44e-05 ***   |
| Time24:HumidityM      | -0.0009450      | 0.0002218         | 0.000146 ***   |
|                       |                 |                   |                |
| <b>Random Effects</b> | <b>Variance</b> | <b>Std. Dev.</b>  |                |
| Ind (Intercept)       | 1.606e-06       | 0.0012674         |                |
| Residual              | 1.527e-07       | 0.0003908         |                |

**Table 2.** Summary table for linear mixed effect model testing effect of initial mass (grams), acclimation status (yes or no), time (measured in hours) and humidity (low or medium) on mass lost over 36 hours with mass as the response variable and time, humidity, acclimation, initial mass as fixed effects and individual ID as a random effect. Data from time measurements >0 hours were used.

| <b>Fixed Effects</b>                     | <b>Estimate</b> | <b>Std. Error</b> | <b>P-value</b> |
|--|-----------------|-------------------|----------------|
| (Intercept)                              | 1.160e-03       | 5.507e-04         | 0.041427 *     |
| Time                                     | -3.663e-05      | 8.328e-06         | 1.69e-05 ***   |
| Humidity (Medium)                        | -2.115e-04      | 2.184e-04         | 0.338697       |
| Acclimation (Yes)                        | -4.755e-04      | 2.126e-04         | 0.030880 *     |
| Initial Mass                             | 8.620e-01       | 5.693e-02         | < 2e-16 ***    |
| Time:Humidity (Medium)                   | 1.006e-05       | 3.303e-06         | 0.002591 **    |
| Time:Acclimation (Yes)                   | 1.117e-05       | 3.215e-06         | 0.000614 ***   |
| Humidity (Medium):Acclimation (Yes)      | 3.715e-04       | 2.996e-04         | 0.222116       |
| Time:Initial Mass                        | -5.937e-04      | 8.609e-04         | 0.491125       |
| Time:Humidity (Medium):Acclimation (Yes) | -4.342e-06      | 4.531e-06         | 0.338941       |
|  |                 |                   |                |
| <b>Random Effects</b>                    | <b>Variance</b> | <b>Std. Dev.</b>  |                |
| Individual (Intercept)                   | 1.854e-07       | 0.0004306         |                |
| Residual                                 | 4.576e-08       | 0.0002139         |                |

**Table 3.** Summary table for linear mixed effects model testing effects of acclimation and humidity on mass over 36 hours. Mass (in grams) is the response variable and time (in hours), humidity level (low or medium), and acclimation status (yes or no) are fixed effects with individual ID as a random effect. Full data from time =0 hours for acclimation no beetles was used. And data from the true time =24 hours and up for acclimation yes beetles was used (time was shifted 24 hours for these individuals to represent the time at which they begun desiccation treatments).

| <b>Fixed Effects</b>                     | <b>Estimate</b> | <b>Std. Error</b> | <b>P-value</b> |
|--|-----------------|-------------------|----------------|
| (Intercept)                              | 9.212e-03       | 4.239e-04         | <2e-16 ***     |
| Time                                     | -4.382e-05      | 2.621e-06         | <2e-16 ***     |
| Humidity (Medium)                        | -4.880e-04      | 5.995e-04         | 0.4212         |
| Acclimation (Yes)                        | -5.379e-06      | 5.843e-04         | 0.9927         |
| Time:Humidity (Medium)                   | 8.130e-06       | 3.706e-06         | 0.0291 *       |
| Time:Acclimation (Yes)                   | 3.848e-06       | 3.612e-06         | 0.2877         |
| Humidity(Medium):Acclimation (Yes)       | 1.319e-04       | 8.264e-04         | 0.8742         |
| Time:Humidity (Medium):Acclimation (Yes) | 1.032e-06       | 5.108e-06         | 0.8401         |
|  |                 |                   |                |
| <b>Random Effects</b>                    | <b>Variance</b> | <b>Std. Dev.</b>  |                |
| Individual (Intercept)                   | 1.587e-06       | 0.0012599         |                |
| Residual                                 | 8.109e-08       | 0.0002848         |                |

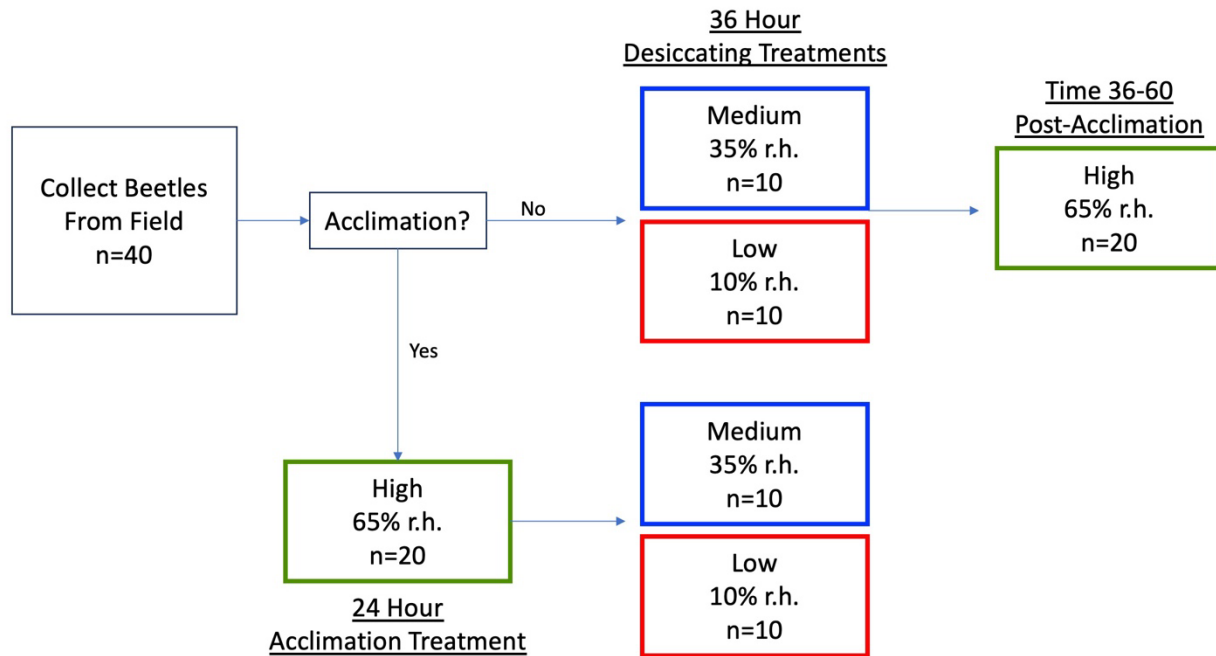
**Table 4.** Final model, summary output of linear mixed effect model examining the effect of low and medium humidity treatments alone on mass lost over 36 hours. Mass (in grams) is the response variable and time (in hours) and humidity level (low or medium) are fixed effects with individual ID as a random effect. Full data from time =0 hours for acclimation no beetles was used. And data from the true time =24 hours and up for acclimation yes beetles was used (time was shifted 24 hours for these individuals to represent the time at which they begun desiccation treatments).

| <b>Fixed Effects</b>   | <b>Estimate</b> | <b>Std. Error</b> | <b>P-value</b> |
|------------------------|-----------------|-------------------|----------------|
| (Intercept)            | 9.209e-03       | 2.843e-04         | < 2e-16 ***    |
| Time                   | -4.179e-05      | 1.807e-06         | < 2e-16 ***    |
| Humidity (Medium)      | -4.186e-04      | 4.020e-04         | 0.304509       |
| Time:Humidity (Medium) | 8.673e-06       | 2.555e-06         | 0.000794 ***   |
|                        |                 |                   |                |
| <b>Random Effects</b>  | <b>Variance</b> | <b>Std. Dev.</b>  |                |
| Individual (Intercept) | 1.505e-06       | 0.0012268         |                |
| Residual               | 8.139e-08       | 0.0002853         |                |

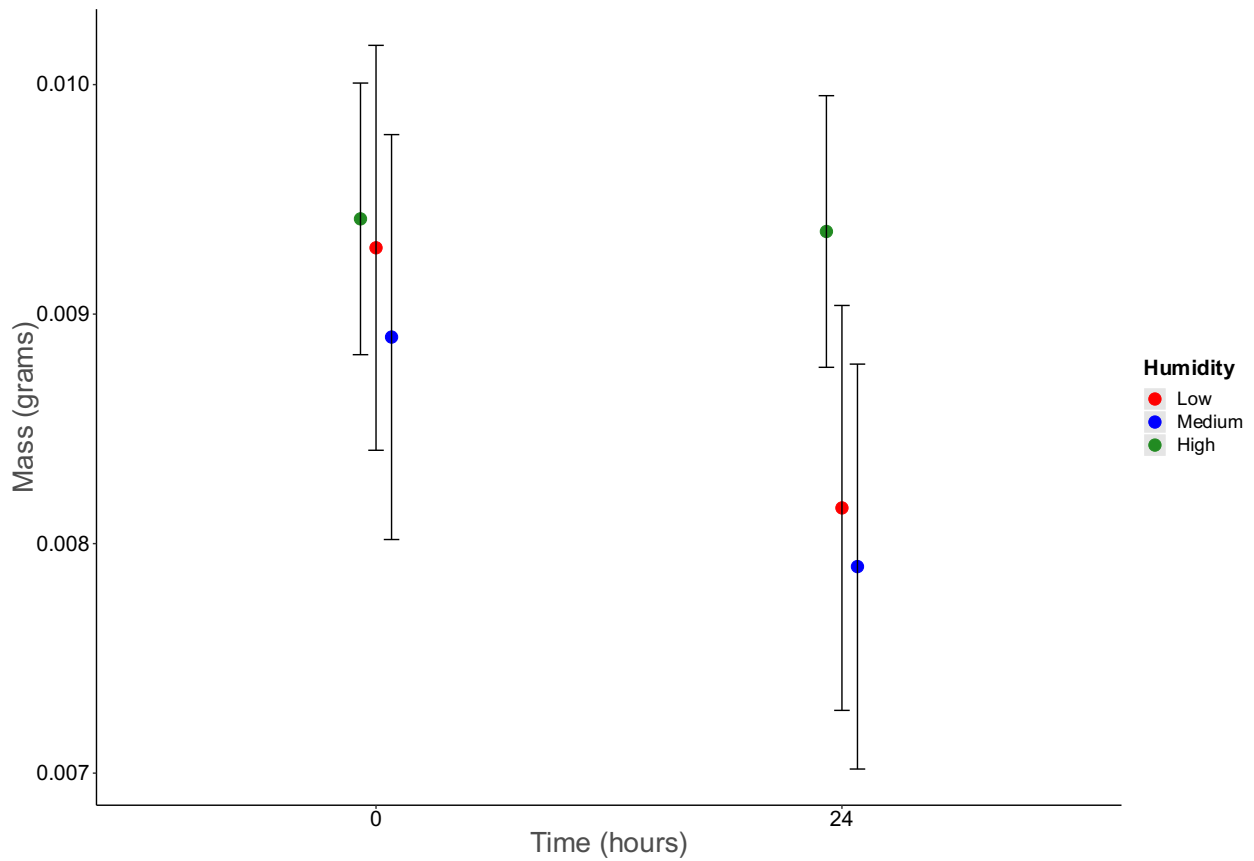
**Table 5.** Summary table for linear mixed effect model comparing differences in average mass at time 36 and time 60 between beetles originating in the no acclimation/ low and medium humidity treatments who were transferred to the high (acclimation chamber) treatment for 24 hours. Mass (in grams) is the response variable and time (as a factor in hours) and humidity history (low or medium) were fixed effects with individual ID as a random effect.

| <b>Fixed Effects</b>         | <b>Estimate</b> | <b>Std. Error</b> | <b>P-value</b> |
|------------------------------|-----------------|-------------------|----------------|
| (Intercept)                  | 0.0076778       | 0.0004743         | 1.52e-11 ***   |
| Time (60)                    | 0.0002889       | 0.0001148         | 0.0229 *       |
| Humidity (Medium)            | -0.0001778      | 0.0006707         | 0.7943         |
| Time (60): Humidity (Medium) | -0.0002000      | 0.0001624         | 0.2358         |
|                              |                 |                   |                |
| <b>Random Effects</b>        | <b>Variance</b> | <b>Std. Dev.</b>  |                |
| Individual (Intercept)       | 1.965e-06       | 0.0014019         |                |
| Residual                     | 5.931e-08       | 0.0002435         |                |

## 8. FIGURES

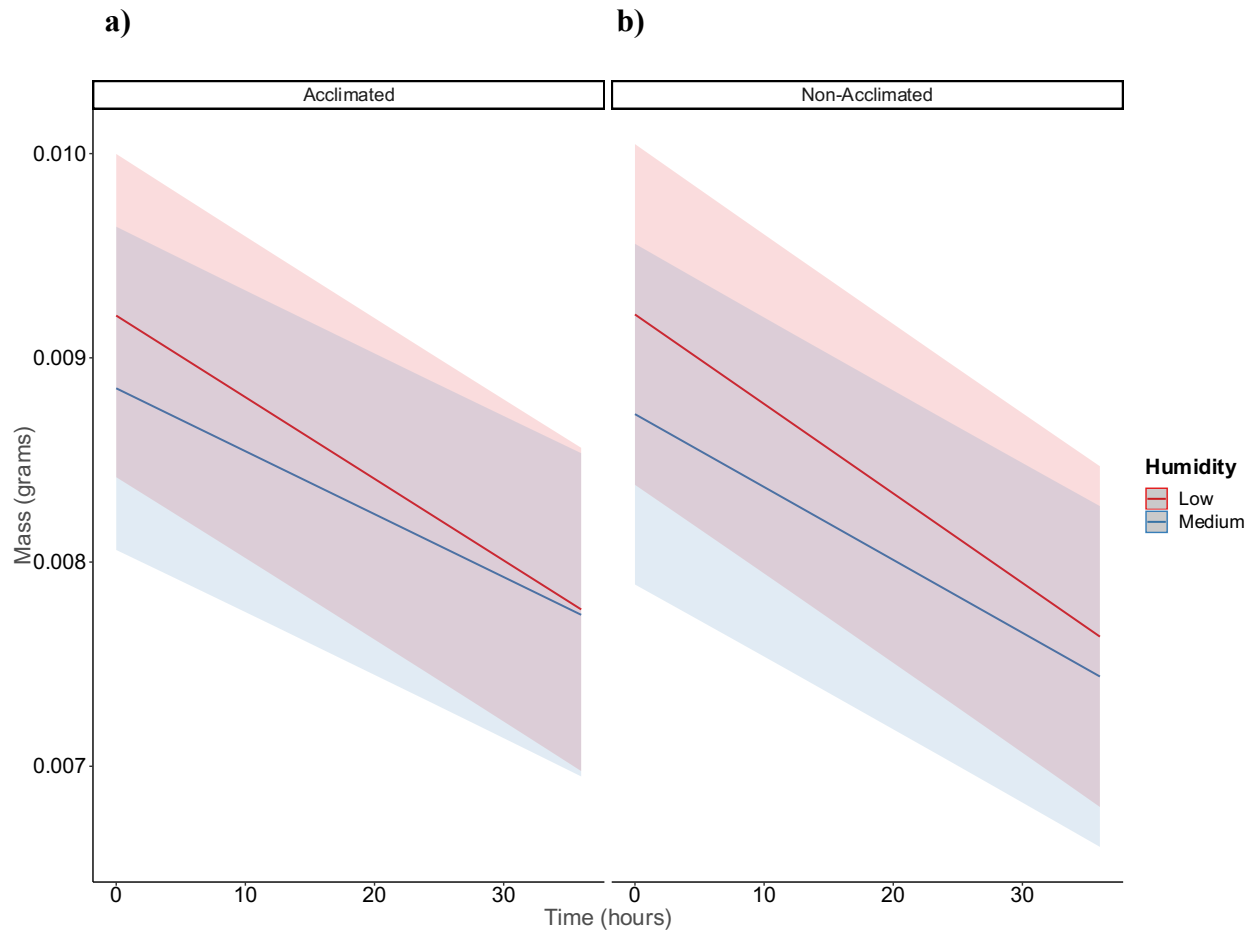


**Figure 1.** Schematic portrayal of study design with colored boxes representing the different humidity treatments.

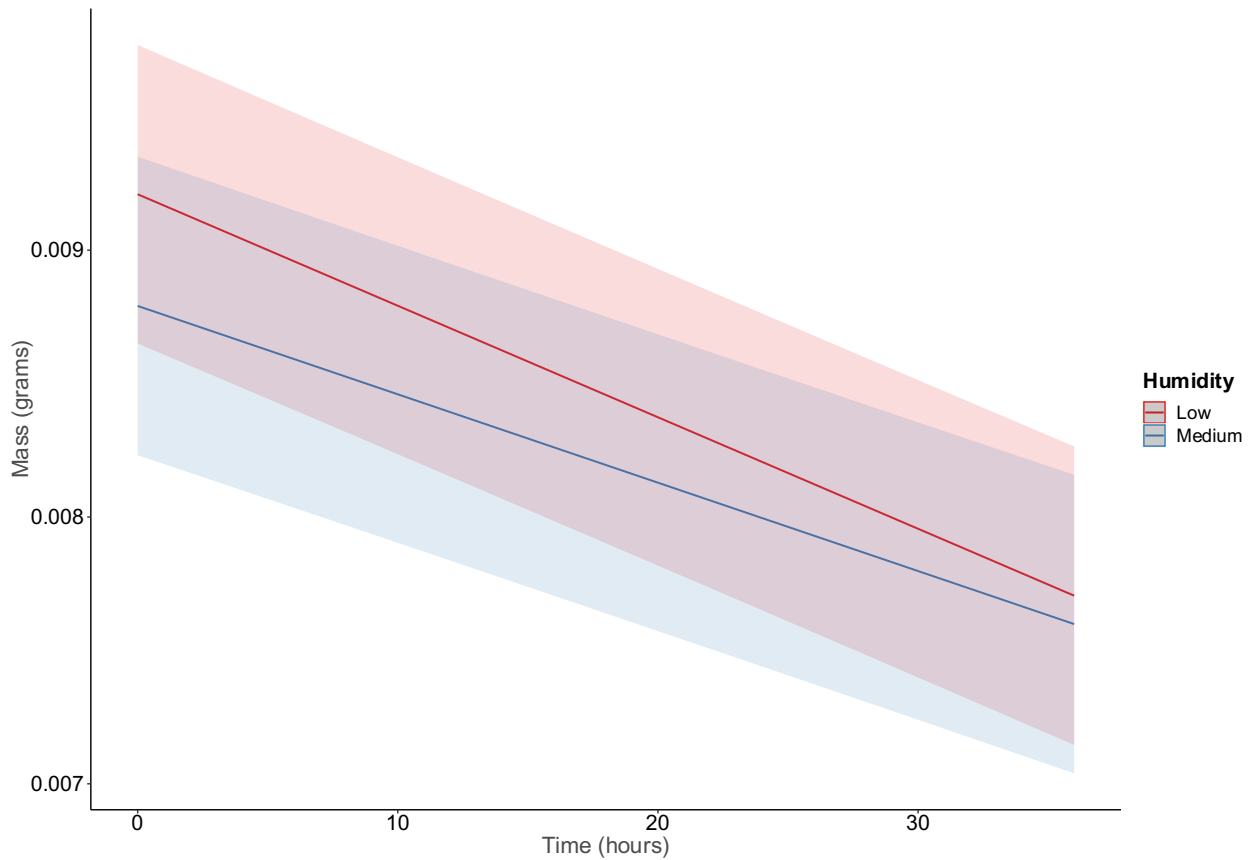


**Figure 2.** Differences in average mass at time 0 and time 24 between the low, medium and high (acclimation) humidity treatments. The average mass at time 24 for the high humidity group was significantly higher than the other two groups (acclimation beetles have not been time shifted forward 24 hours yet at this point). Line bars represent the 95% confidence interval.

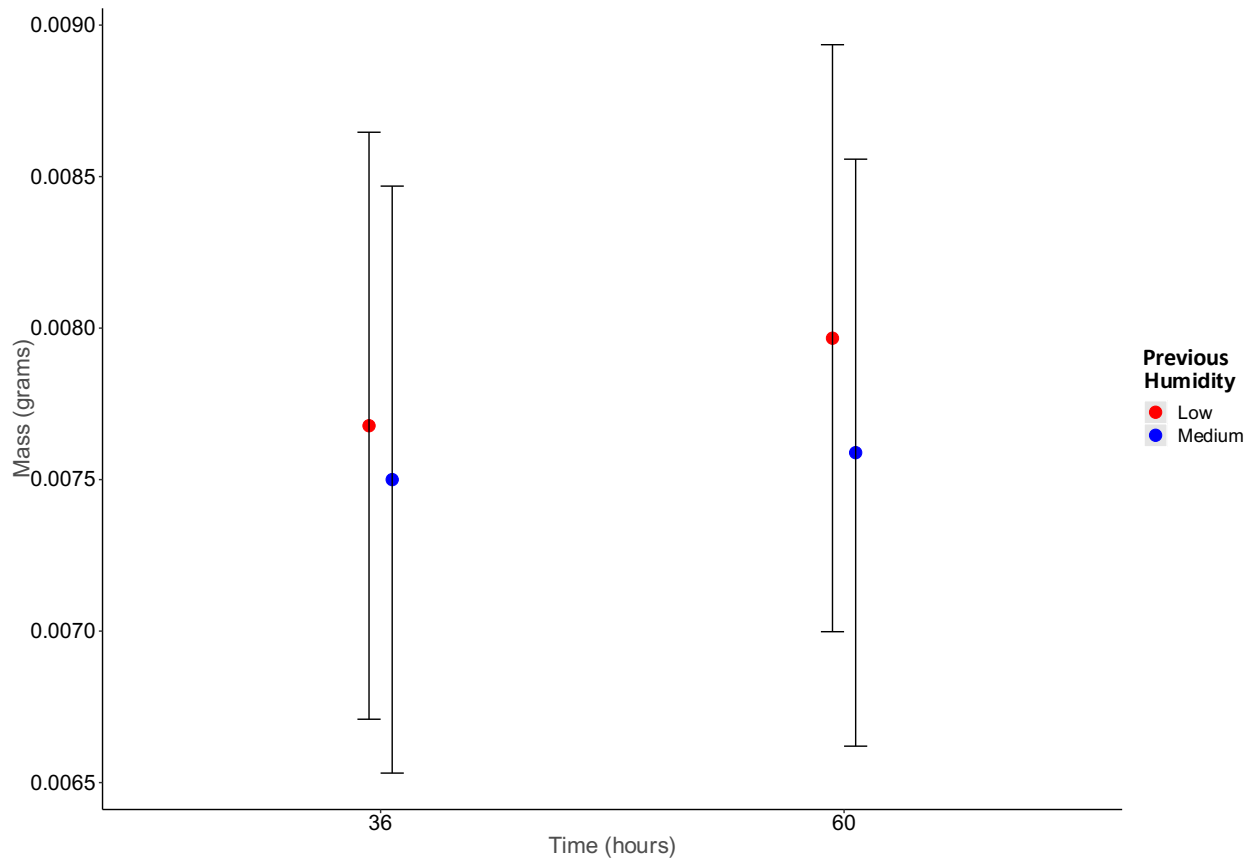




**Figure 3.** The effect of acclimation and different humidity treatments on mass loss. a) Changes in mass for beetles acclimated to high humidity and then transferred to low and medium humidity treatments (acclimated beetles have been time shifted forward 24 hours). b) Changes in mass loss for non-acclimated beetles in response to low and medium humidity. Shaded areas represent the 95% confidence interval.



**Figure 4.** Effect of time on mass loss between low and medium humidity treatments of all beetles (acclimated beetles have been time shifted forward 24 hours). The slope for medium humidity is significantly more shallow than for low humidity, indicating that insects in the medium humidity lose mass at a slower rate than those in the low. Shaded areas represent the 95% confidence interval.

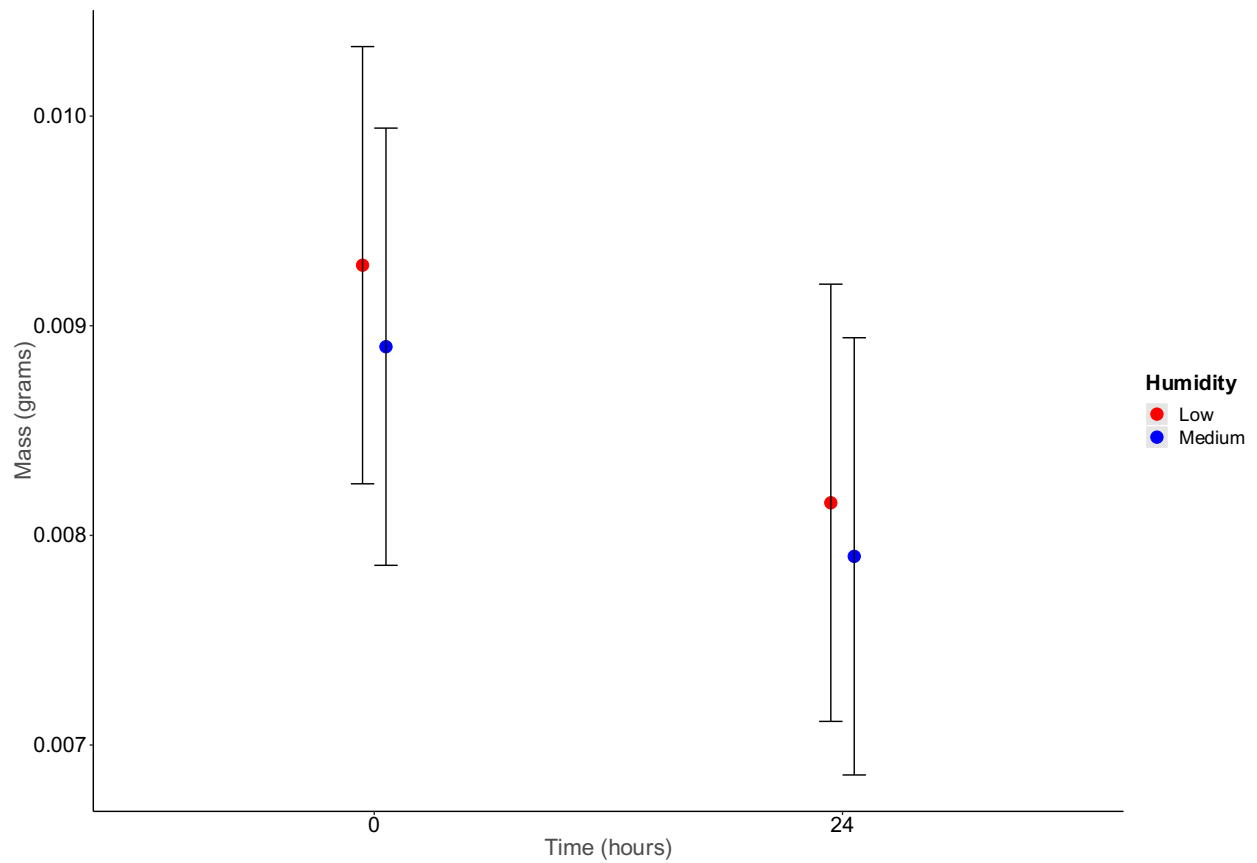


**Figure 5.** Differences in average mass at time 36 and 60 in the high humidity (post-acclimation) treatment for the non-acclimated beetles (transferred from the low or medium humidity desiccation trials) into high humidity. The average mass at time 60 was significantly higher than at time 24 for both groups. Average mass between groups did not differ from each other at either time. Line bars represent the 95% confidence interval.

## 9. APPENDICES

**Appendix 1.** Summary results of linear mixed effect model between low and medium humidity treatments to verify they are not statistically different from each other at time 0 and 24. Mass (in grams) is the response variable and time (as a factor in hours) and humidity level (low or medium) were fixed effects with individual ID as a random effect.

| <b>Fixed Effects</b>               | <b>Estimate</b> | <b>Std. Error</b> | <b>P-value</b> |
|------------------------------------|-----------------|-------------------|----------------|
| (Intercept)                        | 0.0092889       | 0.0005106         | 8.30e-13 ***   |
| Time (24 hours)                    | -0.0011333      | 0.0002160         | 7.99e-05 ***   |
| Humidity (Medium)                  | -0.0003889      | 0.0007221         | 0.597          |
| Time (24 hours): Humidity (Medium) | 0.0001333       | 0.0003055         | 0.668          |
|                                    |                 |                   |                |
| <b>Random Effects</b>              | <b>Variance</b> | <b>Std. Dev.</b>  |                |
| Individual (Intercept)             | 2.137e-06       | 0.0014617         |                |
| Residual                           | 2.100e-07       | 0.0004583         |                |



**Appendix 2.** Figure depicting average mass at time 0 and time 24 between the low and medium humidity treatments. The average mass at time 0 and 24 is not statistically different from each other (see Appendix 1 above). Line bars represent the 95% confidence interval.



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