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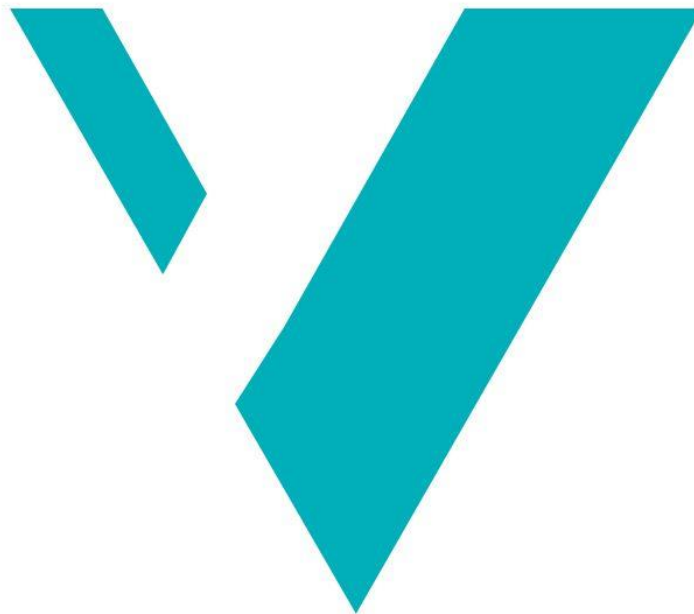
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# Lepidoptera Larvae Responses to Induced Chemical Defenses in Bilberry (*Vaccinium Myrtillus*) Grown in Two Climatic Conditions



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Master Thesis in Climate Change Management

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I confirm that the work is self-prepared and that references/source references to all sources used in the work are provided, cf. Regulation relating to academic studies and examinations at the Western Norway University of Applied Sciences (HVL), § 10.





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to Induced Chemical Defenses  
in Bilberry (*Vaccinium Myrtillus*)  
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Master thesis in Climate Change Management

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## Preface

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The only true wisdom lies in knowing you know nothing  
-Socrates-

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**THANK YOU VERY MUCH**

to

Main Supervisor

Dr. Mark Andrew Kusk Gillespie

for the help designing and conducting experiments,  
analysing data and reviewing my chapters

Co-supervisor

Dr. Stein Joar Hegland

for the inputs in previous literature, analysis and eco-coffees

from

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Admiral butterfly, Håkon Heiestad 2017



## Abstract

Host plant and herbivorous insect relationships are complex and intricate as they involve interactions influenced by many biotic and abiotic factors, leading to varying and often unpredictable patterns of insect populations and plant defoliation. The climate is changing and is predicted to bring higher mean temperatures globally, in addition to more frequent extreme weather, potentially affecting interactions between species and communities depending on each species unique response to the changes. If the climate affects the nutritional and chemical variations within the host plant and insect relationships, maybe this can be used to our advantage within agricultural practices, or maybe there are potential hazards. This thesis aims to gain a better understanding of this relationship by examining herbivorous lepidoptera larvae preference and performance on wild bilberry plants (*Vaccinium Myrtillus*) exposed to methyl jasmonate induced chemical defences and elevated temperature. Methyl jasmonate (MeJa) is a phytohormone used in signalling the defensive genetic expression in many plants. The preference and performance experiments demonstrated that the collective larvae and separated morphotypes did not react significantly to the MeJa induced defences, but rather had a significant increase in Relative Growth Rate (RGR) when eating the warmed bilberry plants compared to the larvae that were fed ambiently grown bilberry plants. This effect from warming on RGR was present from both MeJa treated and water treated plants. The slight elevation in temperature may have altered the chemical composition of the bilberry plant to become a more nutritious food for the larvae. This can alter herbivorous insect population dynamics and plant defoliation with potential economic consequences for crops and natural habitats.



## Sammendrag på Norsk

Vertsplante og insektforhold er komplekse og intrikate, da de involverer interaksjoner påvirket av mange biotiske og abiotiske faktorer som fører til uforutsigbare mønstre innen insektpopulasjoner og plantemengder. Klimaet er i endring, og det forventes høyere gjennomsnittstemperaturer globalt, i tillegg til hyppigere ekstremvær, som potensielt påvirker samspillet mellom arter og samfunn avhengig av hver art sin unike respons til endringene. Hvis klimaet påvirker ernæringsmessige og kjemiske variasjoner i forholdet mellom vertsplante og insekt kan dette kanskje brukes til vår fordel innen landbrukspraksis, eller kanskje det finnes potensielle farer. Denne oppgaven tar sikte på å få en bedre forståelse av dette forholdet ved å undersøke planteetende lepidoptera larver sin preferanse og ytelse på ville blåbær (*Vaccinium Myrtillus*) utsatt for metyljasmonat induisert kjemisk planteforsvar og forhøyet temperatur under vekst. Metyljasmonat (MeJa) er et fytohormon som brukes til å signalisere det defensive genetiske uttrykket i mange planter. Preferanse- og ytelseseksperimentene viste at de kollektive larvene og separerte larve gruppene ikke reagerte på MeJa-indusert planteforsvar, men hadde heller en betydelig økning i relativ vekst rate (RGR) når de spiste de varmedebehandlede blåbærplantene sammenlignet med larver som ble matet blåbærplanter dyrket under åpen himmel. Denne effekten fra varming på RGR var tilstede fra både MeJa-behandlede og vannbehandlede planter. Temperaturøkningen under vekst perioden til blåbærplanten kan ha endret den kjemiske sammensetningen av blåbærplanten til å bli en mer næringsrik mat for larvene. Dette kan forandre insektpopulasjons dynamikk og plantemengde med potensielle økonomiske konsekvenser for avlinger og naturlige habitater.

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## 1.0 Introduction

Plants may seem helpless when attacked by herbivores, but they actually possess several defence mechanisms against attackers. Plants do not have immune systems and senses similar to animals but have rather developed defences designed to detect potential threats and hinder critical damage through repelling or inhibiting attackers (Freeman and Beattie 2008). These mechanisms usually involve physical barriers such as spines, trichomes, surface wax and tough cuticles for making the plant matter harder to access for herbivores, but also involves a chemical line of defence through the synthesis of defensive compounds within the plant (Freeman and Beattie 2008). Plants and herbivorous insects have been in a co-evolutionary arms race for over 350 million years (Johnson and Züst 2018), and these host plant and insect relationships are usually intricate as they involve interactions between many biotic and abiotic factors and other trophic levels, leading to variations and oscillating patterns of insect populations and plant defoliation, where for example predation, food quality and local weather patterns play an important role (Ossipov et al. 2014, Kollberg et al. 2015).

In recent decades, the earth's climate has seen relatively rapid changes in temperature and carbon concentrations and according to projected climate models, the warming today is minor compared to what is expected in the end of this century (Pachauri et al. 2014). Global mean temperatures are predicted to increase by 1.9 to 5.2°C, and atmospheric carbon concentrations are projected to reach from 430 to beyond 1000 parts per million depending on socioeconomic pathways (Tolvanen and Henry 2001, Pachauri et al. 2014). With a higher mean temperature globally, drought and heavy rain are likely to increase, and interactions between species, communities and changes in community structures will likely be affected by individual species unique responses (Werkman and Callaghan 2002, Kollberg et al. 2015). Insects can gain increased development rates and number of generations per year with longer seasons (Buckley et al. 2017, Santamaria et al. 2018), and the nutrient content in plants will also change, potentially having cascading effects on herbivorous insects feeding on these plants, altering population dynamics and outbreaks both positively and negatively (Franzke and Reinhold 2011, Kollberg et al. 2015).

The producer trophic level consists of plants which bring energy from the sun into organic forms, providing many organisms on earth with a rich source of nutrients. There is strong evidence of ecological responses to climate change (Tolvanen and Henry 2001, Walther 2010, Rogora et al. 2018), and the changing climate has been projected to cause more frequent extreme weather events resulting from a global mean temperature rise (Pachauri et al. 2014), and the resulting changes affects specie resource availability, as the nutrient content within the plants also will change (Tolvanen and Henry 2001, Franzke and Reinhold 2011, Kollberg et al. 2015, Santamaria et al. 2018). As host plant and herbivore relationships in optimal conditions have complex interactions with defensive chemicals, these dynamics are also likely to be affected by the change in climate and nutrient availability, as over 200 000 defensive secondary metabolites are known from the plant kingdom, and single plant species can produce thousands, in addition to insects producing chemicals for resistance against these (Pappas et al. 2017). There are few studies that have assessed the relationships between plant defences and climate change, and there are no studies to my knowledge combining live herbivores with simulated host plant warming and induced plant defences as factors.

If a changing climate can affect the nutritional and defensive chemical variations within host plants and herbivores, assessing and understanding how these mechanisms work could be essential for future food security (Pappas et al. 2017, Johnson and Züst 2018). For example, the agricultural sector operates

closely with the climate, crop and herbivorous insect relationships, often using synthetic pesticides to kill herbivores in and around crops in addition to depending on precipitation (Freeman and Beattie 2008). Global food security relies heavily on the control of herbivorous pests, though many pesticides have been banned. We use plants extensively for food, fuel, wooden and fibrous materials, cosmetics, dyes, inks, rubber and medicines (Freeman and Beattie 2008), and consumer demands are increasing (Bruce et al. 2017, Pappas et al. 2017). In addition, some herbivorous insects are gaining resistance to these substances, and some of the chemicals are passed on to our food, potentially damaging our health (Nicolopoulou-Stamati et al. 2016, Bruce et al. 2017). In order to decrease the negative anthropogenic impacts on the environment and wildlife, clean and sustainable ways of energy, food, and material production must be implemented (Pachauri et al. 2014). All these factors have led to a higher interest in alternative pest controls such as the use of plant defensive hormones, or exogenously applied ‘elicitors’ as an alternative to the synthetic chemical pesticides (Awang et al. 2013, Bruce et al. 2017, Pappas et al. 2017). It is important to find new and innovative ways to sustain both modern human civilizations and a rich wildlife on earth. However, applying elicitors is a much more complex alternative and little is known about how these elicitors perform under a changing climate.

This thesis aims to gain a better understanding of the host plant and herbivore relationship in a changing climate by examining lepidoptera larvae preference and performance on wild bilberry plants exposed to induced chemical defences and elevated temperature. Methyl Jasmonate (MeJa) is a naturally occurring plant hormone (phytohormone) and is also an ‘elicitor’ used in exogenous signalling of the defensive genetic expressions of many plants (Farmer and Ryan 1990, Soares et al. 2016), which will be used in this thesis. The first, “choice” experiment tests the herbivores consumption preferences when given a choice of the four different plant treatments, which should indicate if the larvae can detect a difference and show a preference between the MeJa induced defence or temperature treatments. The second, “no-choice” experiment compares the weight gain or loss of the larvae after a week of consuming a specific diet or plant treatment, which should indicate how the larvae performs on each treatment.

I expect the larvae to detect the bilberry plant with MeJa induced defences as they have been sprayed with a relatively strong solution of MeJa, in theory causing the bilberry plant to have highly active defences (Orrock et al. 2017). If the larvae can detect the defences, they can avoid these and choose another food source for better fitness. The bilberry plant may have increased growth or decreased defences in a simulated warming condition (Buttler et al. 2015, Gavrichkova et al. 2017), and the larvae weight gain or loss after a week on a specific treatment will indicate which diets are the most detrimental or beneficial for the larvae.

## 2.0 Literature Review

### 2.1 Plant Defences

Plants defence mechanisms include physical constitutive defences like wax, cuticles and bark which act as hindrances for herbivores and especially neonates (Freeman and Beattie 2008, Despland 2017). If this physical defence is breached and the plant is attacked by a pathogen or herbivore, chemical defences within the plant will be activated as a response (Tianzi et al. 2018). For the plant to perceive attackers, it uses pattern recognition receptors upon contact with chemicals associated with the attackers, either microbial, pathogenic or herbivore associated molecules (Johnson and Züst 2018). Plant chemical defences are mainly regulated by three phytohormones called Jasmonic Acid, Salicylic Acid, and Ethylene. The Jasmonic Acid (JA) pathway activates the plants resistance towards chewing herbivores, while the Salicylic Acid involves resistance of pathogens. The JA signalling pathway is characterized by inducing defensive secondary metabolites called allelochemicals. These include polyamines, quinones, terpenoids, alkaloids, phenylpropanoids, glucosinolates, and antioxidants, in addition to the plant synthesizing polyphenol oxidase and protease inhibitors (Farmer and Ryan 1990, Escobar-Bravo et al. 2017). In order to activate the synthesis of these chemicals, a phosphorylation cascade is triggered through physical or chemical cues, causing the biosynthesis of JA phytohormones (Tianzi et al. 2018). The JA is transported locally from area of wounding or infection, diffusing through cellular fluids or the vascular system (Farmer and Ryan 1990). The JA is detected by receptors that stimulate the expression of JA-responsive genes, leading to the synthesis of the allelochemicals. These defensive chemicals aid the plant in deterring herbivores through oxidative stress or disrupting the digestive system of the attacker (Johnson and Züst 2018, Tianzi et al. 2018), reallocating compounds and reducing nutritional value of the plant tissue to the herbivore (Orrock et al. 2017). In addition to signalling other plants, volatile elicitors can attract the herbivores natural enemies through olfactory cues (Orrock et al. 2018). Different signal levels initiate different defence levels in different temporal responses in the plant (Orrock et al. 2017). For example, while some defensive chemicals are present within 30 minutes to 24 hours in the plant, trichomes have a more delayed response, appearing later in newly formed leaves. Trichomes are the chemical factories responsible for producing the secondary metabolites listed in the introduction (Escobar-Bravo et al. 2017), in addition to being physical barriers for neonate insects (Despland 2017).

Methyl Jasmonate (MeJa) is the volatile version of JA and is used as a volatile signalling compound for communication across plants, even across different plant species (Farmer and Ryan 1990, Ueda et al. 2012), effectively allowing a plant to warn nearby plants that it is being attacked through emitting MeJa to its surroundings (Karban et al. 2010). This volatile organic compound has in other words been identified as a language between plants and neighbouring plants, plants enemies and enemies of plants enemies. MeJa has been used as a perfume fragrance for decades, and has been identified in many plant families (Farmer and Ryan 1990). Airborne cues like MeJa are usually the strongest inducer of chemical defences, though the plant can also use mechanical stimulation on its trichomes, mechanical wounding, fungal inoculations and recently even kairomones as cues (Lombardero et al. 2013, Orrock et al. 2018). Kairomones are chemicals not directly associated with attacks but still signals the presence of potential herbivorous insects, such as mating pheromones and other communicative chemicals, saliva, oviposition and even frass by the herbivores (Orrock et al. 2018). Potential attacks are important for the plant to discover as early as possible to appropriately mount chemical defences, but at the same time not risk a significant reduction in growth on false alarm. Plants with induced chemical defences are forced to perform a trade-off within their composition as chemicals used for photosynthesis, growth and

reproduction are decreased when redirecting energy for synthesizing defensive chemicals (Orrock et al. 2018), and is found to be proportional to amount of hormone signalling (Paudel et al. 2014). A snail passing by can cause a kairomone cue associated with possible attacks, and in the absence of any actual attacks can lead to unnecessary costly defences for the plant (Orrock et al. 2018).

Although this is the general outline of plant defence through the JA and MeJa phytohormone signalling pathways, the subject is more complex as there are unique variations between species reactions and interactions with induced plant defences (Lombardero et al. 2013, Kollberg et al. 2015). Given what we know about MeJa and its general signalling pathway in plants, it is expected that the bilberry plant will have induced defences which I expect the larvae to either avoid or be nutritionally affected by.

## 2.2 Insect and Host Plant Interactions

Most mortality in larvae populations occur in the early instars, and if many early instar larvae survive, heavy defoliation of the host plant will likely occur some weeks later. Early instar mortality is extremely variable and does not present any clear patterns (Despland 2017). Some larvae outbreaks occur with a decade or so in between, as heavy defoliation causes plants to mount chemicals strongly for the next season. This kills many of the next seasons neonate larvae, leading to less defoliation that season, leading to reduced defences the next season. Defences decline through time, which again leads to more defoliation, and the cycle starts again (Ossipov et al. 2014, Despland 2017).

One of the main causes of early instar mortality is failure to establish on plants and arthropod predation. In other words, host plant traits, natural enemies, microbes, and environmental factors usually interact in complex species-specific ways that can generate unpredictable dynamics in outbreaks (Kollberg et al. 2015, Li et al. 2016, Orrock et al. 2018). Within the population, young larvae may cannibalise eggs and later instar larvae may cannibalise younger instar larvae, which interacts with dynamics within mortality and nutrition, which is influenced by factors such as population density, nutritional quality, and synchrony with host plant (Despland 2017). Two effects that have been identified as predictors for larvae population dynamics however, is spring weather and prior defoliation (Despland 2017). Inducing the plant defences by larvae attack can occur rapidly within a few hours or days, affecting colonizing and developing herbivores early, or delayed in weeks or months to affect subsequent establishments (Escobar-Bravo et al. 2017).

Insect can also adapt to diets through antioxidant enzymes to counter host plant defence chemicals (Tianzi et al. 2018). The antioxidant enzymes based in the gut of the larvae can be upregulated to protect against oxidative radicals generated by ingesting the pro-oxidant allelochemicals of the plant. The role of the antioxidant enzymes is to prevent oxidative stress by scavenging for reactive oxygen species such as these pro-oxidant allelochemicals and bind them instead of damaging cells (Tianzi et al. 2018). Neonate larvae may be more vulnerable to plant defences though, as the young larvae have different metabolism, digestive physiology and gut microbiota, in addition to having weaker mandible and cuticle toughness. They are therefore more vulnerable to defensive compounds that may have little effect on older individuals, as they have a more limited set of digestive enzymes (Despland 2017).

Many adapted herbivores also produce something called effectors, orally secreted proteins which can suppress or manipulate the defensive pathways of the plant. Aphids in particular are effective in triggering the SA pathway rather than the JA pathway, suppressing plant defences through phytohormonal crosstalk. However, SA defensive pathways also produce potent herbivore resistance chemicals in many plants (Johnson and Züst 2018). Allelochemicals and nutrients can also affect the insects susceptibility to

infection both positively and negatively, involving a fourth trophic level in addition to natural enemies influencing the population dynamics (Despland 2017). It is possible that plants can enhance the persistence of insect pathogens on the plant surface, and potentially expose the insect to a higher susceptibility of infection. Plants can also passively transport insect virus through the phloem, functioning as a vector of the insect pathogen, potentially increasing plant fitness (Escobar-Bravo et al. 2017).

As the larvae in this thesis are of a few different species in different instars, there may be differing reactions to the plants with MeJa and temperature treatments (Kollberg et al. 2015, Despland 2017). Separating the larvae in morphotypes will account for some of this variability through having the bigger, most likely later instar larvae in one morphotype group and the smaller larvae in another group. I expect to see the larvae in general to avoid MeJa induced defences in the bilberry plants, though some of the older larvae may be resistant because of more digestive enzymes.

### 2.3 Insect and Host Plant Interactions in a Changing Climate

Increased temperature and CO<sub>2</sub> causes changes in Carbon to Nitrogen (C/N) concentrations within plants because of changes in photosynthesis and respiration (Santamaria et al. 2018). Seasonal patterns of C/N shows that N concentrations are higher early in the season when the plants are in an active growth period, and declines through the season (Tolvanen and Henry 2001). This C/N concentration in plant tissue correlates positively with the tissue decomposition rates of the plant, in addition to nutritional value to herbivores (Santamaria et al. 2018). Plant quality as food for herbivorous insects is determined by proteins, carbohydrates and secondary metabolites, though the C/N ratio is a fairly robust indicator of plant nutritional quality, and in general the plants with a higher N content, or lower C/N ratio, is more palatable to herbivores (Gherlenda et al. 2016, Li et al. 2016). Carbon elevation could decrease the nutritional value for insects as it decreases N concentrations, increasing the C/N ratio (Li et al. 2016). C elevation has been observed to extend developmental time and reduce survival and fitness for insect herbivores through plant nutrition (Gherlenda et al. 2016). At the same time, increased temperature has generally been predicted to increase insect performance (Kollberg et al. 2015), and the natural enemies of the herbivores may also become more efficient in a warmer climate. It has been suggested that temperature sensitivity increases with trophic levels, potentially causing a higher top-down regulation of herbivorous insects in a higher temperature (Kollberg et al. 2015).

Since the bilberry plants in this thesis have only been exposed to increased temperature without elevated CO<sub>2</sub> concentrations, I expect the plants to have a higher photosynthesis rate, potentially increasing N compared to the Ambient treatment, which may lead to a more nutritious plant for the larvae. This effect under MeJa treatment should be reduced however, as plant defences reduces photosynthesis in addition to reallocating nutrients and mounting allelochemicals (Mathur et al. 2018).



## 2.4 Experimental Design and Hypothesis

Other studies that have been conducted regarding the relationship between induced defences, host plants and insects show varying results as many factors are playing a role in the interactions (Franzke and Reinhold 2011, Lombardero et al. 2013, Orrock et al. 2017). To gain a better understanding of how climate is affecting these relationships, one can perform controlled experiments and simulate climate change within the host plant and herbivore interactions, observing which different factors have the most significant effects and evaluate potential uses and solutions.

A key tool for understanding vegetation responses to climate change is the experimental warming of plants through Open Top Chambers (OTC) (Werkman and Callaghan 2002). Direct effects of increased temperature by OTC's and elevated nitrogen were small on the heathers in one study (Werkman and Callaghan 2002), and another heather plant in Canada from a 15 year simulated warming study was resistant to the warming, and the OTC did not alter nutrient availability or soil water content (Hudson and Henry 2010). In another study from the same area, aboveground growth increased with OTC temperature in almost all species of dwarf shrubs, forb and sedge (Tolvanen and Henry 2001). OTC's were shown to affect microclimatological variables in another study, affecting factors such as moisture, light, and wind speed, and even lowered soil temperature as a result of increased evapotranspiration. The OTC grown bilberry plants in this thesis were expected to have greater growth and be lusher and more nutritious, with potentially more C and N content. Both OTC treatments had more leaves (Gillespie, unpublished data), indicating that the bilberry plants are different when grown with OTC temperature increase, though C and N contents were not measured.

A couple of example studies using tomato plants as host plant for lepidoptera larvae in relation to JA or MeJa induced defences demonstrate the results I expect to see. The larvae fed with MeJa induced plants avoided the plant and rather turned to cannibalism in one of the studies, leaving the plant much less defoliated. In addition, the growth rate of the larvae was slower in MeJa treated plants in both experiments, and the plants with induced defences also had a decreased growth and reproduction (Paudel et al. 2014, Soares et al. 2016), which was also seen in the bilberry plants used in this study (Gillespie, unpublished data). This indicates that the bilberry plants have performed a trade-off in growth for defensive chemicals, which the larvae should notice.

I will assess the larvae reactions to MeJa induced defences in bilberry plants and see if the temperature differences in plant growth condition can influence this. Plant defences need to be considered when predicting responses in herbivores to climate change, as this may become increasingly important (Kollberg et al. 2015). My hypothesis is that the larvae will avoid the MeJa treated bilberry plants and show a different growth rate when fed with MeJa induced plant diets (Orrock et al. 2017). The temperature increase could decrease these defensive traits if evapotranspiration in soil increases, leading to drier soil, and could also show a better performance in larvae (Santamaria et al. 2018). These combined interactions can aid in further understanding the intricate relationships between herbivorous insect and host plants in a changing climate.

## 3.0 Methods

The procedure of collecting larvae, collecting plant samples and performing experiments were done in two identical sessions lasting two weeks each during the June and July months of 2017. I collected 30 larvae over three days in each monthly session, resulting in 60 larvae of about 7-8 varying unidentified lepidoptera species.

### 3.1 Field Work 1: Collecting Larvae Samples

The larvae were collected through sweeping a net through bilberry plants growing on a sunny south/south-east facing hillside close to the entrance to Solvorn town, Western Norway, at around 220-250 meters above sea level. During the larvae collection, I kept all the larvae in the large glass jar together with a good amount of seemingly neutral cut bilberry plants from their habitat. I then separated the larvae when I returned home, in smaller aerated containers, feeding them with bilberry plants from their habitat and labelling each larvae container and their different morphotypes. The larvae were separated into three morphotypes: Green Big Larve (GBL), Green Small Larvae (GSL), and Stick Pretender Larvae (SPL). Within these morphotypes there were probably 2-3 species of larvae, some with black stripes along the side in GBL and GSL. The Stick Pretender Larvae were either green or brown with similar camouflage pattern, identical body shapes, with the common trait of stiffening and attempting to camouflage as sticks whenever disturbed.

### 3.2 Field Work 2: Collecting Plant Treatments

Mark Gillespie at the Western Norway University of Applied Sciences in Sogndal is responsible for the bilberry plant treatments used in this study. The four plant treatments were treated and collected from a South facing hillside of Storehaugfjellet near Kaupanger, Western Norway, at about 450-475 meters above sea level, close to Haukaasen Airport (Picture 1). The bilberry plants were grown in six separate block formations labelled from A to F, which were established in May 2016. In the corners of each of the 16x16m blocks were marked 2x2m grids labelled 1-4. In each block, one of four treatments was randomly assigned to the four plots, and was replicated six times for each treatment combination. The four treatments conducted were:

Ambient Water (AW): Open air control plants which were only treated with water spray.

Ambient MeJa (AM): Open air plants which were sprayed with water containing 10 mMol MeJa solution.

OTC Water (OTC\_W): Plants placed within Open Top Chambers, only treated with water spray.

OTC MeJa (OTC\_M): Plants placed within Open Top Chambers, sprayed with water containing 10mMol MeJa solution.

The MeJa was applied with a 5l knapsack sprayer, sprayed steadily over the MeJa plots twice with 10mMol solution in water on 2<sup>nd</sup>, 10<sup>th</sup> and 19<sup>th</sup> of June 2016. The increased temperature treatment was achieved with Open Top Chambers (OTC; Picture 1), which were removed during the snow season. The OTC's were measured to increase the temperature by an average of 1°C in the treated plots and were placed on the same day as the first water and MeJa applications in 2016.



Picture 1: Hillside in Kaupanger where plant treatments were conducted and collected, with an OTC in the lower right corner.

Shoots from each plot in every block were collected in plastic zip-lock bags and labelled with a permanent marker. All shoots were then placed in separately labelled water pots in the biology laboratory at HVL to remain relatively fresh and replaced when needed through the experiments. Samples were collected in middle of June for the first round of experiments, and in the middle of July for the second round of experiments.

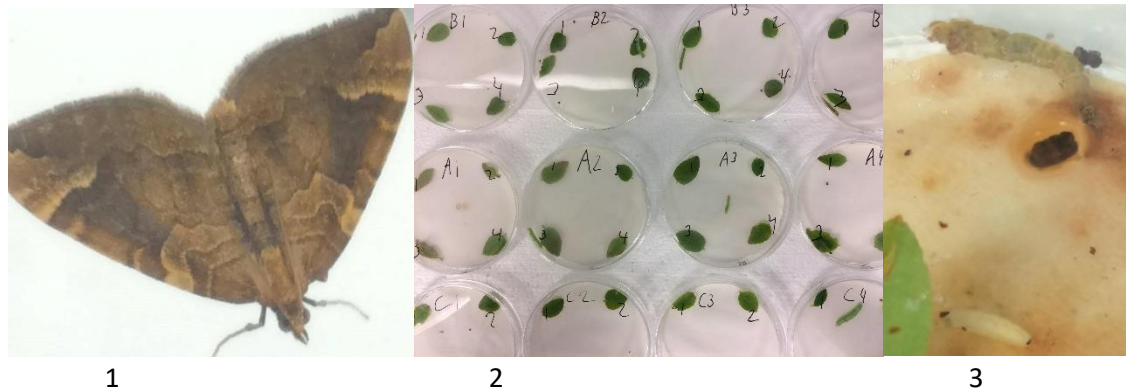
### 3.3 Lab Work, Experiment 1: Larvae Preference

I placed filter papers in the 9cm diameter petri dishes and moistened each paper with a little water from a pipette. I then measured the size of one leaf per treatment (1-4), from each block (A-F), with graph paper. For each block, a leaf from each treatment was placed in each petri dish equal distances apart near the edge of the prepared petri dishes. I wrote treatment and block labels on the lid, and then randomly placed each larva in the center of each petri dish, handling the larvae with the soft bristle tips of two paint brushes. I closed the lids and placed each dish in the temperature control room and ensured that air could move in and out of the dishes (Picture 2). The temperature-controlled room held a temperature of 20 – 22°C, with a light cycle of 16/8 hours day/night. During the first 30 minutes, I checked and recorded each larva's first choice. After 4 hours, I wrote a few notes on activity, and after 24 hours I ended the experiment. Each leaf was then measured with a graph paper, and put up against its original size, estimating the percentage eaten from each leaf.

### 3.4 Lab Work, Experiment 2: Larvae Diet Performance

I assigned a randomized diet to each larva from the preference experiment, I then placed filter papers in new 9cm diameter petri dishes and moistened them with water from a pipette, and labelled the lids tops with the treatments and blocks. I wrapped a moist cotton wool piece in plastic foil around the cut root of each shoot to maintain plant freshness and placed the prepared shoot in the prepared petri dishes. I recorded each larvae's initial weight and placed them in their corresponding assigned petri dishes, to be placed in the temperature and light controlled room. I observed their consumption over one week, replacing each shoot as they were consumed, in addition to replacing the filter papers when deemed too dirty. I also noted anomalies such as parasite attack (Picture 2), fungal attack, skin shedding, pupating, nest spinning, and color change. After one week on the diet, I recorded the final weight of each larvae, and subtracted the initial weight to see total weight gain or loss.

After I finished weighing the larvae, I placed them in separate aerated containers with neutral bilberry plants for the larvae to grow, pupate and transform to moth (Picture 2) or butterfly so that I could attempt to identify the different species.



Picture 2: From left: 1) Reared moth from Stick Pretender Larvae 2) Larvae first choice experiment 3) Parasite attack.

### 3.5 Statistical Analysis

In the experiment regarding consumption preference, a three-way ANOVA was conducted, with percentage eaten from each leaf after 24 hours as the dependent variable, and the treatments of temperature and MeJa and the three larvae morphotypes as the independent variables. All two-way interactions were also included in the initial model to test the hypotheses that the morphotypes had different responses to the treatments, and that the preferences for MeJa treated leaves were different under different temperature treatments. The initial model was simplified by sequentially removing terms that were not significant at the  $p=0.05$  level. The final model was then the one with only significant terms remaining. Both initial and final models were checked for validity by inspecting the residuals graphically for normality and equality of variance. In addition, the first choices of the larvae were tabulated to perform a chi-square test to see if there was a correlation between larvae morphotype and treatment preference.

For the experiment regarding larvae performance, a three-way ANOVA was performed in the same way as above, but the dependent variable was the larvae weight gain or loss, represented by the relative growth rate (RGR) which is the average daily growth gain or loss of larvae, given by the formula

$$RGR = [\ln(W_f) - \ln(W_i)]/D \quad [1]$$

where  $\ln$  is the logarithmic transformation of the final weight  $W_f$ , minus the logarithmic transformation  $\ln$  of the initial weight  $W_i$ , divided by  $D$  which is the seven days passed between weight measurements (Gillespie and Wratten 2011).

All statistical analyses and Figure 1, 4 and 5 were conducted with R studio version 3.3.1, and Figure 2 and 3 and the data sets were conducted with Microsoft Excel 365. All randomizations for assigning larvae to petri dishes were done through using the service from [www.random.org](http://www.random.org). The complete data sets from the experiments can be found in the appendix. The data sets include 52 larvae observations for first choice, 48 larvae observations for eaten leaf percentage and 39 larvae observations for performance experiment out of the 60 larvae originally collected, also containing which of the two months in which the larvae were collected, though this was not considered in the statistical analysis.

## 4.0 Results

### 4.1 Larvae Preference

In the first experiment, there were no significant effects of MeJa treatment, temperature or morphotype on the percentage of leaf material eaten in 24 hours (Figure 1). The means are too similar, and the standard errors of all the eaten leaf percentages are overlapping too much to indicate a significance from the treatments.

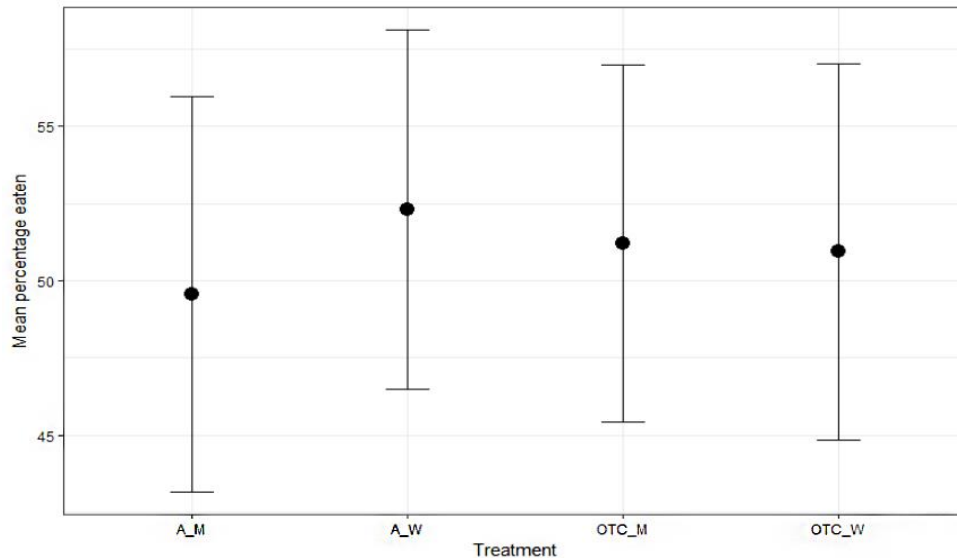


Figure 1: Means of percentages eaten of each treatment for all larvae morphologies (black dots). On the x-axis A = Ambient temperature treatment, OTC = Open Top Chamber temperature treatment, M = MeJa treatment, W = Water treatment. The error bars are  $\pm 1$  standard error.

### 4.2 Larvae Leaf Treatment First Choice

For the experiment regarding first choice, the data in Figure 2 suggests a trend towards an initial preference of the control plants (AW; Ambient temperature, Water treatment).

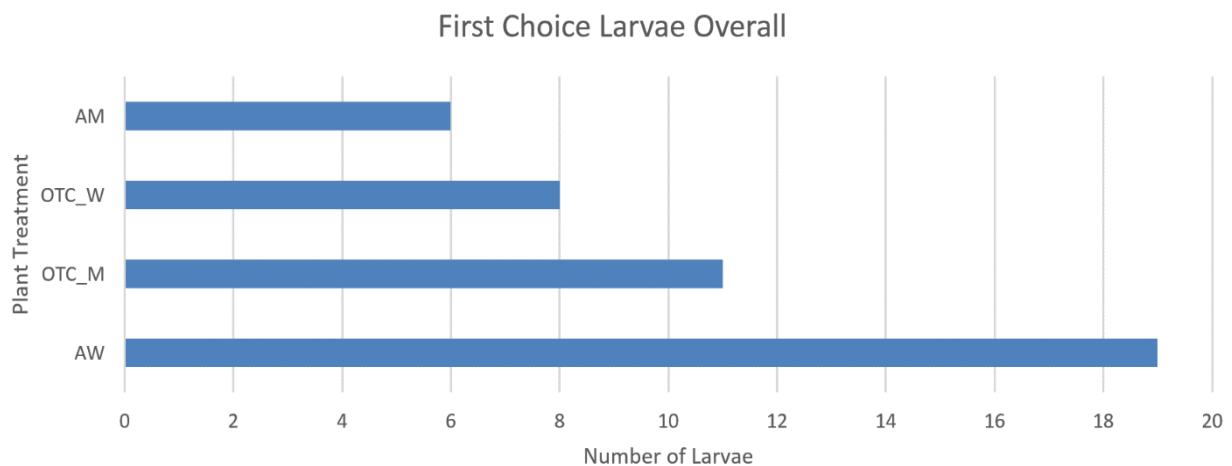
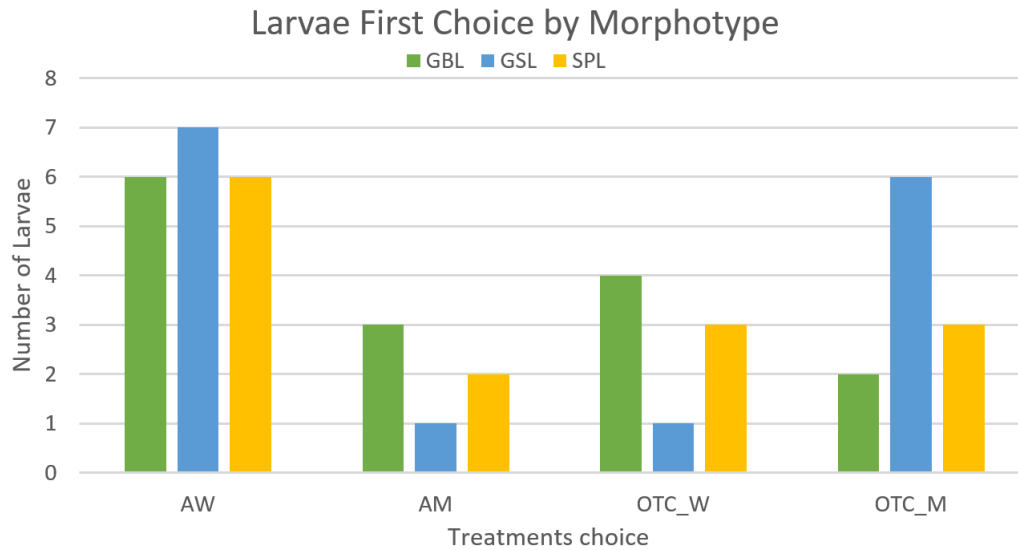


Figure 2: First choice of treatment for all larvae morphotypes after one hour. On the y-axis A = Ambient temperature treatment, OTC = Open Top Chamber temperature treatment, M = MeJa treatment, W = Water treatment. Total number of larvae is 44, and 8 larvae had no first choice.

The data also indicate a preference for the control treatment when separating morphotypes (Figure 3).

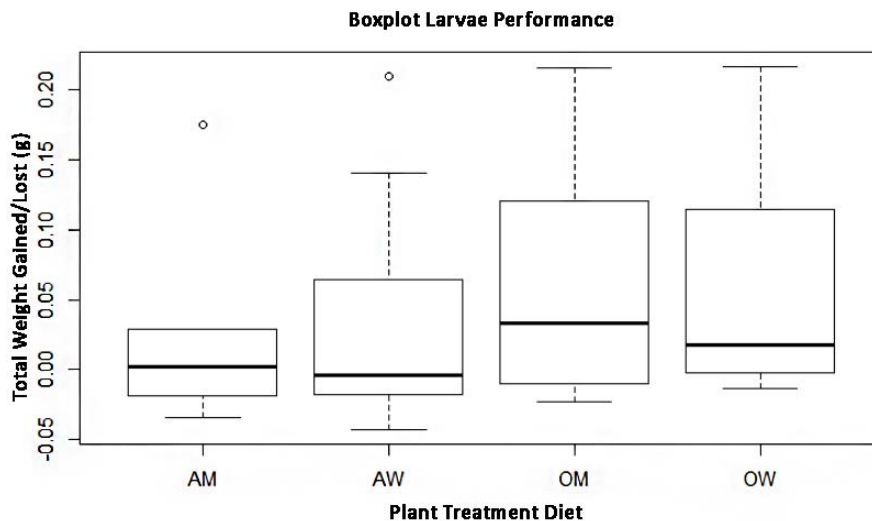


**Figure 3: Number of larvae from each morphotype first choice of treatment in first hour of experiment.** Morphotype Legend: GBL = Green Big Larvae, GSL = Green Small Larvae, and SPL = Stick Pretender Larvae. On the x-axis A = Ambient temperature treatment, OTC = Open Top Chamber temperature treatment, M = MeJa treatment, W = Water treatment.

However, the results from the chi-squared test indicate that there was no correlation between the larvae first choice and the plant treatments in this experiment (x-squared = 5.069, df = 6, p = 0.535).

### 4.3 Larvae Diet Performance

For the experiment regarding larvae performance on the four different treatment diets, the data is spread over ~0.25g, showing variations in all treatments seen in Figure 4.



**Figure 4: Boxplot of total weight gained or lost for all larvae on each treatment.** On the x-axis A = Ambient temperature treatment, O = Open Top Chamber temperature treatment, M = MeJa treatment, W = Water treatment.

Comparing the RGR of the larvae with the temperature and MeJa treatments showed that there was no significant effect from the MeJa ( $p=0.21339$ ), but rather the temperature treatment showed a significant effect ( $p=0.02314$ ) on the RGR with all larvae included collectively, though morphotype had a higher significant effect ( $p=0.00166$ ).

**Table 1: Multiple ANOVA of RGR compared to morphotype, temperature and MeJa treatment.**

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Morphotype	2	0.13618	0.06809	7.987	0.00166
Temperature	1	0.04885	0.04885	5.73	0.02314
MeJa	1	0.01378	0.01378	1.616	0.21339
Morph:Temp	2	0.00289	0.00144	0.169	0.84502
Morph:MeJa	2	0.00021	0.0001	0.012	0.988
Residuals	30	0.25575	0.00853		

Separating and analysing larvae in the 3 morphotype groups with MeJa and temperature, the Green Big Larvae and Green Small Larvae had a significant effect on RGR ( $p \leq 0.05$ ) when temperature was almost significant ( $p=0.798$ ), but not the Stick Pretender Larvae ( $p=0.1048$ ). Simplifying the statistical model by removing MeJa while keeping interaction of Morphotype and Temperature yielded no significance, and then removing morphotype and temperature interaction resulted in that the morphologies still had a more significant effect on the RGR ( $p=0.00083$ ) than temperature growth condition, though temperature still had a significant effect on the RGR ( $p=0.017084$ ) (Table 2).

**Table 2: Simplified model of multiple ANOVA**

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Morphotype	2	0.13618	0.06809	8.742	0.000833
Temperature	1	0.04885	0.04885	6.271	0.017084
Residuals	35	0.27263	0.00779		

The larvae feeding on the Open Top Chamber grown bilberry plants had an almost threefold increase in RGR compared to the larvae fed with ambient grown bilberry plants (Figure 5), though the true unlogged difference may be slightly lower or higher.

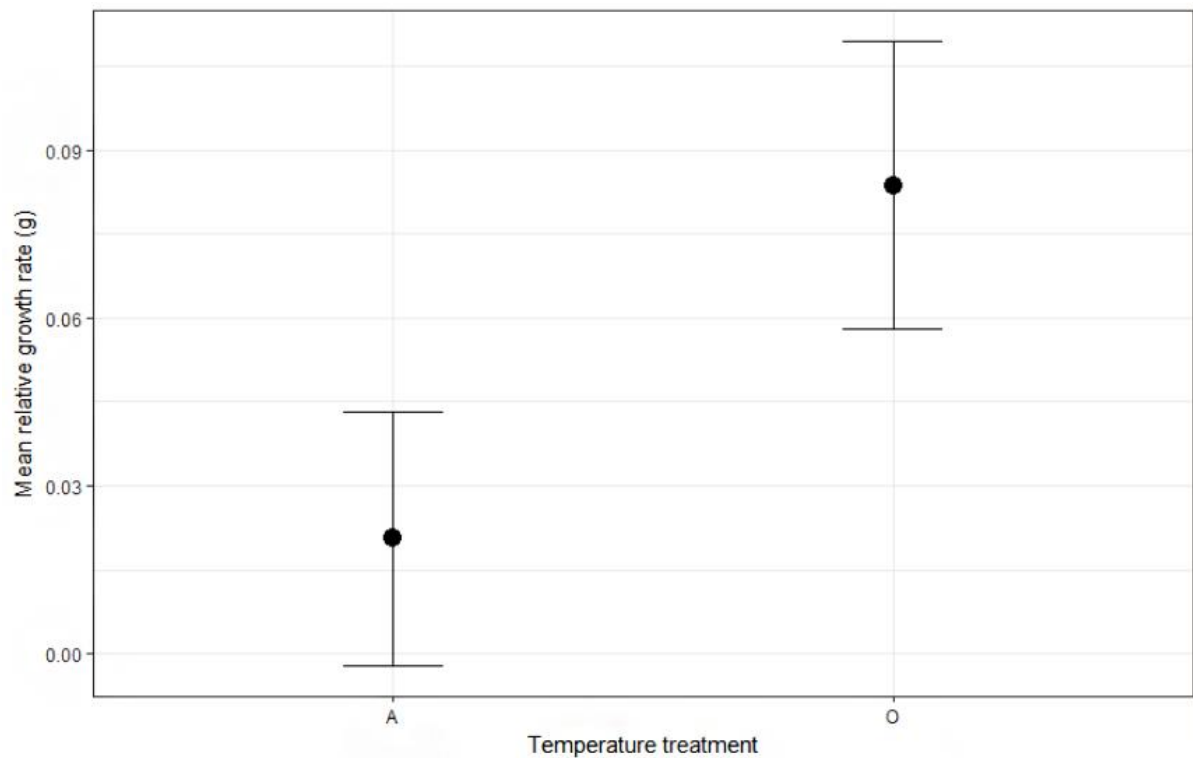


Figure 5: The mean relative growth rate of all larvae in grams (black dots), plotted against temperature growing condition of the bilberry plants. X-axis: A = Ambient, O = Open Top Chamber (warmed treatment). The error bars are  $\pm 1$  standard error. The relative growth rate is based on Equation 1.

## 5.0 Discussion

### 5.1 MeJa Induced Defences and Resistance in Larvae

This study demonstrated that the collective larvae and separated morphotypes did not react to the MeJa induced defences, neither in preference or performance. There were no significant differences in growth or leaf percentage consumed between larvae eating water treated or MeJa induced plant diets. This is inconsistent with much research regarding MeJa induced defences and led me to reject my hypothesis reasoned in chapter 2.2 of larvae avoiding and performing worse on MeJa treated plants. Several of the analyses indicated a slight trend in the hypothesized directions, but not enough to conclude with statistical significance. The MeJa treated bilberry plants used in this experiment had less growth and less herbivory (Gillespie, unpublished data), which is consistent with what is expected of plants reactions to MeJa but this did not translate to herbivore resistance in this experiment.

As to what factors could have caused the larvae to not show expected reactions to MeJa are many, such as differences in larvae development or differences in bilberry plant defences. The diversity of larvae species and age groups may have clouded the results as there were several instar levels and morphological types of larvae used in this experiment, which may have differing abilities to tackle the plant defence chemicals. For example, if the older larvae have the ability to upregulate their digestive



enzymes to cope with the defensive chemicals (Tianzi et al. 2018), they would likely consume every plant treatment. If this upregulation occurred before or during the experiment, this may have led to the scattered results without trends. Some of the smaller, potentially younger larvae (SGL) refused to eat anything, rather spinning nests under the petri dish paper, which may have been a reaction to being in captivity or requiring younger leaves. If these are young larvae, then in addition to having a more limited set of digestive enzymes, many species require young expanding plant foliage with generally less toughness and higher nutritional value (Despland 2017), which may have hindered them to eat the bilberry plant leaves even if they could tell a difference in treatments.

Many leaves in the preference experiment were completely consumed or completely avoided after 24 hours with no clear pattern, which contributed to the result in Figure 1. The percentages for each leaf eaten perhaps should have been measured at an earlier time point than 24 hours, before the larvae had a chance to completely consume one or more leaves. The fact that there was no significant difference is however consistent with the performance experiment which also showed no significant effect from MeJa induced defences.

Alternatively, the bilberry plant leaves used in the experiment were not completely controlled in terms of their application of MeJa, suggesting that they could have had variations in mounted defensive chemicals at the time of collection, about one year after application of 10mMol MeJa. Depending on the genotype, time lapsed and other stimuli such as field herbivory after application of MeJa, the bilberry plant leaves may have contained different amounts of naturally activated defensive chemical after a year of growing in slightly different conditions. However, defensive chemicals should be present as the MeJa induced bilberry plants showed a general lower growth and reproduction (Gillespie, unpublished data) in the field, in addition to being induced with a relatively strong solution of 10mMol MeJa.

The control treatments could have been affected to induce defences on their own through mechanical wounding, kairomones or other sources of volatile MeJa signalling (Lombardero et al. 2013, Orrock et al. 2018), activating similar resistance as the MeJa treated plant. Cutting the bilberry plants in order to transport to the lab may have also activated defensive pathways, although this should not be nearly as strong as direct application of 10mMol MeJa (Orrock et al. 2018), and I think the larvae should have noticed this difference. In order to remove uncertainty like this, the bilberry plants could have been grown in pots in controlled conditions, having treatments and larvae directly applied without mechanical damage to prevent the activation of defensive pathways in the control plants.

## 5.2 Temperature Growth Condition of Plant and Larvae RGR

Rather than being reactive to MeJa induced defences, the larvae yielded some interesting results regarding the temperature in which the bilberry plants were grown. Comparing the speed at which the larvae grew relative to size showed that there was a significant difference between plants grown in warmed and ambient conditions. Feeding on a diet of OTC grown bilberry plants, regardless of MeJa or water application, the larvae had an almost threefold increase in RGR compared to the larvae that were fed ambiently grown bilberry plants. The slight warming of the bilberry plant growth conditions may have altered the chemical composition of the plant to become more nutritious for the larvae. This is an interesting find, but the experiment was only done over 7 days of feeding, which could have been extended for a more robust result.

Elevated temperature increases the photosynthetic rate in plants to a certain threshold, and it has been suggested that increased photosynthesis, if nutrient uptake is also enhanced, leads to higher growth

rates in the plant but can also lead to increased C/N ratio if growth does not increase (Tolvanen and Henry 2001). The C/N content in the bilberry plants may also have been affected by the time of collection in June or July, as N is higher early in the season. The bilberry plants treated with OTC's had more leaves, indicating an increase in growth as a result of warming, though C and N concentrations were not measured. The OTC temperature increase only affected growth in the water treated bilberry plants without MeJa induced defences (Gillespie, unpublished data), but this did not translate to the larvae performance as they grew faster on both water and MeJa treated OTC treatments, which indicates that the bilberry plant defences were not effective on the larvae. This rejects my hypothesis of larvae performing worse on MeJa induced and warmed bilberry plants, where I suggested that MeJa would decrease photosynthesis and reduce nutritional value even when warmed.

The basic material for soft tissues and integument of insects are available N in the plants in terms of proteins. In general, the plants with a higher N content, or lower C/N ratio, is more palatable to herbivores (Li et al. 2016). A meta-analysis comparing hundreds of insect experiments found that under elevated N, the overall insect herbivore performance in trees was enhanced by over 15% and increased insect performance with higher insect density, survival rates, body mass, reproductive rate and resistance to pathogens, with a shorter development time (Li et al. 2016). Lepidoptera larvae benefit from the same increased available N level in their food (Franzke and Reinhold 2011).

Previous studies have shown that another effect somewhat related to increased temperature, drought, makes the plant more susceptible and suitable to herbivory through reducing the effectiveness of the plant defences and causing a higher soluble protein content (Franzke and Reinhold 2011). The OTC's could have altered the evapotranspiration in the soil of the bilberry plants, causing a slight reduction in soil water content (Buttler et al. 2015), potentially causing a similar but smaller effect to drought, which could have increased the soluble protein content within the bilberry plants. As the defences of the plants are also affected by the drier soil, the larvae showed a general increased RGR even when feeding on bilberry plants with induced defences. Another study regarding drought demonstrated that mites feeding on drought stressed plants with induced defences performed better on this plant, compared to the watered plants without defences (Santamaria et al. 2018), which is the same result I have regarding higher RGR on one of the MeJa induced plants. Proline has also been reported to cause insects to grow faster, an amino acid that was available in significantly higher amounts in the drought stressed plants, which might be present in the bilberry plants (Franzke and Reinhold 2011).

This is interesting in relation to climatic factors and plant defences as it demonstrates better larvae performance, although not preference, when fed plants grown in elevated temperature even with induced plant defences. Increased temperature has also in general been predicted to improve performance for insects (Kollberg et al. 2015). If temperatures increase and herbivorous insect metabolism and performance increases together with it, in addition to making plants more palatable to herbivores with reduced plant defences (Franzke and Reinhold 2011, Santamaria et al. 2018), there may be increased frequencies and/or magnitude of herbivore outbreaks (Despland 2017) with potentially negative economic consequences for both society and ecology. With a faster development of insects and longer seasons, more individuals may survive predator or parasitoid attacks and even allow a second generation in the same season, likely resulting in higher herbivory pressure and outbreaks. Herbivorous insect population dynamics will however, fluctuate through the relative impact of bottom-up and top-down processes influenced by temperature (Kollberg et al. 2015). When these tri-trophic interactions are influenced separately by temperature, it causes complex patterns in insect population and plant

defoliation (Kollberg et al. 2015), making it hard to predict what will happen with increased temperature in general as every outcome can be different depending on many factors.

I connect the larvae having a faster RGR to the nutritional value of the bilberry plants, which I think has increased with the OTC treatment regardless of MeJa induced defences, as the warmth either caused a direct elevated N content or indirectly through altering soil moisture of the bilberry plants. Although, none of the C/N concentrations, nutritional values or secondary metabolites were measured in any way in the bilberry plants, and this is all based on a combination of information from literature reviews with interpretation of my own treatments and results.

### 5.3 Potential of MeJa as an Alternative to Conventional Pesticide

The use of plant elicitors as an alternative to chemical pesticides has seen increased interest in recent years. The evolution of pesticide resistance in insects and changes in legislations are limiting conventional pesticide options for farmers, in addition to increasing global food demands (Bruce et al. 2017, Pappas et al. 2017). As the MeJa in this experiment did not show any significant effect on the larvae's preference or performance, but rather the growth condition of the plant, then purely as a pesticide alternative, plant defences show mixed results. However these experiments were performed on wild bilberry plants and not cultivated crops, in a small-scale lab setting and may translate differently.

A study from Malaysia using chilli compared JA and conventional pesticide against a pathogen called leaf curl disease. It demonstrated that JA started with low impact but had a slow increase in effectiveness while conventional pesticide started with a high impact that had a slow decrease in effectiveness. In the end of the experiment, the JA was almost as effective as the conventional pesticide (Awang et al. 2013). The same experiment however, showed that Salicylic Acid which is the phytohormone activating the defensive signalling pathway related to pathogen resistance (Johnson and Züst 2018), was actually less effective against the leaf curl disease in this instance (Awang et al. 2013). There is more inconsistency as well though, as demonstrated in another study by Bruce et al (2017). The JA treatment reduced the disease initially at 2 and 3 weeks after treatment, then lost effectiveness after 4 weeks, and again reduced disease after 5 weeks. A repeat was done of the experiment and no significant reduction in infection levels after 4 or 5 weeks were found (Bruce et al. 2017), demonstrating inconsistent resistance. Other experiments using MeJa and JA against herbivores such as Orrock et al. (2017) and Paudel et al. (2014) show consistency with repelling herbivores, and adding drought conditions apparently worsens the MeJa induced defences substantially as demonstrated in the experiments by Franzke and Reinhold's (2011) and Santamaria et al. (2018).

The larvae in this experiment were of different species in different instars which affects the larvae susceptibility to the defensive chemicals (Despland 2017), likely causing the non-significant effect on preference and performance. Plant defences are dependent on plant genetics and physiology and can be altered by the environmental context as well (Bruce et al. 2017), as herbivorous mite and grasshoppers were shown to perform better in plants exposed to drought, even with plant defences activated. This is consistent with the larvae fed warmed bilberry plants in this experiment, who also showed an increased RGR and effectively ignoring the MeJa induced defences. This combination of biotic and abiotic factors can potentially lead to large losses in crops exposed to these stressors simultaneously in a future with more extreme weather.

It is most likely much more effective to integrate elicitors in Integrated Pest Management (IPM) strategies and to have a range of options at hand, and not rely solely on one tactic. Induced resistance is

non-specific and typically protects plants against a range of different pests, though breeding could also potentially create specie specific defences and even reduce trade-off in plant defences (Bruce et al. 2017). The elicitors applied in the field should also make the herbivores more susceptible to pathogens, which are often absent in lab experiments.

If elicitors can be understood in order to give consistent results and is combined with biological control through natural enemies, they could act additively and synergistically in reducing pest levels if the net impact is positive when the plant defences do not impair the natural enemies (Pappas et al. 2017).

## 5.4 Improvements and Further Study

The larvae in this experiment were of different species in different instars and of unknown age which affects the larvae susceptibility to the defensive chemicals produced by the plant. Standardizing the larvae species, instar and age will reduce this uncertainty, for example through rearing larvae in the laboratory. The plants used in the experiments should be disturbed as little as possible to not activate any additional defensive processes in the control plants, as for example the cutting of the bilberry plants in this experiment may have contributed to the MeJa not showing a significant difference. Having the larvae consume a plant treatment diet for two or three additional weeks may also enhance the precision of the growth rate data. In addition, when measuring percentages of leaf eaten from each treatment, give less time than 24 hours for the larvae to eat as they can likely blur the result when consuming entire leaves, though this will depend on the age of the larvae and rate of consumption. Evidently, these experiments are small scale, and scaling them up are required for translating the research into field settings.

## 6.0 Conclusion

With a changing climate at hand, cascading effects on ecological processes affecting insect population dynamics and plant defoliation will occur with potentially economic consequences. MeJa activates the defensive pathway synthesizing chemicals within the plant for inhibiting and deterring herbivores, though little is known about how climate change affects this defensive process. The MeJa induced defences in this study did not affect the lepidoptera larvae in either preference or performance. In addition, the larvae grew faster from eating bilberry plants grown in experimentally warmed conditions regardless of induced plant defences. This indicates and emphasizes that the processes involved in host plants, herbivores and climate are not black and white but rather complex interactions between biotic and abiotic factors with several possible outcomes. Potential for elicitors as alternative pesticide show mixed results and adding elicitors to an IPM strategy is likely the most effective method in alternatively controlling pests. Many unique interactions between individual species, several trophic levels, together with top-down and bottom-up factors interacting individually with climate causes this subject to be relatively hard to replicate in lab studies and need to either be scaled up in size or frequency. Either way, plant defences need to be considered when predicting responses in insects and plants to climate change as this may become increasingly important for understanding ecological processes potentially affecting future food security and resource availability for both humans and wildlife.

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## 8.0 Appendix

### 8.1 Data: 52 larvae, 3 morphotypes: 1<sup>st</sup> choice, Leaf % percentage eaten, weight gain/loss.

June	-	July	2017								
Morphotype	Larvae ID	1st choice	OTC_W	OTC_M	A_W	A_M	Diet	Weight1	Weight2	Gain/Loss	
JUNE	A1	A_M	7.142857143	0	33.33333333	41.66666667	B4 am w	0.0308	0.0247	-0.0061	
Green	A2	OTC_W	60	25	12.5	20	A4 otc w	0.0738	0.2904	0.2166	
Big	A3	-	100	100	100	100	B2 am m	0.0257	x (F5)	-	
Larvae	A4	A_M	14.28571429	100	100	55.55555556	A1 am w	0.0801	0.0421	-0.038	
	B1	-	100	8.333333333	100	100	C2 am m	0.0122	0.041	0.0288	
	B2	OTC_M	90	91.66666667	92.85714286	87.5	A2 otc m	0.0727	0.2885	0.2158	
	B3	OTC_W	9.090909091	91.66666667	36.36363636	11.11111111	B3 otc m	0.036	0.1649	0.1289	
	B4	-	100	100	100	100	C1 otc m	0.0781	x (C1)	-	
	C1	OTC_M	10	50	45.45454545	0	A3 am m	0.0267	0.049	0.0223	
	C2	OTC_W	90	9.090909091	100	100	C4 am w	0.0208	0.1612	0.1404	
	C3	OTC_W	90	7.142857143	40	100	C3 otc w	0.0369	0.1834	0.1465	
	C4	-	100	100	100	100	D1 am w	0.1861	0.3136	0.1275	
	D3	A_W	57.14285714	27.27272727	60	15.38461538	B1 otc w	0.0832	0.1663	0.0831	
JULY	F1	A_W	0	100	100	100	-	-	-	-	
Green	F2	A_W	0	54.54545455	100	14.28571429	F3 am m	0.1795	0.3548	0.1753	
Big	F3	A_W	0	83.33333333	8.333333333	90.90909091	F1 am w	0.1444	0.3542	0.2098	
Larvae	F4	A_W	57.14285714	81.81818182	15.38461538	93.75	-	-	-	-	
	F5	A_W	0	0	36.36363636	92.30769231	-	-	-	-	
	F6	A_M	100	66.66666667	47.36842105	100	-	-	-	-	
JUNE	D1	OTC_M	16.66666667	90	100	100	F1 am w	0.0366	0.03	-0.0066	
Green	D2	OTC_M	100	90.90909091	100	100	D2 am m	0.0434	0.0273	-0.0161	
Small	D4	OTC_M	100	72.72727273	100	100	D3 otc m	0.0213	0.0547	0.0334	
Larvae	E1	A_W	100	90	40	100	D4 otc w	0.0363	0.0235	-0.0128	
	E2	A_W	100	100	44.44444444	100	E2 otc w	0.0476	0.034	-0.0136	
	E3	A_W	100	90.90909091	77.77777778	100	E1 am m	0.0498	0.029	-0.0208	
	E4	A_W	100	100	60	100	E3 am w	0.0213	0.0175	-0.0038	
	F1	A_M	100	100	100	5	E4 otc m	0.0461	0.0359	-0.0102	
JULY	D1	OTC_M	100	100	100	100	D4 otc w	0.083	-	-	
Green	D2	A_W	100	100	0	100	D3 otc m	0.0929	0.2134	0.1205	
Small	D3	-	0	0	0	0	E2 otc w	0.0665	-	-	
Larvae	D4	OTC_M	100	100	37.5	100	D1 am w	0.1217	-	-	
	E1	OTC_M	0	0	0	14.28571429	E4 otc m	0.062	-	-	
	E2	A_W	9.090909091	41.66666667	100	0	E3 am w	0.0443	0.04	-0.0043	
	E3	OTC_W	8.333333333	0	0	0	E1 am m	0.076	0.047	-0.029	
	E4	A_W	0	15.38461538	0	0	D2 am m	0.0515	-	-	
JUNE	F2	A_W	0	28.57142857	33.33333333	92.30769231	F3 am m	0.019	0.0481	0.0291	
Stick	F3	-	16.66666667	8.333333333	9.090909091	16.66666667	F4 otc m	0.0125	0.0638	0.0513	
Pretender	F4	-	100	16.66666667	91.66666667	9.090909091	F2 otc w	0.031	0.0515	0.0205	
Larvae	F5	A_W	42.85714286	18.18181818	84.61538462	6.25	B2 am m	0.0516	0.0173	-0.0343	
	F6	A_W	100	100	63.63636364	7.692307692	C1 otc m	0.0108	0.0217	0.0109	
	C1	OTC_M	41.66666667	50	0	14.28571429	C3 otc w	0.0742	0.0898	0.0156	
	C2	OTC_M	50	65	0	0	A1 am w	0.1292	0.0867	-0.0425	
	C3	OTC_W	40	35	18.18181818	28.57142857	B3 otc m	0.061	0.0485	-0.0125	
	C4	OTC_M	10	46.66666667	0	0	B1 otc w	0.1155	-	-	
JULY	A1	A_W	0	33.33333333	52.63157895	0	A3 am m	0.0483	0.0425	-0.0058	
Stick	A2	A_W	43.75	22.22222222	21.42857143	18.18181818	C4 am w	0.099	0.07	-0.029	
Pretender	A3	A_W	43.75	0	8.333333333	50	A2 otc m	0.0962	0.0735	-0.0227	
Larvae	A4	A_M	50	57.14285714	0	8.333333333	C1 otc m	0.0437	-	-	
	B1	OTC_W	0	0	91.66666667	72.72727273	C2 am m	0.0346	0.0371	0.0025	
	B2	-	0	0	0	0	B4 am w	0.0609	0.0631	0.0022	
	B3	OTC_W	44.44444444	20	25	0	B2 am m	0.0171	0.0456	0.0285	
	B4	A_M	0	0	30	90	A4 otc w	0.061	0.0686	0.0076	