

EFFECTS OF TEMPERATURE ON PEA APHIDS, THEIR HOST PLANTS, AND THEIR  
PARASITOIDS

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Title

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**DOCTOR OF PHILOSOPHY**

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## ABSTRACT

Temperature has the potential to alter every aspect of an organism's biology. This is especially true when we focus on small ectotherms such as insects. Understanding the effects of temperature on insects is particularly important given that climate change scenarios predict changes in temperature across the globe.

In Chapter 1 we explored the effects of heat shocks on a discrete host-parasitoid interaction, specifically asking what happens if the heat shock happened before, during or after the interaction. We found that heat shocks had a stronger negative effect when they occurred while the wasp was actively foraging. In a follow-up behavioral experiment, we observed that this result is likely caused by the heat shock quickly rendering the majority of wasps inactive.

In Chapter 2 we tested how variation in temperature affects pea aphid population size and how the effect changes with average temperature. We compared the population size of pea aphids under constant and fluctuating temperature profiles across a cool temperature range (20C and 16C/24C) and a warm temperature range (28C and 24C/32C). We saw that in the cooler range, pea aphids in the constant and fluctuating temperature treatments had the same population size. However, the same was not true for the warmer temperatures. In that case, fluctuating temperature profiles produced smaller populations compared to the constant temperatures.

In Chapter 3 we focused on the possible indirect effects of temperature on pea aphids mediated by the aphids' host plants. We performed five experiments where we manipulated the exposure temperature (16C, 24C, and 32C) for plants and aphids.

While temperature had strong direct effects on aphids and also affected plant size, temperature had little to no indirect effects on pea aphid fecundity.

While the idea of temperature change can seem straightforward, temperature effects on insects are not straightforward. Timing and variation of temperature change are important. Indirect effects though direct effects on hosts also are important. My work shows a number of approaches for investigating these different temperature effects to better understand what might happen to insects when climate changes.

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## **DEDICATION**

To my lovely wife, without whom I would not had the strength to finish this thesis.

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## INTRODUCTION

This dissertation focuses on the effects that temperature can have on insect herbivores, their host plants, and their parasitoids. The first chapter studies effects of timing of sudden large increases in temperature, known as heat shocks, on the relationship between parasitoid wasps (*Aphidius ervi*) and pea aphids (*Acyrtosiphon pisum*). The second chapter focuses on the effect of temperature variation on pea aphid population size at different temperature ranges. The third chapter addresses the indirect effects that temperature has on pea aphid fecundity that are mediated by direct effects on host plants (*Vicia faba*).

In the first chapter, we quantified the impact of an abiotic stress, a heat shock, on a behavioral interaction, a parasitoid wasp stinging a pea aphid. The effect of heat shocks on host-parasitoid interactions has been extensively studied, mostly at the population level (Bannerman et al., 2011; Cayetano et al., 2013; Hance et al., 2007; Harmon et al., 2009; Sentis et al., 2013, 2017). However, neither the heat shock nor host-parasitoid interaction are constant through time. They instead are discrete events, so the relative timing of heat shock and the host-parasitoid interaction may become important. Since the frequency and intensity of heat shocks is expected to increase under climate change scenarios (Field et al., 2012; Seneviratne et al., 2012), it is crucial to understand how heat shocks can alter species interactions. Using a before-during-after approach, we tested if the heat shock happened before the interaction, during the interaction, and after the interaction. We used pea aphids and the parasitoid wasp *Aphidius ervi*. Since we saw a strong effect of the heat shock while the interaction was

occurring, we carried out a follow up behavioral experiment to understand what behaviors were driving the change in the outcome of the interaction.

In the second chapter, we explored how the effects that changes in the daily average temperature and daily temperature variation influence population growth in pea aphids. Both are expected to increase under future climate change scenarios (Easterling et al., 2000; IPCC, 2007, 2012; Jian-Bin et al., 2017; Oreskes, 2018; Rahmstorf and Coumou, 2011; Rummukainen, 2012). There is a large body of knowledge on the effects of increases in average temperature on terrestrial organisms (Frazier et al., 2006). Less is known about the effects of increases in temperature variation (Vázquez et al., 2017). Since both are predicted to change in the future, it is crucial to understand how they interact with each other. To study this, we compared small pea aphid populations under constant and fluctuating temperatures, and we repeated the comparison over two different ranges of temperature. By using changes in population size as our response variable we were able to incorporate multiple demographic processes such as development time, fecundity, survival, etc., each of which can be influenced by temperature variation.

In the third chapter, we made an attempt to differentiate between the direct effects of temperature on insect herbivores and the indirect effects of temperature on insect herbivores that occur through direct effects on host plants.. When we study insect herbivores, such as pea aphids, it comes at a high physiological cost to separate the pea aphid from its host plant, especially if they are separated for more than a short time period (Kopco, 2017; Nelson, 2007). Since temperature can affect plants (Rowland and Gusta, 1977; Thompson, 1974; Thompson et al., 1977; Veteli et al., 2002), when we do



temperature experiments with pea aphids we normally cannot differentiate the possible direct effects of temperature on the aphids from the possible indirect effects through the host plants. To focus on the plant-mediated indirect effects of temperature on pea aphids we performed five complimentary, manipulative, controlled experiments in the laboratory where we manipulated at which temperature the plants and the aphids feeding on them were exposed to. Results suggest that plant-mediated indirect effects of temperature are relatively minor, especially when compared to the strong direct effects of temperature on pea aphids.

### **Study organisms**

#### **Pea aphids**

Aphids (Family: Aphididae) are one of the most important agricultural insect pests in the world (Dixon, 1998; van Emden and Harrington, 2017). Pea aphids (*Acyrtosiphon pisum*) in particular are also used as biological models for ecological, developmental and evolutionary studies, such as insect-plant interactions, symbiosis, or virus vectoring (Brisson and Stern, 2006). Pea aphids reproduce asexually during spring and summer seasons (Kenten, 1955). During this time, female pea aphids produce parthenogenetic clones. When autumn begins (shorter days and lower temperatures) pea aphids switch to sexual reproduction producing sexual male and female offspring. The number of offspring produced by pea aphids is greatly affected by several factors, such as environment temperature, aphid age, and host plant quality (Lamb, 1961; Morgan et al., 2001). For this reason, aphid offspring production, is a useful and common metric to measure impacts of temperature (Bieri et al., 1983; Murdie, 2009; Siddiqui et al., 1973). Pea aphids can feed on several legume plants (Family:

Fabaceae), however broad beans (*Vicia fava*) can act as their universal host. The aphids used for this thesis are always reared using broad fava beans. If undisturbed, pea aphids feed constantly and are normally found on the lower sides of leaves, buds and pods, ingesting phloem sap through its sucking mouth parts.

### **Microbial symbionts**

All aphids harbor an obligate (primary) symbiont (*Buchnera aphidicola*) which allows them to properly digest plant sap (Wilson et al., 2010). But some species of aphids can also harbor a diversity of facultative (secondary) symbionts, that can have an array of effects (Oliver et al., 2010). In the case of pea aphids, some of these effects include altering the temperature effects or parasitoid resistance. Since the experiments in this thesis tested various effects of temperature, we made sure that the aphids that we used did not harbor any facultative symbionts.

### **Parasitoid wasps**

Aphids are usual prey for a number of natural enemies such as ladybugs, wasps, or pirate bugs (van Emden and Harrington, 2017). Common aphid natural enemies are some wasps of the Braconidae family. These wasps can be aphid parasitoids (insects that complete their larval development inside a single aphid, killing it in the process). All known parasitoids of aphids are solitary, meaning that they just oviposit one egg inside one aphid. After that, the wasp larva develops inside the aphid feeding in its body. After a week (although this is a very temperature dependent process) the aphid dies and the wasp larva becomes a pupa. After 2 or 3 more days, a fully formed adult wasp emerges and starts the cycle again. *Aphidius ervi* wasps are a common natural enemy for pea aphids. Pea aphids and these parasitoid wasps are used extensively to study

temperature effects on insect biology and ecology (Jefferies and Lewis, 2013; Le Ralec et al., 2010; Meisner et al., 2007, 2014). The wasps that we used were always naive wasps, meaning that they never encountered an aphid of any kind before being used for the experiments. This is because *Aphidius ervi* wasps can show changes in aphid preference depending on aphid instar, aphid color, and wasp age (Langley et al., 2006; Lin and Ives, 2003).

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into the amino acid relations of the pea aphid, *Acyrtosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect Mol. Biol.* 19, 249–258.



# THE ROLE OF TIMING IN THE HEAT SHOCK EFFECTS ON A HOST-PARASITOID INTERACTION

## Abstract

How abiotic factors affect individual species is now a well-studied topic, but their effects on interacting species are harder to determine. In host-parasitoid systems, for example, an altered abiotic factor may affect both species directly, while also affecting their interactions. Moreover, if the abiotic effects and species interactions are not constant through time, but instead have discrete events, the timing of abiotic effects and species interactions may become important. One such discrete abiotic effect associated with climate change is the increase of heat shocks (short-term large increases in temperature). This study investigates the role of timing in relation to how those heat shocks affect a host-parasitoid system. We tested how the timing of a heat shock (increase temperature from 22C to 38C for 4 hours) affects successful attack and reproduction of a parasitoid wasp (*Aphidius ervi*) attacking its host, the pea aphid (*Acyrtosiphon pisum*). We tested three treatments: 1) heat shock before wasp attacks the hosts, 2) heat shock while the wasp is foraging, and 3) heat shock after the wasp has attacked hosts. Our response variable was wasp mummy production. Our results showed that heat shock had the largest effect when it occurred while wasps were actively foraging, with very few mummies produced in these conditions. Follow-up behavioral tests indicate that the cause of this was cessation of wasp behavior during heat shocks. When heat shocks were applied 3 days before or after the foraging, mummy production was only slightly lower than the control treatments where everything was kept at a constant temperature. These results show the potential importance of

timing when considering the effects of an altered abiotic factor, especially when considering relatively discrete events.

## Introduction

Timing is crucial for understanding effects of climate change, especially changing temperature. For example, many studies demonstrate how changing temperatures can alter the timing of when species are active, potentially causing phenological mismatches between interacting species (DeLucia et al., 2012; Yang and Rudolf, 2010). However, discrete abiotic events such as flash floods, cold snaps, or heat shocks can also critically affect species and their interactions (Jentsch et al., 2007). Given their discrete nature, such events might have different effects when they occur at different times. If so, their timing could ultimately be important to the point of determining if there is a large effect or no effect at all.

Heat shocks are discrete events that can alter species interactions (Bannerman et al., 2011; Cayetano et al., 2013; Harmon et al., 2009; Le Lann et al., 2014; Schreven et al., 2017; Sentis et al., 2013, 2017). Heat shocks are short periods of high temperature, often lasting just a few hours. The frequency and intensity of heat shocks is expected to increase under climate change scenarios (Field et al., 2012; Seneviratne et al., 2012). Aphids, including the pea aphid *Acyrtosiphon pisum*, have been used to study the effects of heat shocks. Heat shocks can substantially alter both aphids (Will et al., 2017) and their interactions with natural enemies (Bensadia et al., 2006; Harmon et al., 2009). Heat shocks can also negatively affect aphid microbial mutualistic symbionts (Heyworth et al., 2016), which can have a negative effect on aphid biology (Oliver et al., 2003, 2010).

Previous research has found that aphid-parasitoid interactions can also be influenced by heat shocks (Bensadia et al., 2006). Like heat shocks, interactions between aphids and parasitoid wasps are fairly discrete in that there is an important point when the wasp attacks the aphid and lays an egg inside its host. Given that both the attack and heat shocks are relatively discrete events, the timing between the two could play a large role in how heat shocks alter the interaction. There is considerable research on the effects a heat shock can cause while the parasitoid is attacking (Ismaeil et al., 2013; Roux et al., 2010), and some after the attack has happened, when the wasp is in larval or pupal form (Chihrane et al., 1993; Jerbi-Elayed et al., 2015). These results suggest that the relative timing of heat shock and parasitoid attack might be important. However, there is still a need for a direct comparison of heat shock effects at different stages of the host-parasitoid interaction.

When considering the timing of heat shock and parasitism, we can differentiate when the heat shock occurs in relation to the parasitoid attacking and stinging the host. This helps us establish three clear phases of when the heat shock occurs: before the stinging, while the stinging is occurring, and after the stinging.

When a heat shock happens before the stinging, the environmental temperature may alter how suitable an aphid is as a host. For example, a heat shock has the potential to alter the aphid so that its fitness as a host and resistance to the parasitoid changes (Bensadia et al., 2006; Cayetano et al., 2013). Besides potentially altering the performance of parasitoids in these hosts, such changes in host fitness may alter whether the parasitoid chooses to sting that aphid (Colinet et al., 2005; Ma et al., 2015).

When stinging happens while a heat shock is underway, the behavior of both aphid and parasitoid may change. Changes in insect behavior due to heat shocks may lead to significant changes in trophic interactions (Schmitz et al., 1997). In this case such changes could come about through changes in parasitoid foraging behavior. Previous examples have shown that temperature can alter parasitoid fitness and fecundity, although behavior was not observed in these studies (Ismaeil et al., 2013; Jerbi-Elayed et al., 2015; Roux et al., 2010). Moreover, temperature might affect the aphid and its behavior, including changes in defense behavior (Ma and Ma, 2012; Sentis et al., 2017).

When the heat shock occurs after the stinging happened, it could directly affect the host and the developing parasitoid inside the host. At this stage, the parasitoid progeny cannot change its behavior to escape the stressful environmental conditions, thus a heat shock could more easily alter parasitoid development (Chihrane et al., 1993; Jerbi-Elayed et al., 2015; Wang et al., 2014). In addition, some hosts may be more vulnerable to extreme abiotic conditions when they are parasitized (Hoang, 2001), which could mean higher mortality for both host and the parasitoid developing inside it. This effect can occur even if the heat shock just shortens the lifespan of the host such that the parasitoid cannot complete its development (Ballman et al., 2012).

Given these different potential ways heat shocks may influence aphids, we predict that timing will alter the ultimate effect of heat shocks on the host-parasitoid relationship. To test this prediction, we tested the effect of a heat shock on a host-parasitoid system in three different scenarios, with heat shock applied before, during, or after a host-parasitoid interaction.

## Methods

We tested the effect of heat shock timing on a host-parasitoid interaction using three separate experiments where we exposed aphids and sometimes parasitoid wasps to a heat shock. We exposed just aphids when shocking before host-parasitoid interactions, both aphids and adult wasps when shocking during the host-parasitoid interaction, and aphids and developing wasps when shocking after the interaction. As a follow up experiment, we also tested how a heat shock alters the behavior of aphids and parasitoid wasps.

### Pea aphids

Pea aphids (*Acyrtosiphon pisum*) are hemimetabolous (incomplete metamorphosis) insects. They go through 4 nymph stages (instars) before becoming adults. The instars resemble the adults in shape and form. Parasitoid wasps have different preferences for different instars (Hawthorne and Via, 2001; He et al., 2011; Henry et al., 2005; Lin and Ives, 2003), so to control for wasp preference in our experiments we used same-age aphids.

Pea aphids are a good model organism to study trophic interactions and temperature effects. They are very convenient to rear and work in laboratory conditions, and have a diverse set of natural enemies that are also easily reared on laboratory conditions, such as parasitoid wasps or coccinellid beetles. Their biology is well known (International Aphid Genomics Consortium, 2010), and are an important pest in some legume crops (van Emden and Harrington, 2007).

Pea aphids and parasitoid wasps are used extensively to study temperature effects on insect biology and ecology (Jeffs and Lewis, 2013; Meisner et al., 2014; Le

Ralec et al., 2010). The effects of extreme temperatures on aphids and their parasitoids are also well known at a population level, however there is little known about the mechanisms that temperature may influence (Bannerman et al., 2011; Gillespie et al., 2012; Ismaeil et al., 2013; Jerbi-Elayed et al., 2015; Roux et al., 2010).

### **Parasitoid wasps**

Given that the experiments we present are about timing, it is useful to consider the timing of the wasp life cycle. This wasp species, *Aphidius ervi*, is a solitary endoparasitoid of aphids, meaning the wasp oviposits a single egg inside its host, in this case, the aphid. The egg hatches and the larva develops inside the still-living aphid before eventually killing the host and forming a mummy containing the developing pupa. This process normally takes seven to nine days. Two or three days after the mummy formation, an adult wasp emerges and starts mating. Female wasps will then look for more aphids to oviposit.

Female wasps can show changes in aphid preference depending on aphid instar, aphid color, and wasp age. Females will attack all pea aphid instars, although they have highest attack rates on second and third pea aphid instars (Ives et al., 1999). For all the experiments, we used mated, naïve wasps to avoid any potential bias that wasps may have (Colinet et al., 2005; Langley et al., 2006; Lin and Ives, 2003). This means that the wasps had never encountered an aphid as an adult.

### **Insect rearing**

Pea aphids were reared using common methodologies developed in previous experiments (Kopco, 2017; Kraft et al., 2017). Pea aphids used for the experiments were reared in a laboratory colony in the Department of Entomology, North Dakota

State University (Fargo, ND), and were originally collected from several alfalfa (*Medicago sativa*) fields near campus. The colony was created in 2013 and every summer pea aphids from fields in the same area were added to the colony. Aphids were maintained on fava bean (*Vicia faba*) plants of the Broad Windsor variety (Territorial Seeds, Oregon). Pea aphids collected from alfalfa readily establish on fava bean in the laboratory and provide a good host plant for experimental work on pea aphids (Meisner et al., 2014). Aphid colonies were reared in mesh cages (collapsible cube mesh cages. Size: 40cm x 40cm x 40cm. The cages had a tray to hold water, and a clear plastic top to allow for proper lighting.), at  $22 \pm 2C$ , 60–80 % RH under a L16:D8 photoperiod produced with fluorescent growth lights (F14W/T5/865/ECO 14 Watt 6500K Fluorescent Tube made by GE Boston, MA). New plastic pots (10.2 × 10.2 cm, Tessman Seed Co, St. Paul, MN), with 2 or 3 fresh fava bean plants (around 10 cm and a week old) were introduced weekly to the colony, while removing heavily infested plants. The soil used for the colonies was a commercial sphagnum peat moss-based horticultural mix appropriate to grow fava bean plants. The mix included perlite, dolomitic limestone, added nutrients, and a wetting agent (Sunshine Mix LC1, Sun Gro Horticulture, Vancouver, BC).

Adult pea aphids reproduce rapidly and continually, so to obtain a group of young aphids with the same age for experiments we transferred a group of adult aphids to clean uninfested fava bean plants and let them produce newborn aphids for 24 hours. After that we removed all adult aphids and we were left with a group of aphids with the same age (24 hours of difference between youngest and oldest aphids). Parasitism success is also influenced by wasp age, so in the same manner as with the aphids, we

used wasps with the same age (24 hours of difference between youngest and oldest wasps), following the same method that we used for the aphids.

Parasitoid wasps were collected from the same fields and were reared with some pea aphids as above. The only difference is that when older plants needed to be removed, they were cut and kept inside the cage so parasitized aphids could complete development and newly emerged parasitoids could infest aphids on newer plants inside the cage. When we detected low aphid numbers, more aphids were added from the pea aphid colony.

### **Heat shock**

The heat shock consisted of a rapid increase in temperature from laboratory conditions ( $22 \pm 2\text{C}$ ) to  $38\text{ C}$  (Appendix 1), similar to what other have done (Kopco, 2017). The rate of increase of temperature was  $0.5\text{C}/\text{min}$ . Other studies used longer, albeit milder, heat shocks (Jerbi-Elayed et al., 2015), however the temperatures that we used are similar to previous work on pea aphids, parasitoid wasps, and other insects (Bannerman et al., 2011; Cayetano et al., 2013; Harmon et al., 2009; Ma et al., 2015; Martinet et al., 2015; Montllor et al., 2002; Sentis et al., 2017; Wang et al., 2014). Temperature measurements were made using HOBO Pendant Temperature Data Loggers (Onset, Massachusetts) to confirm that induced heat shocks performed as expected. Light conditions were kept the same as the laboratory conditions during the heat shock. The heat shock always occurred in the afternoon (between 12:00 and 16:00), corresponding to the typical time of maximum temperature during summer days. In the population experiments (shock before, during, and after interaction) the



temperature was maintained at 38C for 4 hours (Appendix 1), and in the Behavior experiment temperature was maintained at 38C for one hour (Appendix 1).

### **Shock before interaction experiment**

In the first of the population experiments we exposed aphids to a heat shock before those aphids were exposed to a wasp. This was done to understand the effect that a heat shock could have on the host-parasitoid interactions if the heat shock happens before the interaction.

First, we placed 20 1<sup>st</sup> instar (approximately 1 day old) pea aphids on single fava bean plant enclosed inside a tube cage (Cylindrical tube cage. Height: 26cm, diameter: 12cm. Made of transparent plastic, the cage had two mesh windows on the side and one on the top to allow for air circulation). The plants, pots and soil used were the same as the ones used in the colonies, however just one plant per pot was used in all the experiments (sample unit). We randomly assigned half of the plants to the control treatment, and the other half to the heat shock treatment. After 24 hours to allow the aphids to settle, the plants and aphids in the heat shock treatment were exposed to a heat shock as described before. The plants on the control treatment undergo the exact same conditions as the heat shock plants, with the exception of being exposed to a heat shock. We counted the number of aphids present in the plant right before the heat shock and right after, to detect any kind of heat shock induced mortality. We also counted the number of aphids in the control plants.

Three days after the heat shock we counted aphids on all plants and introduced a naïve female mated wasp in to each cage. We used 3 days as our timing so that we could have the same amount of time between heat shock and stinging (when heat

shock was before stinging) as it was between stinging and heat shock (when heat shock was after stinging) (Figure 1). We did that so that our aphids were still juveniles during both heat shocks and wasp exposure. Previous work has shown that pea aphid-heat shock interactions can vary by life stage (Harmon et al., 2009) and that wasps prefer to encounter 3<sup>rd</sup> and 4<sup>th</sup> instars (Ives et al., 1999; Lin and Ives, 2003).

When foraging, the wasp could access the whole plant and all aphids. After 4 hours (12:00-16:00), we removed the wasp from the cage but left the aphids to measure parasitism. We then waited seven to nine days until wasp mummies formed from stung aphids. We measured absolute wasp offspring (absolute mummy production) and relative wasp offspring (number of mummies divided by the aphids present before the wasp was introduced in the cage) for each plant and compared relative mummy production from cages that had been exposed to a heat shock earlier to those that had been kept in the control. For all three experiments we report relative mummy production, but the outcome is the same if we use absolute mummy production. The total sample size for this experiment was 66 (control = 33, heat shock = 33) over two temporal blocks.

### **Shock during interaction experiment**

In the second experiment, we exposed aphids and adult parasitoids to a heat shock while the wasp was foraging for aphids. This was done to understand the effect that a heat shock can have on host-parasitoid interactions if the heat shock happens while the interaction occurs.

Our goal was to keep the timeline and methodology of this experiment identical to the “shock before interaction” experiment, with the only exception being when the

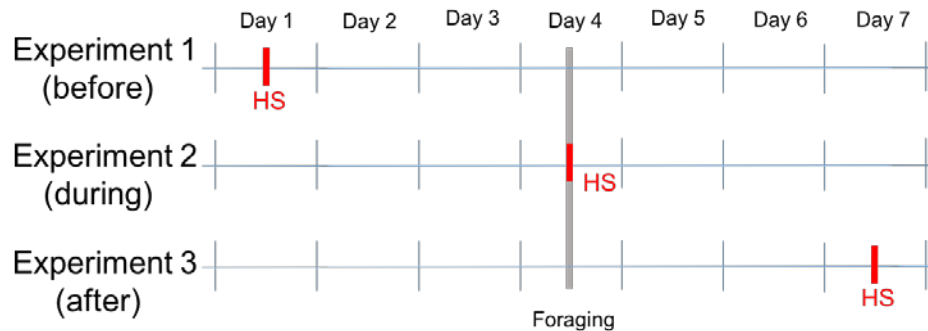
heat shock occurred. To do so, we placed 20 1<sup>st</sup> instar pea aphids on single fava bean plants (on Day 0, Figure 1) and then waited for 4 days before introducing individual wasps to each cage and performing a heat shock. Therefore, in this experiment, the heat shock was three days later than in the first experiment so that it coincided with the wasps foraging for aphids. As before, half of the cages were randomly placed in the heat shock treatment and half were kept as controls at the ambient temperature. The total sample size for this experiment was 73 (control = 34, heat shock = 39) across two temporal blocks. As before, we counted the number of aphids present in the plant before and after the heat shock, which in this case was the same as the number of aphids present before we introduced the wasp in to the cage. We again compared mummy production from cages exposed to a heat shock and cages kept in the control treatment.

### **Shock after interaction experiment**

In the third population experiment, we exposed aphids to a heat shock three days after the wasp had foraged on the aphids. This was done to understand the effect of a heat shock after the host-parasitoid interaction has occurred.

The timeline and methodology of this experiment is the same as “shock before interaction” and “shock during interaction” experiments, however the heat shock occurred three days after we introduced the wasp to the cage (Figure 1). This means the heat shock occurred seven days after we placed 20 1<sup>st</sup> instar pea aphids on single fava bean plants. All other timing between wasp and aphids were again the same, except that the later heat shock meant that at least some aphids had a developing wasp inside them at the time of the heat shock. As before, half the cages were exposed to

heat shock and half were kept as control. We again counted the number of aphids present in the plant before we introduced the wasp to the cage, and before and after the heat shock. We compared mummy production from cages exposed to a heat shock and cages kept in the control treatment. The total sample size for this experiment was 79 (control = 40, heat shock = 39) divided in two temporal blocks.



**Figure 1.** Diagram of the temporal line of the three heat shock treatments (Before: heat shock 3 days before the foraging event. During: heat shock at the same time as the foraging event. After: heat shock 3 days after the foraging event).

### Behavior experiment

Given the results of the second experiment, we performed a follow-up experiment to quantify potential behavior differences in aphids and wasps when in control and heat shock treatments. The results suggest that the most influential time for the heat shock was when it happened while the wasp was actively foraging for aphids (shock during interaction experiment; see results). Therefore, in this behavioral experiment, we exposed aphids and wasps together to a heat shock, similar to the shock during interaction experiment. However, in this case the aphids and wasps were inside experimental arenas made from a clear cup (Plastic deli cup. Height: 12cm, diameter: 12cm. Made of clear plastic. The lid had a mesh window to allow for air circulation) so we could record their behaviors.

The behavioral experiment consisted of comparing behavioral observations of wasps and pea aphids under control and heat shock conditions. A single mated naive wasp was introduced in a clear cup with 5 4<sup>th</sup> instar pea aphids and a single fresh fava bean leaf. Half of the deli cups were randomly assigned to the control treatment and the other half to the heat shock treatment. We exposed the heat shock cups to a heat shock similar to the “shock during interaction” experiment, but which only lasted one hour.

In our preliminary behavior observations, we observed the following wasp behavior categories: flying, under-leaf, still, walking, stumbling, foraging, stinging, and inactive. We defined the inactive behavior category as wasps that showed no activity at all and presented an unnatural resting position. Before becoming inactive, some wasps showed a stumbling behavior, which was a difficulty to walk or move, and an inability to fly. When wasps showed a complete lack of movement of their legs and antennas and wings not in their natural resting position we defined them as inactive. Inactive wasps usually were laying on their backs or on their sides. In our preliminary behavior observations, we also recorded that a one hour heat shock rendered 90% of wasps inactive, so we decided to use one hour shocks instead of four hours as the previous experiments. In the experiment, we recorded what behavioral category wasps and aphids were exhibiting every two minutes for one hour (30 recordings). A description of the other behavioral categories can be found in Appendix 2. The sample size of this experiment was 56 (control = 23, heat shock = 23) divided in four temporal blocks.

### **Statistical analysis**

To test the hypothesis on Shock before, during, and after interaction experiments we performed ANOVA analyses comparing control and heat shock treatments using R

version 3.4.3 (R Core Team, 2017). Since the experiments were performed over temporal blocks, we included a temporal block effect. In the behavioral experiment, we used Chi Squared and T student tests to test the differences in activity between control and heat shock treatments for wasps and for aphids. We used the ggplot2, dplyr, reshape2, and googlesheets packages to manipulate data and build graphs (Auguie, 2017; Bryan and Zhao, 2017; Cheng et al., 2017; Wickham, 2007, 2009; Wickham et al., 2017).

## Results

Our goal was to look at how different timing scenarios between a heat shock and a host-parasitoid interaction can affect the outcome of that interaction. We performed three different and separate experiments, and although we can't directly compare the data because each experiment was performed separately, our using of the same methodology will allow us to look qualitatively across experiments to better understand how the timing of a heat shock may affect a host-parasitoid interaction. That is why we report shock before, during, and after interaction together in a single section.

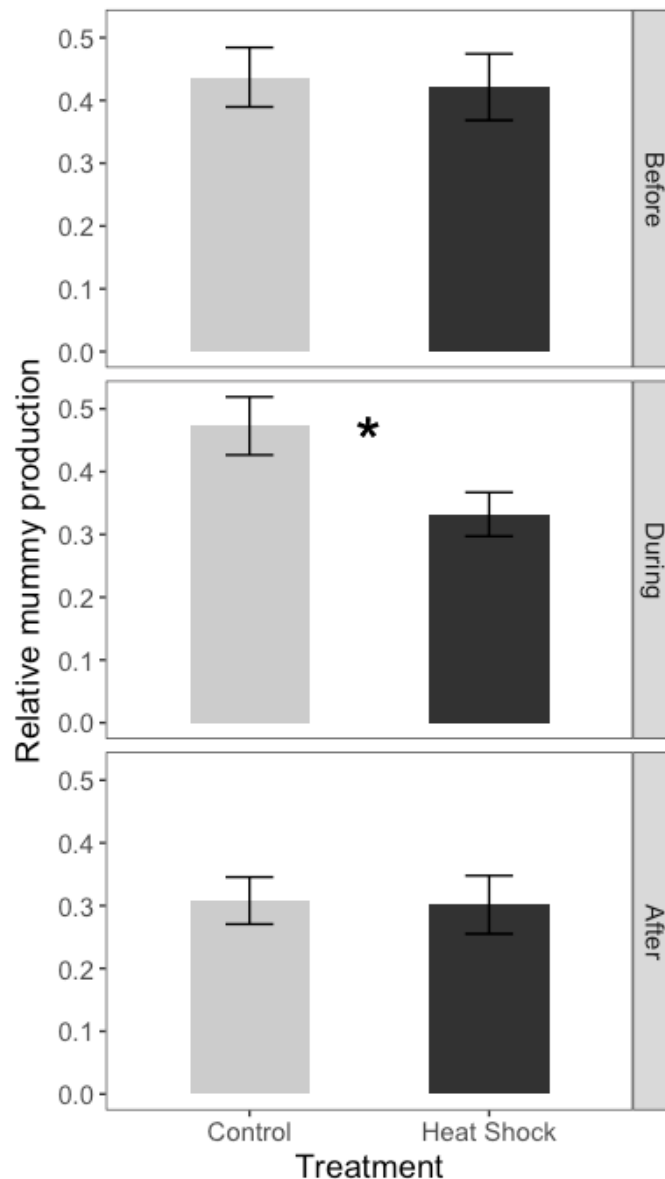
### Heat shock mortality

Since we wanted a strong heat shock but not strong enough to simply kill the pea aphids, we needed to assess heat shock-induced mortality. To do that we counted the aphids present on the plant immediately before and immediately after the heat shock. The number of aphids on plants before the heat shock was not different from the number after the heat shock for any of the three experiments (ANOVA before  $F(1,63)=0.012$ ,  $p=0.93$ ; ANOVA during  $F(1,70)=1.128$ ,  $p=0.29$ ; ANOVA after  $F(1,68)=0.225$ ,  $p=0.63$ ). In the first experiment (before), we also assessed the possible

long-term effects of pea aphid mortality due to heat shock by comparing the number of aphids present in the plant before the heat shock and the number of aphids present in the plant before the stinging 3 days later. Yet again, there was no difference between control and heat shock treatments ( $F(1,63)=0.024$ ,  $p=0.87$ ).

### **Shock before, during, and after interaction**

The only treatment where we found a heat shock effect on mummy production was when the heat shock occurred while the wasps were foraging. Relative mummy production was not different between the control and heat shock treatments when the heat shock occurred before the wasp foraged ( $F(1,64)=0.6$ ,  $p=0.44$ ) or when the heat shock occurred after the wasp foraged ( $F(1,70)=0.013$ ,  $p=0.91$ ) (Figure 2). However, the control and heat shock treatments were different when the heat shock occurred while the wasp was foraging ( $F(1,76)=8.985$ ,  $p=0.0038$ ) (Figure 2). Wasps had a reduced relative mummy production (effect size=29.7%) when exposed to the heat shock treatment while it was foraging.



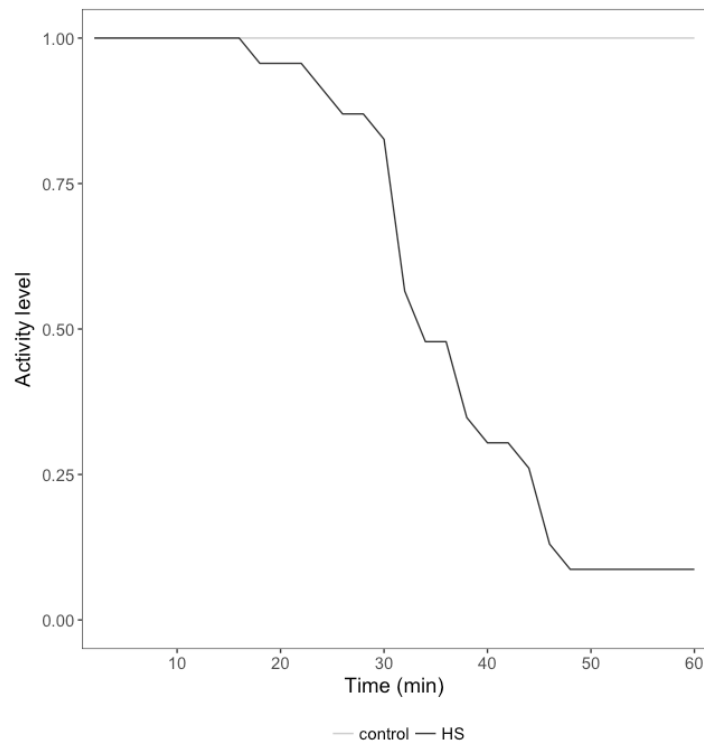
**Figure 2.** Wasp mummy production divided by the number of adult pea aphids present in the plant before introducing the wasp (average  $\pm$  standard error). The wasps in the shock during interaction (\*) treatment experienced a reduced production of mummies when exposed to the heat shock treatment while foraging. In the experiments shock before and after, wasp mummy production in control and heat shock treatments was not significantly different.

### Behavioral experiment

Wasp behavior differed under control and heat shock treatments. In the heat shock treatment 21 out of 23 wasps became inactive by the end of the experiment, but

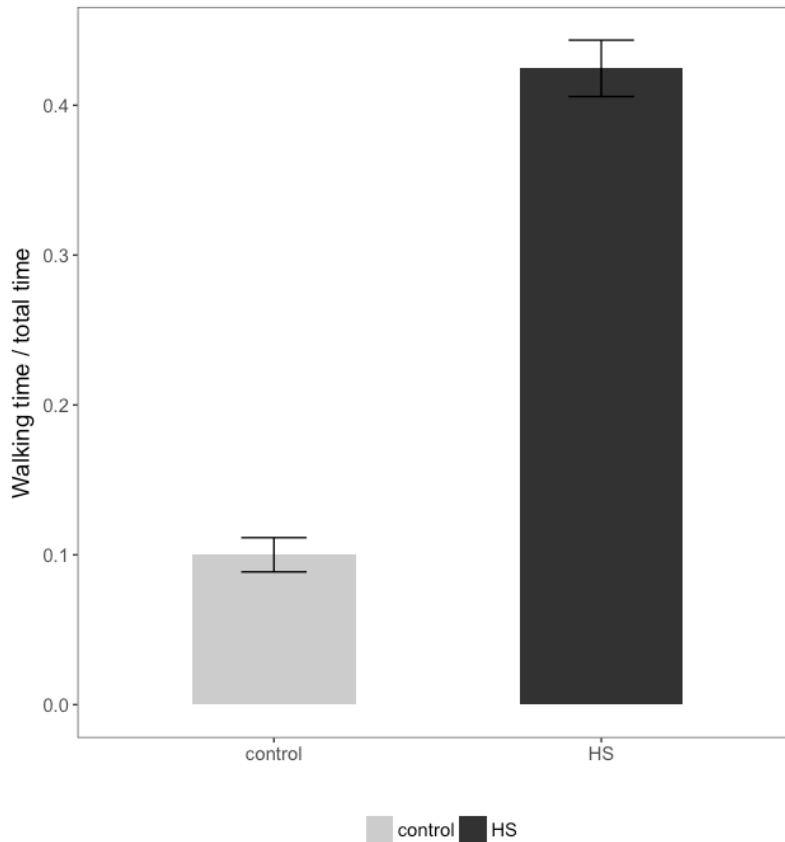


no wasps became inactive in the control treatment (Pearson's Chi-squared: 35.048,  $df=1$ ,  $p<0.001$ ). Average latency to cessation of movement was  $37.3 \pm 2.1$  minutes (Figure 3).



**Figure 3.** Average proportion of inactive wasps under heat shock conditions and control conditions in the behavioral follow up experiment. Activity level was calculated as the average number of active wasps compared to inactive in the duration of the experiment (1hour).

The amount of time that pea aphids spent walking under control and heat shock treatments was also different. Aphids spent four times more time walking under heat shock conditions compared to control ( $t(35.49) = -6.5449$ ,  $p < 0.0001$ ) (Figure 4).



**Figure 4.** Amount of time (average  $\pm$  standard error) that pea aphids spent walking under control and heat shock conditions in the behavioral follow up experiment. Aphids were divided in two categories: walking and not walking.

## Discussion

We predicted that timing would alter the effect of a heat shock on a host-parasitoid relationship since heat shocks can influence the relationship in different ways depending if it occurs before, during, or after a host-parasitoid interaction. Overall, when the heat shock occurred while the wasp was foraging on aphids, mummy production was lower in the heat shock treatment compared to the control. On the other hand, when the heat shock occurred before or after wasp foraging it did not alter wasp mummy production in our experiments. Follow up behavioral observations suggest that

reduced mummy production could be related to movement of aphids or the enormous increase in inactive wasps during heat shocks.

The heat shock had no effect on wasp mummy production when it happened before the host-parasitoid interaction. As far as we know, no one has previously studied if heat shocks can influence aphids in a way that alters parasitoid host preference. However, several papers have shown how heat shocks can influence pea aphids alone. Strong heat shocks like we performed in this study are known to cause changes in aphid survival, reproductive output, and resistance to parasitoids (Bensadia et al., 2006; Dunbar et al., 2007; Trotta et al., 2018). Another side effect of heat shocks is the change of the mutualistic facultative symbiont pool that insects can harbor (Cayetano et al., 2013; Heyworth et al., 2016), causing an array of effects, depending on the symbiont affected (Montllor et al., 2002; Oliver et al., 2003, 2010). Despite these potential changes also happening to the experimental aphids, we did not see any ultimate effect on wasp mummy production.

One factor that could explain this result could be that wasps were exposed to a limited number of aphids. Even if a wasp was less likely to sting previously shocked aphids because they are perceived as a lower quality host, it is possible that in our experiment, this difference simply altered how long it took for the wasp to be willing to accept the shocked aphid. Given the time frame, the lack of choice in hosts, and enclosed conditions of our experiment, it is possible we could not detect any differences in wasp preference.

There are other factors that could also alter the results. It could be that the three days between the aphids being exposed to a heat shock and the wasp foraging are

enough recovery time for the aphids. In Trotta et al. (2018), the authors propose the hypothesis that younger pea aphid instars are more resistant to heat shocks, and that this can be due to the low mobility that young instars have compared to late instars and adults (Ben-Ari et al., 2015). Since young instars are not able to physically escape unfavorable temperature conditions, they may have to be more adapted to microclimatic extreme temperatures. It is also possible that some of the aphids used in this paper harbor mutualistic facultative symbionts, however the presence of symbionts and its interaction with the heat shock did not alter the host-parasitoid interaction. It could be possible that shocked aphids had a reduced load of secondary symbionts, but it was still effective against parasitoids (Oliver et al., 2003).

When the heat shock occurred while the wasp was foraging on the aphids we saw a strong and negative effect of a heat shock on wasp mummy production. This could be caused by wasp and/or aphid factors. First, aphids moving around more could cause an increased parasitism defense/avoidance (Ma and Ma, 2012). Second, wasps could be stressed by the extreme temperature and not able to properly forage (Ismaeil et al., 2013; Jerbi-Elayed et al., 2015).

In addition, stressed aphids are more susceptible to alarm pheromone signals from other aphids, thus making them more aware of predators (Le Lann et al., 2014; Schwartzberg et al., 2008). Even if not aware of the wasp presence, aphids tend to move around more when experiencing a heat shock, as we saw in the behavioral experiment, where aphid walking was significantly higher when exposed to a heat shock (Figure 4). On average, pea aphids on the experimental arenas that were exposed to a heat shock spent 32% more time moving compared to control aphids, which has also

been shown in other research using this system (Kopco, 2017). This movement could cause difficulty for wasps, since they normally need to antennate the aphid to assess host quality when foraging (Rehman and Powell, 2010).

Although a change in aphid behavior alone could affect mummy production, it seems likely that differences in shock during interaction experiment were at least influenced by the large effect the heat shock had on wasps. After approximately 40 minutes the majority of wasps were showing signs of heat stupor. Roux et al. (2010), tested the effects of a similar heat shock on another parasitoid wasp, *Aphidius avenae*. Those shocked wasps were also negatively influenced, which was seen in the form of reduced production of offspring and a sex-dependent change in lifespan. In our experiment, the one-hour heat shock caused heat stupor signs in the majority of female wasps. Although not lethal, the heat stupor point is very close to the upper lethal temperature limit in insects (Vannier, 1994). Before the onset of stupor, wasps were walking and flying when under heat shock, but it is unclear if they were trying to forage or seeking thermal refuge from the harsh conditions (Scheffers et al., 2014), which would prevent them from foraging.

There was no effect of a heat shock when this occurred after a wasp parasitized the aphids. We had several reasons to expect a strong heat shock effect on parasitized aphids, since a heat shock would be stressing already stressed (parasitized) aphids. Moreover, wasp larvae developing inside a host might not be able to behaviorally react to heat stress. Limited research conducted on heat shocks and immature insects suggests that heat shocks negatively affect development and adult fitness of mosquitoes and *Trichogramma* parasitoids (Chihrane et al., 1993; Mourya et al., 2004;

Ramesh and Baskaran, 1996; Wang et al., 2014). Another study used milder but longer heat shocks on late larval and early pupal stages of an aphid parasitoid (Jerbi-Elayed et al., 2015). The authors exposed wasps to a heat shock, accompanied by a desiccation stress. This caused reduced life span and egg load on the emerging parasitoids. Our results showed wasps under a heat shock and control treatments had a similar relative mummy production rate, which may have been due to several reasons.

The heat shock may have debilitated aphids and the developing wasps, but just enough to create a fragile equilibrium. The developing wasp might have had reduced survival due to heat stress, but increased survival due to weaker aphids; for example easier time overcoming the aphid immune system. It has been shown that wasps can more easily overcome the immune systems of aphids who have been heat shocked (Bensadia et al., 2006; Blumberg, 1991; Thomas and Blanford, 2003). Especially with a short-time change in temperature, the aphid body could act as a temperature buffer, shielding the wasp larvae from the full effect of the heat shock. Compared to Jerbi-Elayed et al. (2015), we shocked an earlier stage of the parasitoid wasp larva, probably between egg and first instar larva (Martinez et al., 2016). Although the immune attack by the wasp on the pea aphid system starts during the deposition of the egg (Beckage and Gelman, 2004; Kraaijeveld and Godfray, 2009), the egg is likely the developmental stage that is more resistant to heat stress (Howe, 1967).

The methodological approach that we used has some limitations. Aphids were of different ages when they were heat shocked in the different experiments. Aphid instars have different sensitivities to heat stress (Trotta et al., 2018). However, we also knew parasitoid wasps have a strong preference and these preferences match the instars that

result in the best wasp performance for particular instars (Colinet et al., 2005; He et al., 2011). In our experimental design, we could not keep the ages constant for both things and ultimately decided to keep the aphids that the wasp encountered the same age

The timing of the heat shock in relationship to foraging also imposed some limitations to the experimental design. If we were not concerned with having the same duration of time between heat shocks and foraging we could have used a later heat shock which would have affected an older developing wasp larva. At 3 days, the parasitoid inside the aphid body is likely still in its egg form or first instar larva (Martinez et al., 2016; Pennacchio and Digilio, 1989). With a later heat shock, our design would be similar to that of Jerbi-Elayed et al. (2015), where heat shock had a strong and negative effect on developing parasitoid wasps. Another option would be to use a host with a longer lifespan, which would allow us for bigger time windows to implement a similar before-during-after methodology.

Our results show that timing of large, discrete abiotic events is important . The use of a before-during-after methodology approach may be a helpful intermediate step between individual experiments and population-level experiments, especially when the mechanisms vary with timing. In this instance, the results appear to be driven by heat shocks having stronger direct effects on adult parasitoids while foraging than at other times. Due to the discrete nature of heat shocks, this could mean that these shocks only impact a small percentage of parasitoids. However, since many abiotic effects alter organisms through both direct and indirect effects, it may be that continued investigation of both the relative strength and the timing of different mechanisms will be extremely

helpful for better understanding the ultimate effect of changing abiotic effects on this and other interactions.

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## HOW PEA APHIDS HANDLE THERMAL VARIATION: CONSTANT VERSUS FLUCTUATING TEMPERATURES

### Abstract

Insect performance is affected by temperature, with changes in daily average temperature (DAT) and changes in amplitude of daily temperature variation (DTV) both having potential effects. Changing both of these factors simultaneously can produce interactive effects on insects, which may be helpful to understand when variation of temperature is important. To understand that interaction we asked how insects are affected by a constant temperature compared to fluctuating temperatures (change in DTV) when in different temperature ranges that have different average temperatures (change in DAT). We tested the response of pea aphids (*Acyrtosiphon pisum*) on fava bean plants (*Vicia faba*). To properly test for differences in DTV only, the constant and fluctuating treatments had the same DAT within a given temperature range. One set was in a cooler range (constant: 20C and fluctuating: 16C/24C). The second set was in a warmer range (constant: 28C and fluctuating: 24C/32C). After 9 days, we compared aphid population sizes reared under temperature profiles with the same DAT. In the cooler range (20C vs 16C/24C), we obtained similar sized populations. However when the fluctuations occurred in the warmer range (28C vs 24C/32C), populations were half the size compared to populations under constant temperatures. Our experiment showed that insect response to DTV is not constant for all temperature ranges; DTV was strongly detrimental to aphids at higher DAT. Given that future climate scenarios predict both increases in DAT and DTV, this might indicate even stronger effects of climate



change on pea aphids than would have been predicted from changes in constant average temperatures alone.

## **Introduction**

Climate change is altering temperatures, including the abnormally rapid increase in average temperatures across the globe (IPCC, 2007). While this trend has been known for some time (Oreskes, 2018), recent scenarios also show a dramatic increase in temperature variation (Easterling et al., 2000; IPCC, 2012; Rahmstorf and Coumou, 2011; Rummukainen, 2012; Stoks et al., 2017). Daily temperature variation (DTV) is the amplitude of temperature fluctuations in each day to help determine how variation can alter organisms, including how such effects can be different from changes in the daily average temperature (DAT) (Deutsch et al., 2008; Kingsolver et al., 2013; Paaijmans et al., 2013). While changes to DAT and DTV can be related, they are not the same. Both can influence organisms separately. In fact, the interaction between DAT and DTV may pose a greater risk for biodiversity than either factor alone (Ma et al., 2015; Vasseur et al., 2014). Despite this potential importance, we cannot yet easily predict how DAT and DTV may interact in a given system. More work is needed to better understand their effects in different systems and why they occur.

The effects of DAT and DTV have been studied individually. Numerous studies have shown the effects of DAT by comparing organisms' performance at different constant temperatures (Brust, 1967; Duyck et al., 2004; Force and Messenger, 1964). More recently, studies have looked for effects of DTV by comparing organisms that are exposed to variable temperatures, with different treatments altering how much the temperature fluctuates (Stoks et al., 2017). The most straight-forward of such studies

compares organisms exposed to some amount of DTV with organisms experiencing a constant temperature (no DTV) (Ismail et al., 2010; Pike et al., 2005; Torres et al., 2002; Yeargan et al., 1978). An important lesson from this work is the need to control for the average temperature when exposing organisms to different amounts of DTV. If not, we confound possible effects of DAT and DTV (Colinet et al., 2015; Stoks et al., 2017).

Studies looking for effects of DTV have thus far failed to provide a single consistent answer. Some research concludes that DTV does not matter. In these studies, organisms exposed to fluctuating temperatures perform the same as organisms exposed to a constant temperature, as long as the DAT is the same for both treatments (Auad et al., 2015; Behrens et al., 1983). Other studies, however, have shown that DTV is important for performance, even when both treatments have the same DAT (Htwe et al., 2013; Ullah and Lim, 2015). Still other studies found variable results about the possible effects of DTV (Egwuatu and Taylor, 1977; Hagstrum and Leach, 1973; Hagstrum and Milliken, 1991).

One factor that could be contributing to these different results is the particular range of temperatures used for constant and fluctuating temperature treatments (Ma et al., 2015; Stoks et al., 2017; Vasseur et al., 2014). While some studies have compared constant and fluctuating temperature treatments over different temperature ranges, their results are still variable (Mironidis and Savopoulou-Soultani, 2008; Radmacher and Strohm, 2011; Torres et al., 2002; Walgenbach et al., 1988). However, one of the main trends that has emerged is that fluctuating temperatures seem to negatively affect organisms compared to a constant temperature when the two are compared over

relatively warmer temperature ranges compared to milder or colder temperature ranges (Bahar et al., 2012; Chown et al., 2015; Joshi, 1995).

The goal of this study is to understand the possible effects of DTV on pea aphids by comparing their performance in constant versus fluctuating temperatures, and to do so over two different ranges of temperature. We want to evaluate if any effect of DTV changes in a cooler versus warmer range of temperature. To perform these tests, we will look at aphid population size as our primary metric. Typically, DTV studies are performed looking at a single, specific demographic metrics, such as development time. By using changes in population size and running the experiment over a longer time frame were able to incorporate multiple demographic processes such as development time, fecundity, and survival -each of which may be influenced by DTV.

## **Methods**

### **Pea aphids**

Pea aphids (*Acyrtosiphon pisum*) are hemimetabolous insects (undergo an incomplete metamorphosis) that feed on plant sap of the bean family (Fam: Fabaceae). Under relatively long days and warm temperatures in a laboratory environment, pea aphids maintain parthenogenetic reproduction indefinitely and their embryos always develop from an unfertilized egg. Offspring are female clones that share the same genetic background as the mother.

There is extensive research on how pea aphids are influenced by temperature (Bieri et al., 1983; Campbell and Mackauer, 1975; Lamb et al., 1987; Montllor et al., 2002; Morgan et al., 2001; Murdie, 1969; Siddiqui et al., 1973). Population size is expected to change when performing temperature experiments due to the temperature

sensitivity of multiple demographic parameters, including fecundity, mortality, and developmental time (Bieri et al., 1983; Campbell and Mackauer, 1975, 1977; Lamb et al., 1987; Morgan et al., 2001; Murdie, 1969, 2009; Siddiqui et al., 1973; Will et al., 2017).

Pea aphids can express broad diversity in their facultative (secondary) symbionts (Oliver et al., 2010), genotype (van Emden and Harrington, 2017; Simon et al., 2003), and color morph (Caillaud and Losey, 2010; Losey et al., 1997). Some of these factors, such as the facultative symbiont *Serratia symbiotica*, can interact with temperature (Chen et al., 2000; Heyworth et al., 2016; Montllor et al., 2002). To reduce possible complicating effects from such factors in our experiments, we used animals from a single clone (line 82B-AB) of pink-morph aphids that harbored no known secondary symbionts. This aphid line was obtained from researchers at the University of Georgia (Athens, GA) in the summer of 2013. While using this aphid line helped us reduce individual variation and other possible complicating factors, our experimental results could potentially be only relevant to this specific aphid line.

### **Insect rearing**

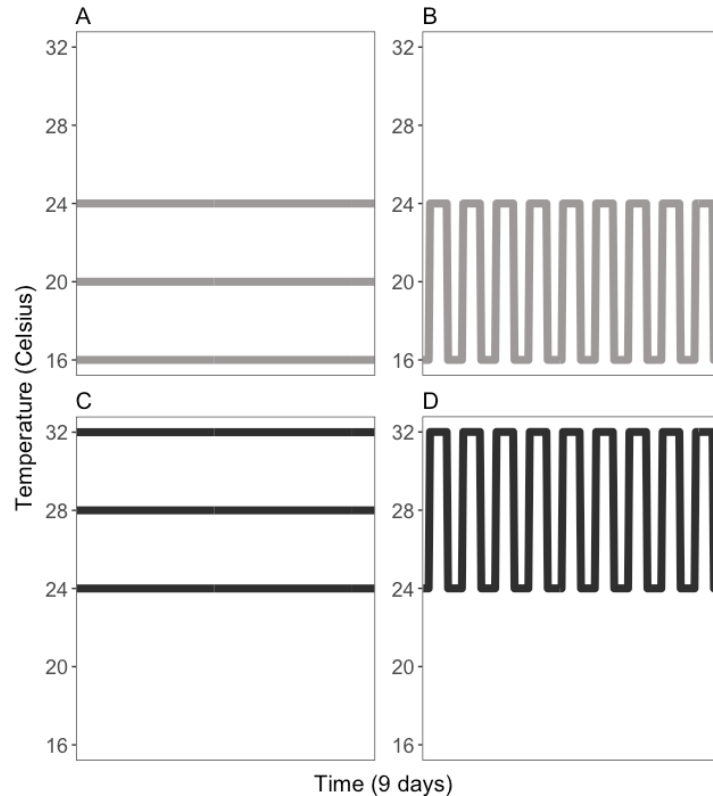
We reared aphid colonies in collapsible mesh cages (40cm x 40cm x 40cm, BugDorm, Taiwan). Each cage contained a tray to hold water and had a clear plastic top to allow for light. We held all cages at relatively constant environmental conditions:  $20 \pm 2$ C, 60–80 % RH under a L16:D8 photoperiod produced with fluorescent growth lights (F14W/T5/865/ECO 14 Watt 6500K Fluorescent Tube made by GE, Boston, MA). We planted 2 or 3 fava bean seeds (Broad Windsor, Territorial Seeds, Oregon) in plastic pots (10.2 x 10.2 cm, Tessman Seed Co, St. Paul, MN) each week, and used the plants

to replace heavily crowded plants to minimize effects of overcrowding (Murdie, 1969; Watt and Dixon, 1981). We planted bean seeds in a commercial sphagnum peat moss-based horticultural mix that included perlite, dolomitic limestone, added nutrients, and a wetting agent (Sunshine Mix LC1, Sun Gro Horticulture, Vancouver, BC).

### **Common methodology**

To test the effect of DTV on pea aphid population size at different temperature ranges, we carried out two experiments with different temperature ranges (cool and warm ranges). First, we planted a single fava bean in each pot (10.2 × 10.2 cm, Tessman Seed Co, St. Paul, MN) and let it germinate. When the plants reached an average of 7cm, we infested each plant with fifteen pea aphids. To create small populations that included a range of different aged aphids, we infested with five 1<sup>st</sup>-2<sup>nd</sup> stage instars, five 3<sup>rd</sup>-4<sup>th</sup> stage instars, and five adults. To prevent the aphids from escaping, we covered each plant with a cylindrical tube cage (26 cm high x 12cm diameter) made of transparent plastic with mesh windows for air circulation.

We randomly assigned each plant to one of four different temperature treatments: constant low, constant medium, constant high, and fluctuating (Figure 5). The exact temperature of each treatment was the main difference between the two experiments (described below). However, for both experiments the medium constant temperature is the DAT of the fluctuating treatment. Therefore, comparing those two is the proper comparison between a treatment with DTV and a treatment with no DTV. In both experiments, the constant low and constant high temperatures represent the low and high points of the fluctuating temperature. Therefore, they help provide better context for what the aphids experience at different times in the fluctuating treatment.



**Figure 5.** Temperature profiles of the cool (light gray) and warm (dark gray) experiments. The constant (A and C) and the fluctuating (B and D) temperature profiles were maintained for nine days.

We randomly assigned plants to treatments and then placed them in growth chambers with their corresponding treatment. Plants and aphids stayed inside the growth chambers for nine days. During this time plants had water available continuously. At the end of nine days, we counted the total number of aphids present on each plant. The response variable for these experiments was the total number of aphids present on each plant after the experiment ended.

### Cool experiment

In this experiment, we tested if small pea aphid populations reared under constant and fluctuating temperatures produced different sized populations. The constant temperature profiles of the cold experiment were 16C (constant low), 20C

(constant medium) and 24C (constant high) (Figure 5A). This range of temperatures is often encountered in the field under conditions when pea aphids perform well (Campbell and Mackauer, 1975, 1977; Lamb et al., 1987). Although there are differences between populations, the optimal temperature for pea aphids tends to be between 22C and 26C (Deutsch et al., 2008; Frazier et al., 2006; Morgan et al., 2001). The fluctuating temperature treatment consisted of 12 hours at 16C and the next 12 hours at 24C (Figure 5B), giving a DAT of 20C. The temperature was low (16C) during the night, switched to high (24C) in the morning (06:00h) and switched back to low in the evening (18:00h). The temperature switch lasted 25 min and the temperature change rate was 0.3C/min. The total sample size of this experiment was 92, with 23 samples for each temperature treatment, divided over three temporal blocks. We switched which growth chambers produced each temperature treatment after each block, to avoid any possible chamber effects being confounded with differences among treatments.

### **Warm experiment**

In this experiment we performed the same comparison of pea aphid population sizes in constant versus fluctuating temperature, but across a warmer range of temperatures. The constant temperature profiles of the warm experiment were 24C (constant low), 28C (constant medium) and 32C (constant high) (Figure 5C). The fluctuating temperature profile consisted of 12 hours at 24C and the next 12 hours at 32C (Figure 5D), giving a DAT of 28C. The temperature was low during the night, switched to high in the morning (06:00h) and switched back to low in the evening (18:00h). The treatments are the same temperature degrees apart from each other and the DTV is the same extent as the cool experiment. The only difference was that all

temperature profiles were 8C higher. For this range of temperatures, the low temperature is near optimal for most pea aphid populations and the high temperature is getting close to the maximum temperature for pea aphids (Deutsch et al., 2008; Frazier et al., 2006; Morgan et al., 2001). The total sample size of this experiment was 120, with 30 samples for each temperature treatment, divided over four temporal blocks. We again switched growth chambers after each block, to avoid confounding treatment and chamber effects.

## **Statistics**

To test the effect of the temperature treatments on pea aphid population size we performed ANOVA comparing each treatment using R version 3.4.3 (R Core Team, 2017). Since the experiments were performed over different temporal blocks, we always included a temporal block effect. Results were then analyzed using Tukey's HSD contrasts to discern differences among individual temperature treatments with the same mean. We used the ggplot2, dplyr, reshape2, and googlesheets packages to manipulate data and build graphs (Auguie, 2017; Bryan and Zhao, 2017; Cheng et al., 2017; Wickham, 2007, 2009; Wickham et al., 2017).

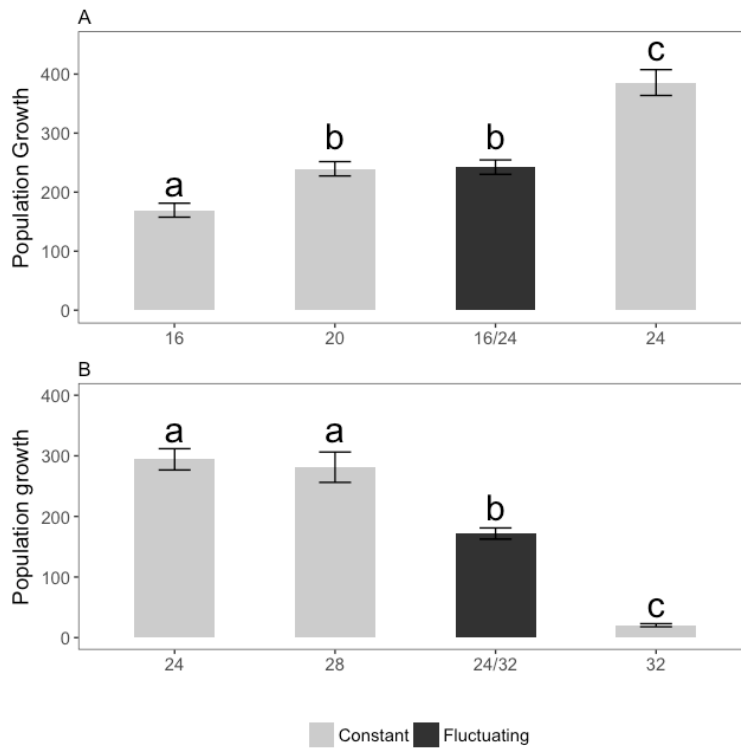
## **Results**

### **Cool experiment**

Aphid populations were strongly affected by temperature with aphid populations at the highest constant temperature being more than twice the size of the populations in the lowest temperature and the other treatments being in between (Figure 6A). This lead to a significant effect of temperature treatment ( $F(3,88)=36.09$ ,  $p<0.0001$ ). Despite these treatment effects, we did not find any significant differences when comparing the



medium constant temperature and the fluctuating temperature treatment, which had the same DAT (20C and 16C/24C;  $p=0.99$ ).



**Figure 6.** Aphid population size ( $\pm$  standard error) in the cold (A) and warm (B) environments. The light bars represent the population size of pea aphids exposed to constant temperature profiles and the dark bars aphids exposed to fluctuating temperature profiles. The letters indicate significant differences between treatments (Tukey’s HSD post hoc analysis ( $p<0.05$ )).

### Warm experiment

We again found that temperature treatment had a large and significant effect on the size of aphid populations at the end of the experiment (Figure 6B;  $F(3,118)=66.1$ ,  $p<0.0001$ ). However, over this range of temperatures the largest population was found in the lower temperatures with smaller populations in the high and fluctuating temperature treatments. However, our main comparison was again between the medium constant temperature treatment and the fluctuating temperature treatment with the same DAT. Unlike the previous experiment, DTV seemed to be important over the

warm range of temperatures as pea aphid populations at constant 28C were 60% larger compared the populations under the fluctuating treatment (28C and 24C/32C;  $p < 0.0001$ ).

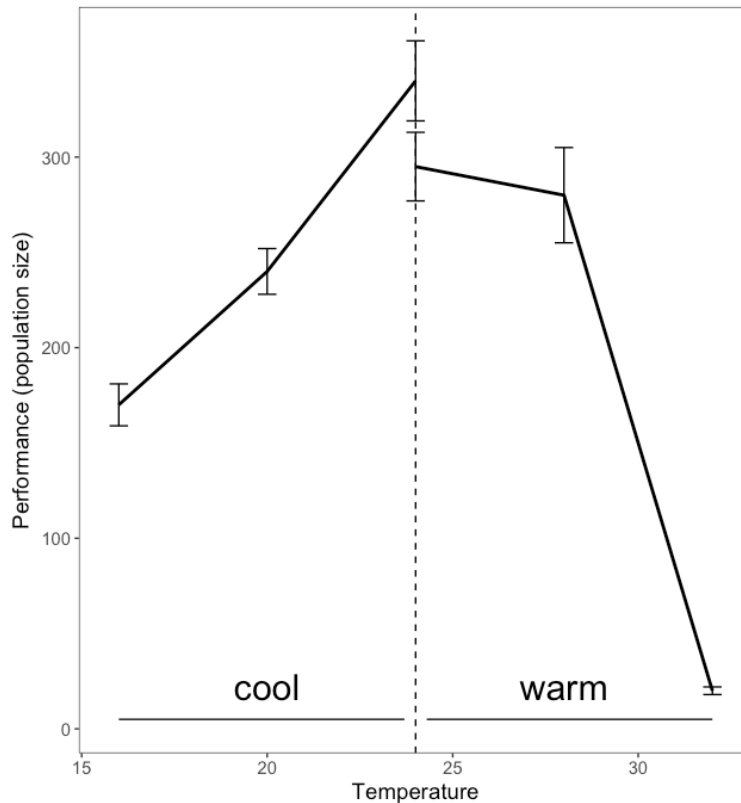
## Discussion

The main goal of our experiments was to understand if DTV was important by comparing pea aphid population size in constant versus fluctuating temperatures when both treatments had the same DAT. We found that our results depended on what range of temperatures we used. In a cool environment, DTV was not important, as there was no difference between populations in the constant compared to fluctuating temperature treatments, similar to what some authors have found (Auad et al., 2015; Behrens et al., 1983). However, DTV had a large detrimental effect on pea aphid population size when tested across a warmer range of temperatures.

To explore how an organism's performance changes with temperature, usually constant temperature, scientists use thermal performance curves (TPC) (Stoks et al. 2017). A TPC describes the relationship between performance and temperature for a given trait of an organism, and they are a useful tool to visualize and predict temperature effects. Using a TCP in our case may help us understand why we saw a difference of the effect that DTV had at different temperature ranges. Empirical evidence suggests that TPCs tend to take the same general shape (Schulte et al., 2011). A typical TPC begins at the lowest temperature where performance occurs (minimum temperature or  $T_{min}$ ). As temperature increases, performance also increases such that the TPC has a linear or potentially exponential increase in slope until it reaches the highest point where performance of the given metric is greatest (the optimal

temperature or  $T_{opt}$ ) (Stoks et al., 2017). In a typical TPC we usually see a plateau around  $T_{opt}$ , rather than a sharp switch between the ascending and the descending slope (Schulte et al., 2011). After the area around  $T_{opt}$ , there is a rapid decline in performance, usually quickly reaching the point where the organism can no longer live (maximum temperature or  $T_{max}$ ) (Colinet et al., 2015; Stoks et al., 2017; Vasseur et al., 2014).

To help understand how temperature range influenced the differences in DTV we used the results from our constant temperature treatments to build a TPC (Figure 7). Our TPC was based on population sizes across different constant temperatures and shows a similar shape to a typical TPC. However, there are some key differences. First, the resolution of our curve is not very fine since we did not originally design our temperature treatments for this purpose. Secondly, both our cool and warm experiments used a constant 24C treatment, but they did not get the exact same results. These differences are likely due to the two experiments being done separately at different times. This can cause differences in aphid performance due to differences in environmental factors like humidity or transient differences in the aphids themselves due to the exact state of the aphid colonies at the time of the experiment. all six of the treatments demonstrate what an overall TPC for our pea aphids might look like while being true to the two separate experiments we performed.



**Figure 7.** Thermal performance curve of the pea aphid population size relationship with temperature. We used the population size values (average  $\pm$  standard error) obtained in the six constant temperature treatments. The left side of the figure is the cool range and the right side of the figure is the warm range.

Over the cool range of temperatures (left part of the graph), our TPC has a positive, approximately linear slope. This means that if we increase the temperature from 16C to 24C (+8C) as in the fluctuating treatment, we would expect an increase in performance that is twice as much as when we increase from 16C to 20C (+4C) or from 20C to 24C (+4C). The consequence of that is that we would expect that the performance of aphids at a constant 20C would be the average of their performance at 16C and 24C, which is fairly close to our observations.

We can then ask whether aphid performance in a variable temperature treatment is merely the cumulative response of what happened when held at each of the constant

temperatures they were exposed to. This would basically be the case if there were no consequences or carry over effects of switching temperatures at all. For our experiment, this would lead us to suggest that when aphids are at 16C for 12h, their performance is the same as in the constant low (16C) treatment, and when the temperature switched to 24C for the other 12h their performance is the same as the constant high (24C) treatment. Therefore, aphid performance in the fluctuating treatment would again be similar to the average performance of the constant 16C treatment and the constant 24C treatment. This is the same prediction we made for the constant 20C treatment and was, again, close to what we observed (Figure 6A). There has been support for this general idea that when temperature fluctuates around a mean and that fluctuating temperature stays within the linear portion of the slope, performances on the fluctuating temperatures are similar to performances in constant temperatures with the same DAT (Stoks et al., 2017).

The right half of the TPC corresponds to the temperature used in the warm experiment and shows a different shape than the left. Performance was highest at 24C with only a slight decrease at 28C followed by a substantial decrease at 32C. This again follows a standard prediction that organisms have a temperature where performance is maximized ( $T_{opt}$ ), with performance decreasing as temperature increases past some plateau or hump around  $T_{opt}$ . Although  $T_{opt}$  values can vary between populations of the same species adapted to different climates (Lamb et al., 1987), there are multiple reports of pea aphid  $T_{opt}$  around 24C (Morgan et al., 2001), just as we observed.

This difference in the shape of TPC helps us see why the predictions (and results) we saw over cool temperatures no longer hold for the warm temperatures. First,

because the TPC is no longer linear we can see that the average of aphid performance at the constant 24C and 32C treatments is no longer a good predictor for the performance in the constant 28C treatment. However, does that prediction still hold for the fluctuating temperature? As an incredibly rough first approximation for testing this prediction, we can see that the population size at the constant 24C treatment produced around 296 aphids on average and the constant 32C produced close to 20 aphids. The average of those two (158) is similar to the population size of the fluctuating temperature (around 172).

This result is surprising in that it seems to contradict some of the recent work on the effects on DTV, particularly the effects that extreme temperatures can cause on performance (Colinet et al., 2015; Stoks et al., 2017). These authors show that exposure to high temperatures past the  $T_{opt}$  point, tend to cause organisms to no longer react to temperature the same as individuals at constant temperatures, even if the constant temperatures are below the  $T_{opt}$ . However, in our case we do not have evidence that exposure to the 32C treatment for 12h had larger effects on the aphids beyond their poorer performance during that time.

DTV was important over our warmer temperatures. We do not know necessarily know why this is. Perhaps exposure to the high temperatures reduces overall performance such that we can no longer make simple predictions; however, our results do not necessarily support that. Perhaps pea aphids are not harmed as much by high temperatures if they regularly have recovery periods at more optimal temperatures. Similar ideas about the potential beneficial effects of recovery periods have been investigated with insects exposed to otherwise harmful high temperatures (Ma et al.,

2015) and low temperatures (Torson et al., 2017). Alternatively, perhaps the difference between our constant and fluctuating temperature has more to do with relatively similar performance values when around  $T_{opt}$ , which led to the 28C treatment simply being higher than we expected. If so, that would point to the importance of understanding the nature and extent of the TPC area where performance is maximized when picking temperature treatments for manipulative experiments. Future experiments that explore these differences will help us better understand when and why variation is predicted to be important.

DTV is expected to increase in the future due to climate change (IPCC, 2012). Our methods only tested no DTV (constant treatments) versus some DTV (fluctuating treatments), which is a rather extreme contrast. Reproducing a similar experimental design as we did here with different amounts of DTV could be an interesting approach (Bozinovic et al., 2013, 2014; Vázquez et al., 2017). It would also potentially help us gain a deeper understanding of the interactive effects of DTV and DAT, especially if the entire experiment was done over different ranges of temperature. A possible experimental setup could be to test pea aphid performance under three temperature ranges (for example cold, warm, and hot) and with different amounts of DTV, for example 4C, 8C, and 12C.

In conclusion, pea aphids are strongly affected by changes in the constant temperature they are exposed to, but they are not as clearly influenced by DTV. We found that DAT and DTV interacted, such that fluctuating temperature was strongly detrimental to aphids, but only for the warmer range of temperatures. Future climate scenarios predict both increases in global temperatures (increases in DAT) and climate

variability (increases in DTV), which suggests that it may be increasingly likely that DTV will be important in influencing pea aphids, since temperatures will often fluctuate highly around a higher DAT. Our results suggest that this interaction between climate warming and variability is an important phenomenon to study for understanding the ecology and population dynamics of herbivores like pea aphids.

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# THE INDIRECT EFFECTS OF TEMPERATURE ON HERBIVORES MEDIATED BY THEIR HOST PLANTS

## Abstract

Experiments testing the effects of temperature on organisms are key to understand the effects of climate change. Previous temperature experiments where pea aphids (*Acyrtosiphon pisum*) feeding on fava bean plants (*Vicia faba*) were exposed to various constant temperatures showed consistent treatment effects on aphids. However, in almost all such experiments the aphids are necessarily tested while feeding on their host plant. This means that we cannot tell whether treatment effects of temperature come from direct effects of temperature to the aphids or if there are also indirect effects that come from temperature influencing the host plant which causes additional indirect effects to the herbivore. To help address this, we exposed plants and pea aphids to different temperature treatments (16C, 24C, and 32C). This resulted in significant differences in aphid fecundity. In a second experiment, plants were still grown in different temperatures but then moved to a common environment when we added the aphids. Aphid fecundity did not vary across plants grown at different temperatures. In both experiments, temperature influenced plant size. Therefore, in the third and fourth experiments, we tested if temperature-generated differences in plant size affect aphids. Plant size did not affect aphid fecundity. The fifth experiment again exposed aphids to different temperatures and found differences in fecundity. Aphid fecundity was not influenced by how long the aphid's host plant had been exposed to a temperature treatment. Together, these results suggest that temperature likely has a

strong direct effect on aphids and weak, if any, plant-mediated indirect effects of temperature on aphids via direct effects on host plants.

## **Introduction**

Temperature has substantial effects on the ecology of insects (Bale et al., 2002; Cornelissen, 2011; Grainger and Gilbert, 2017). This is particularly well-studied for insect herbivores of economic importance, as crop pests (Cammell and Knight, 1992; Vangansbeke et al., 2015). When we investigate such temperature effects on herbivorous insects, it is sometimes possible to study the insect in isolation from the host plant, sometimes using artificial diets (e.g. Lamb, MacKay, and Gerber 1987; McMillan et al. 2005; Mironidis and Savopoulou-Soultani 2008). In doing so, we can assume that any measured responses are direct effects of temperature on the insect. However, other herbivorous insects have a close association with their host plant, such that when temperature experiments are performed it is preferable to include both the herbivores and their host plants (Asin and Pons, 2001; Barton and Ives, 2014). In these cases, we cannot necessarily differentiate between the direct effects of temperature on the herbivore and the indirect effects of temperature on the herbivore mediated by the host plant. Differentiating the roles of direct and indirect effects in such experiments can improve our mechanistic understanding of temperature effects and help us design and interpret future manipulative experiments, including predicting future effects of temperature on plant-herbivore interactions (Anderson et al., 2001; Boggs and Inouye, 2012; Masters et al., 1998).

Close plant-herbivore associations frequently occur with hemipterans, such as whiteflies, aphids, mealybugs, psyllids, and some planthoppers and leafhoppers

(Douglas, 2006; Li et al., 2015). Of these, aphids are particularly relevant, as crop pests: virtually every crop around the world is attacked by at least one species of aphid (van Emden and Harrington, 2017). Aphids are frequently in the subject of temperature experiments (Meisner et al., 2014; 2010; Wang et al., 2017). As sap-sucking insects, aphids spend most of their time feeding on their host plants (Caillaud and Via, 2000; Dixon, 1985; Wilkinson and Douglas, 1995). Although it is possible to separate them from their host plant, it comes at a high physiological cost, especially if they are separated for more than a short time period (Kopco, 2017; Nelson, 2007). This strong association makes it difficult to perform experiments that test for only direct temperature effects. Artificial diets can help separate aphids from their host plants (Auclair and Cartier, 1963; Douglas et al., 2006; Puterka et al., 2017; Sasaki et al., 1991), and thus give experimental results that are likely dominated by direct effects of temperature. However, this approach is not universally appropriate as it cannot replace the chemical and physical cues aphids need to properly feed (Hopkins et al., 2017; Smith and Chuang, 2014). Moreover, it is unclear how temperature might influence the density and fluidity of artificial diets, especially for studies conducted over days (Yang and Joern, 1994).

Manipulative experiments performed in the laboratory or greenhouse have provided extensive information about temperature effects on aphids, including the pea aphid, *Acyrtosiphon pisum* (Campbell and Mackauer, 1975; Morgan et al., 2001; Siddiqui et al., 1973; Stacey and Fellowes, 2002). In fact, pea aphids have become a model system for a number of research questions, most of which include manipulative, controlled experiments that test the aphid on a host plant like *Vicia faba* (Brisson and

Stern, 2006; Oliver et al., 2010; The International Aphid Genomics Consortium, 2010). Given its prominence in current and previous research, we set out to better understand the role of plant-mediated indirect effects of temperature on the pea aphid-fava bean model system. We do not necessarily assume such results will be transferable to other systems, but we do feel it will be a crucial insight for this common experimental system that may have broader ramifications for similar experimental research.

To look for plant-mediated indirect effects of temperature on pea aphids we performed five complimentary experiments in the laboratory. The experiments varied in how exactly plants were grown. This include exposure to different temperature treatments in order to identify plant-mediated indirect effects on aphids. There is not necessarily any single approach that is best for distinguishing direct and indirect effects without removing the plant from the system, however we feel that the multiple types of evidence we sought here allows us to make a fairly robust conclusion: plant-mediated indirect effects of temperature are relatively minor in the study system, especially when compared to the apparently direct effects of temperature on the aphids themselves.

## **Methods**

### **Pea aphids**

Pea aphids are insects that reproduce asexually during spring and summer seasons, when there are long days and warm temperatures (Dixon, 1998; van Emden and Harrington, 2017). When these environmental conditions are kept constant in a laboratory environment, pea aphids never undergo sexual reproduction, instead maintaining asexual (parthenogenetic) reproduction indefinitely. In these parthenogenetic animals, the embryos develop from an unfertilized egg (Miura et al.,

2003). Therefore, their offspring are clones that share the same genetic background as the mother. In pea aphids, this form of asexual reproduction only produces female aphids, with males only appearing at the end of the summer season (Kenten, 1955). This allows us to create fully monoclonal colonies of aphids that only consist of females.

The number of offspring produced by pea aphids is greatly affected by several factors, such as environment temperature, aphid age, and host plant quality (Lamb et al., 1987; Morgan et al., 2001). For this reason, aphid offspring production, or fecundity, is a useful and common metric to measure impacts of temperature (Bieri et al., 1983; Murdie, 2009; Siddiqui et al., 1973).

Controlled laboratory experiments reduce a potentially large amount of variation across individuals to help provide clearer results for specific study questions related to indirect and direct effects. For example, pea aphids can have a great deal of diversity in their secondary symbionts (Oliver et al., 2010), genotype (van Emden and Harrington, 2017; Simon et al., 2003), or color morph (Caillaud and Losey, 2010; Losey et al., 1997), and there is growing evidence of the interaction between some of these factors and temperature (Chen et al., 2000; Heyworth et al., 2016; Montllor et al., 2002). To help control for some of this variation, we used aphids in these experiments from a single clone (line 82B-AB) of pink-morph aphids that harbored no known secondary symbionts. The aphid line was obtained from researchers at the University of Georgia (Athens, GA) in the summer of 2013, who had previously collected the aphids in an alfalfa (*Medicago sativa*) field near Athens (Georgia). This choice helps us reduce individual variation and helps make sure that our results should not be affected by the

presence of secondary symbionts; yet, there is the tradeoff that our experimental results could change when considering other lines of pea aphids.

Although pea aphids have a wide host range (family: Fabacea), the majority of laboratory experiments are done using fava beans (*Vicia faba*) as host plants (Gwynn et al., 2005; Simon et al., 2011). This is because fava beans have a quick growth rate and are relatively easy to rear in laboratory conditions. Pea aphids found in alfalfa are often transferable to bean plants to form laboratory colonies and perform experiments (Meisner et al., 2014).

### **Insect rearing**

We used commonly practiced rearing techniques for our aphids. Aphid colonies were reared in mesh cages (collapsible cube mesh cages. Size: 40cm x 40cm x 40cm, BugDorm, Taiwan) that each contained a tray to hold water and had a clear plastic top to allow for proper lighting. All cages were kept at relatively constant environmental conditions:  $20 \pm 2^{\circ}\text{C}$ , 60–80 % RH under a L16:D8 photoperiod produced with fluorescent growth lights (F14W/T5/865/ECO 14 Watt 6500K Fluorescent Tube made by GE, Boston, MA). New plastic pots (10.2 x 10.2 cm, Tessman Seed Co, St. Paul, MN) with 2 or 3 fresh fava bean plants (Broad Windsor, Territorial Seeds, Cottage Grove, OR) were introduced weekly to the colony. We removed older plants that were becoming heavily infested to minimize effects of overcrowding (Murdie, 1969; Watt and Dixon, 1981). The growing medium we used for colonies was a commercial sphagnum peat moss-based horticultural mix appropriate to grow fava bean plants. The mix included perlite, dolomitic limestone, added nutrients, and a wetting agent (Sunshine Mix LC1, Sun Gro Horticulture, Vancouver, BC).

## Common experimental methods

To understand how temperature could indirectly affect herbivores through their host plants we performed five separate experiments using fava bean plants, pea aphids, and constant exposure to one of three temperature treatments. Our temperature treatments were set at a relatively low (16C), moderate (24C), or high temperature (32C), all of which are within the basic range of our experimental system where both plant and aphid perform well for at least moderate time periods. Four of the experiments follow the same methodology with minor, but important differences between them that allow us to find slightly different, but complementary pieces of information (Table 1).

**Table 1.** Description of the four experiments with a shared methodology (1 – 4).

#	Name	Planting time	Plant size	Plant temp.	Aphid temp.
1	<b>Baseline</b>	Same	Different	Different	Different
2	<b>Indirect</b>	Same	Different	Different	Same
3	<b>Different size</b>	Different	Different	Same	Same
4	<b>Same size</b>	Different	Same	Different	Same

Planting time explains whether plants in the three temperature treatments were planted at the same time or at different times per treatment. Plant size refers to the size of plants in treatments right before the pea aphid infestation. Plant temperature refers to whether plants in different treatments were each exposed to different temperatures between planting and infestation or if all plants were at the same temperature. The aphid temperature column refers to whether plants infested with aphids were kept in the same environment or were in different temperatures depending on the treatment.

For each of these experiments we first planted one fava bean seed per pot (10.2 × 10.2 cm, Tessman Seed Co, St. Paul, MN) and let the plant germinate. We later transferred four adult pea aphids to each plant and covered the plant with a tube cage to prevent escape (cylindrical tube cage height: 26 cm, diameter: 12 cm) Made of transparent plastic, the cage had two mesh windows on the side and one on the top to

allow for air circulation) to prevent them from escaping. We then measured the number of juveniles produced and the number of adults still alive after 48 hours. The plants had water available at all times via a tray underneath the pots. The factors that changed between experiments include the temperature at which plants and aphids were reared and if the seeds in each treatment were planted at the same time or at different times (Table 1 and methods of each experiment below).

The response variable for these four experiments was pea aphid relative fecundity. Specifically, the total number of offspring present on the plant at the end of the experiment divided by the total number of adults alive at the end of the experiment. By using multiple adults our response averages over potential variation among individuals. Using 48 hours as a response time balances giving the aphids enough time to respond to the plant and treatment while avoiding any potential density-dependent effects on their fecundity (Honek, 1993; Murdie, 1969; Peters and Barbosa, 1977).

### **Baseline experiment (1)**

In this experiment, we asked how aphids were affected by temperature when plants were grown in different temperatures and then aphids were exposed to those temperatures while being on those plants. This basic experiment is meant to mimic a traditional experimental design where any temperature effects could be due to direct effects on the aphid and/or indirect effects mediated by the host plant.

We followed the common methods (see above) with plants randomly assigned to a temperature treatment and placed inside a growth chamber set to one of the three temperature treatments at the time of planting. The plants showed differences in growth rate between temperature treatments (see results), therefore we waited until the



smallest plants reached a minimum size (10cm) and infested all plants at the same time. Plants were placed back in growth chambers after infestation. This means that in this experiment plants were in their temperature treatments for the entire experiment and the aphids were exposed to different temperatures for the 48 hours when we measured their fecundity (Table 1). We performed the experiment over two temporal blocks with a total sample size of 86 plants (16C:31, 24C:28, and 32C:27).

### **Indirect experiment (2)**

In this experiment, aphids were not exposed to different temperature treatments, but plants were still grown in different temperatures before we infested them with aphids. That means that there should no longer be any direct effects of temperature on the aphids because the aphids themselves were not exposed to the temperature treatments. However, by being placed on plants that were grown at different temperatures, some plant-mediated indirect effects were still possible.

We followed the same methods as the baseline experiment (1), however we took the plants out of the growth chambers right before infesting them with aphids. We then placed all plants in a common environment and infested them with 4 adult aphids and gave the aphids 48 hours to produce offspring (Table 1). The laboratory common environment was kept at  $20 \pm 2\text{C}$  and plants were under L16:D8 lighting, the same as inside the growth chambers. We performed the experiment over two temporal blocks with a total sample size of 66 plants (16C:24, 24C:25, and 32C:17).

### **Different size experiment (3)**

In the first two experiments (1 and 2), plants were exposed to different temperatures while growing, and, as a consequence, were of different sizes when

infested with aphids (see results). This means that differences in aphid fecundity could be influenced by differences in plant development and/or other indirect effects of being grown in different temperatures. In previous work, plant stages affected aphid fecundity (Dixon, 1985; Guldmond et al., 2003; Leather and Dixon, 1984). To test the relative role of plant development from other temperature-mediated effects, we carried out two additional experiments (3 and 4) where we controlled for plant development by planting seeds at different times.

In the different size experiment (3), we asked whether differences in plant development alone influence pea aphid fecundity in our experimental set up. To do that we followed the common methodology with the principle exception that all plants and aphids were always kept in the common laboratory environment (Table 1). Plants, and their aphids, were still divided into three plant size treatments, but in this case, the three treatments were designed to mimic the three different points of plant development (measured by plant height) seen in the three temperature treatments in the baseline experiment (1) (Figure 8A). To achieve these differences in plant heights, plants in each treatment were planted at different times.

We determined exactly when seeds in each treatment should be planted by planting 75 fava bean seeds in our common laboratory environment and measuring their growth daily. We found that to mimic the different plant sizes in the baseline experiment (1) plants in 24C treatment had to be planted first, 32C plants 2 days later, and plants in the 16C treatment 2 days after that. Eight days after the last planting, 4 adult aphids were again added and aphids were allowed to produce offspring for 48 hours. The total sample size was 66 with 22 plants used in each of the three plant size

treatments. Over the entire experiment, plants and aphids were kept in the same common environment, so the only difference among treatments was plant size.

#### **Same size experiment (4)**

In the previous experiment, we attempted to separate out temperature-induced differences in plant development from other potential temperature-induced effects by altering plant development but keeping all plants at the same temperature. Here, we perform the complementary experiment where we expose plants to different temperatures but controlled for plant size. To achieve this, we again used our common methodology, including having three temperature treatments that plants were exposed to from planting up until infestation. The major difference is that we planted those seeds at different times so that plants in all treatments would be the same size at infestation (Table 1).

To determine when to plant in each treatment, we again did a preliminary experiment to measure daily plant growth. However, in this case, we did it for plants in each of the three temperature treatments. From that information, we found that we should first plant in the 16C treatment, 4 days later plant in the 32C treatment, and 1 day later plant the plants at 24C. This gave plants that averaged 10cm in each treatment at the time of infestation. Plants were placed in the common laboratory environment at the time of infestation and aphids and plants were kept there for 48 hours. The total sample size was 60 over two temporal blocks with 20 plants used in each of the three temperature treatments. Any differences in aphid fecundity among treatments would indicate temperature-mediated indirect effects of temperature that occur despite plants being roughly equally in their size.

## **Plant time experiment (5)**

To continue our efforts to tease apart potential direct effects of temperature on aphids from potential plant-mediated indirect effects, we performed an additional experiment that modified how long plants were kept in temperature treatments. To do that, we reared plants at room temperature and infested each with a single adult aphid. Each plant was then assigned to one of three temperature treatments (same treatments as early experiments). Within each temperature treatment, half the plants were kept in the same treatment throughout the three days that aphids were exposed to temperature (continuous) and the other half were replaced with plants from the common environment every 24 hours (replaced). Differences among continuous versus replaced plants within the same treatment would indicate that how long plants are exposed to a temperature treatment (72 hours in the continuous versus 24 hours in the replaced treatments) can affect aphid performance.

Our basic methodology was similar to previous experiments. We planted fava bean seeds (one per pot) in the common laboratory environment. Once the seedlings reached an average size of 10cm, we infested them with a single adult pea aphid, and randomly assigned to one of the three temperature treatments: 16C, 24C, or 32C. Since we just used one aphid in each sample unit, we strictly controlled for aphid age. To do that, we infested several plants with fully grown adult aphids a week before the infestation began. After 24 hours, we removed all adult aphids so that the remaining aphids were all the same age (between 0 and 24 hours old). Once those aphids developed into adults, we used them to infest plants in this experiment. After infestation,

we caged plants and introduced them to a growth chamber with the corresponding temperature.

Within each temperature treatment, we exposed half the plants to the temperature treatment for the entire 72 hours experiment (continuous) while the other half were replaced every 24 hours (replaced). Replacement plants were from the same common laboratory conditions as the original plants and had been planted at the same time as the plants used for the rest of the experiment. Every 24 hours all juvenile aphids on every plant were counted and removed from the plant. Adult aphids were also removed; those in the replaced treatment were placed on to a new replacement plant from the common environment and those on the continuous treatment were placed back on to the same plant that had been in the temperature treatment. Aphids can take time to establish and begin feeding once disturbed (Kopco, 2017), which can influence their fecundity (Nelson, 2007). Therefore, it was important that we manipulated all aphids in the same way in every treatment. We transferred each aphid using a wet paint brush, first gently poking them with a bristle, and then “grabbing” them with the brush, and depositing them on a corresponding leaf. The gentle poking was to allow the aphid to remove her stylet from the leaf. Thereafter, we were able to transfer the aphid with as little damage as possible.

The response variable for this experiment was aphid individual fecundity over 48 hours. This was calculated as the addition of the offspring produced after 24 hours of the first plant switch (nymphs laid 24-48 hours after infestation) plus the offspring produced 24 hours after the second plant switch (48-72 hours after infestation). The total sample size was 130 plants across each of the three temperature treatments

(16C:42, 24C:46, 32C:42), and two plant time treatments (replaced:65 and continuous:65), over two temporal blocks.

## **Statistical analyses**

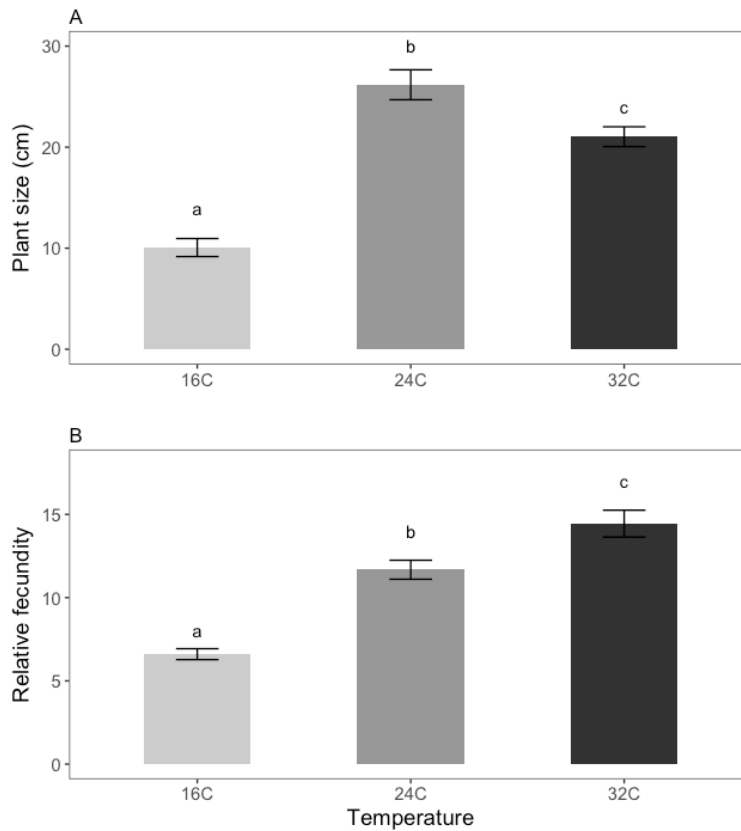
Data analyses were conducted in R version 3.4.3 (R Core Team, 2017). We performed ANOVAs to separately compare plant size and pea aphid fecundity across the three temperature treatments in the first four experiments. For the fifth experiment our analysis included the temperature treatment, the time plants were in the experiment, and their interaction. We also included temporal blocks when needed. Results were then analyzed using Tukey's HSD contrasts to discern differences among individual treatments. We used the ggplot2, dplyr, reshape2, and googlesheets packages to manipulate data and build graphs (Auguie, 2017; Bryan and Zhao, 2017; Cheng et al., 2017; Wickham, 2007, 2009; Wickham et al., 2017).

## **Results**

### **Baseline experiment (1)**

When plants and aphids were exposed to the three temperature treatments there were large differences in both organisms across treatments. At the time of infestation, plant size differed with treatment (Figure 8A;  $F(2,83)=54.29$ ,  $p<0.0001$ ). The smallest plants were found in the lowest temperature, the largest plants in the intermediate temperature, and the plants in the warmest temperature being between those two. Aphid relative fecundity was also different between the three temperature treatments with higher aphid performance as temperature increased (Figure 8B;  $F(2,83)=47.74$ ,  $p<0.0001$ ). Relative fecundity in each treatment was different from each other with aphid

relative fecundity at 32C more than twice of the relative fecundity at 16C. However, the pattern of treatment effects for aphids was not the same as it was for plants.



**Figure 8.** Average ( $\pm$  standard error) fava bean size before infestation (A) and pea aphid relative fecundity (B) in the baseline experiment (1), where both plants and aphids were exposed to different temperature treatments (16C, 24C, and 32C). The relative fecundity was calculated as the total number of offspring present on the plant at the end of the experiment divided by the total number of adults alive at the end of the experiment. The letters a, b, and c indicate significant differences between treatments (Tukey's HSD post hoc analysis ( $p < 0.05$ )).

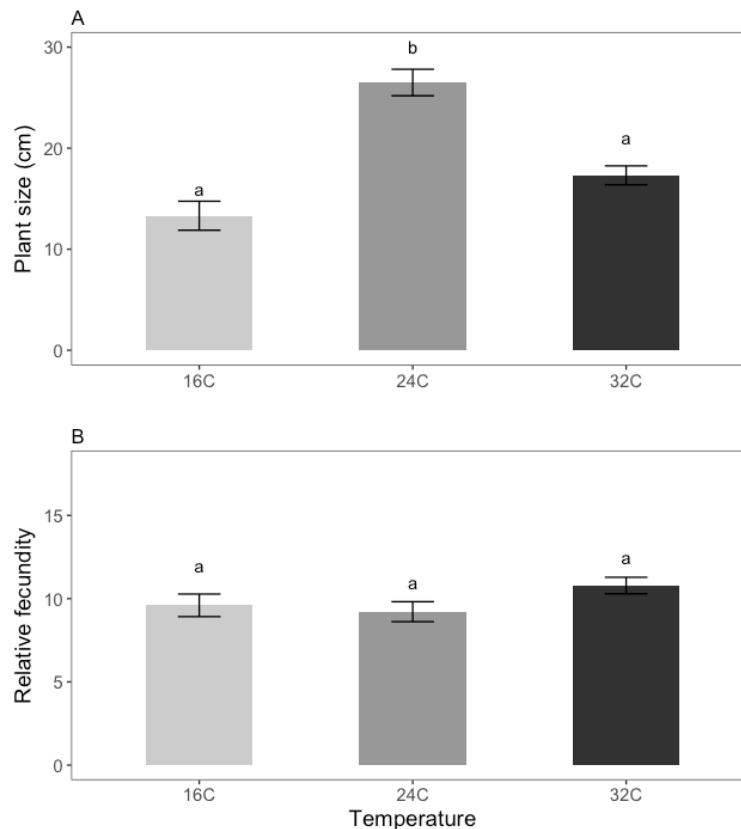
### Indirect experiment (2)

In the second experiment plants were reared under the three temperature treatments, however once infestation occurred, both plants and aphids were kept in a common environment so that aphids were never exposed to different temperatures.

Plant size again significantly varied with temperature treatment (Figure 9A;

$F(2,70)=32.49$ ,  $p < 0.0001$ ) in a similar pattern as the baseline experiment (1). Despite

the differences in what temperature plants were grown in and the subsequent difference in their size, aphid fecundity was fairly consistent and did not show evidence of differences across temperature treatments (Figure 9B;  $F(2,69)=1.505$ ,  $p=0.22$ ).



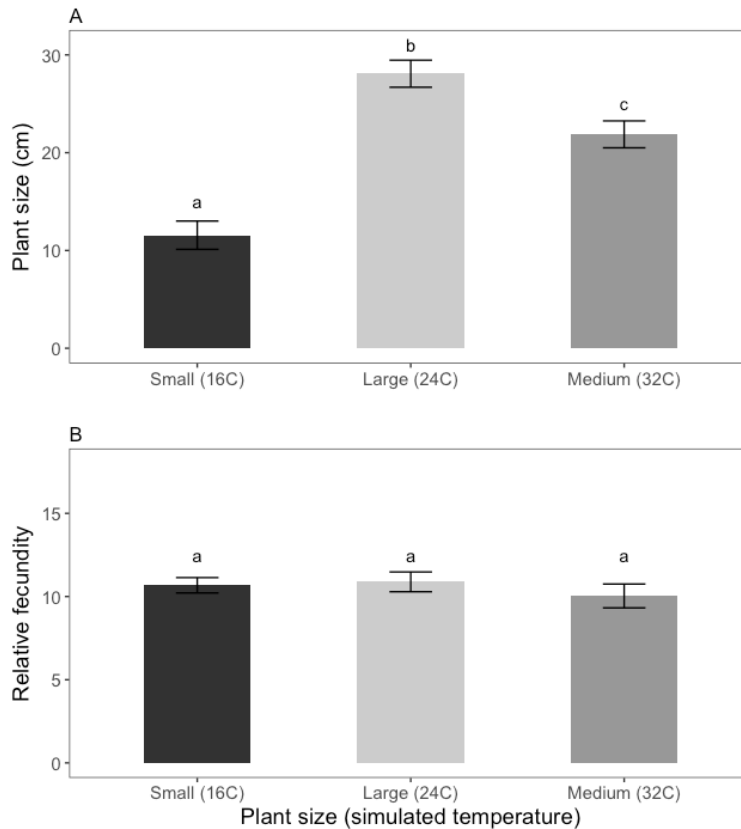
**Figure 9.** Average ( $\pm$  standard error) fava bean size before infestation (A) and pea aphid relative fecundity (B) in the indirect experiment (2), where plants but not aphids were exposed to different temperature treatments (16C, 24C, and 32C). The relative fecundity was calculated as the total number of offspring present on the plant at the end of the experiment divided by the total number of adults alive at the end of the experiment. The letters a and b indicate significant differences between treatments (Tukey's HSD post hoc analysis ( $p < 0.05$ )).

### Different size experiment (3)

The third experiment mimicked the plant size differences seen in the baseline (1) experiment by planting treatments at different times but kept both plants and aphids in the same common environment the entire time. Altering the planting time produced the intended differences in plant size (Figure 10A;  $F(2,60)=33.19$ ,  $p < 0.0001$ ), in the same



pattern as the baseline experiment (1) (Figure 8A). Despite these differences in plant size, aphid relative fecundity was similar across the three plant size treatments (Figure 10B;  $F(2,62)=0.53$ ,  $p=0.58$ ).

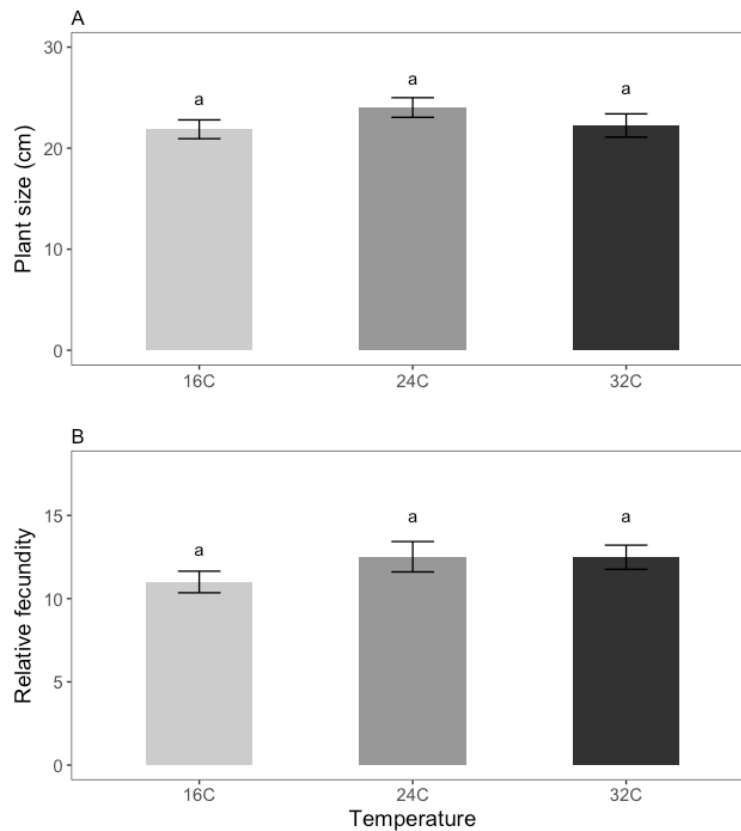


**Figure 10.** Average ( $\pm$  standard error) fava bean size before infestation (A) and pea aphid relative fecundity (B) in the different size experiment (3), where neither plants or aphids were exposed to different temperature treatments. Aphids were reared in plants with different size. The relative fecundity was calculated as the total number of offspring present on the plant at the end of the experiment divided by the total number of adults alive at the end of the experiment. The letters a, b, and c indicate significant differences between treatments (Tukey's HSD post hoc analysis ( $p<0.05$ )).

#### Same size experiment (4)

In the fourth experiment plants were grown in the different temperature treatments, but by planting them at different times they were intended to be the same size when aphids were added. Then both aphids and plants were kept in the common environment. By manipulating planting date, we were able to achieve the intended

similarity in plant sizes across temperature treatments (Figure 11A;  $F(2,57)=1.26$ ,  $p=0.29$ ). However, despite those plants being raised in different temperatures, aphids did not differ in their relative fecundity across treatments (Figure 11B;  $F(2,57)=1.27$ ,  $p=0.28$ ).

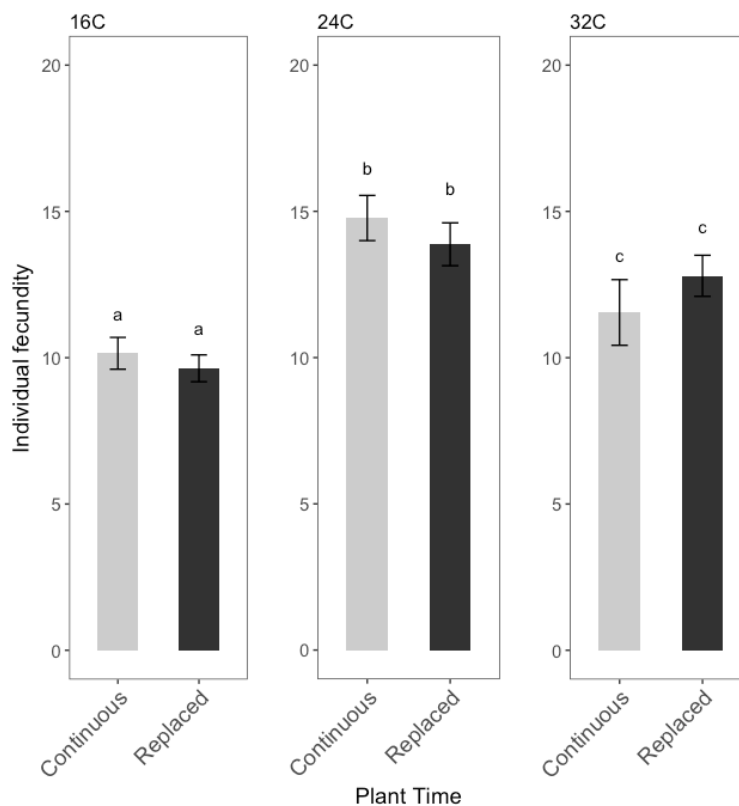


**Figure 11.** Average ( $\pm$  standard error) fava bean size before infestation (A) and pea aphid relative fecundity (B) in the same size experiment (4), where plants but not aphids were exposed to different temperature treatments (16C, 24C, and 32C). Plants had the same size before infestation. The relative fecundity was calculated as the total number of offspring present on the plant at the end of the experiment divided by the total number of adults alive at the end of the experiment. The letter a on all treatments indicates a lack of significant differences between treatments (Tukey's HSD post hoc analysis ( $p<0.05$ )).

### Plant time experiment (5)

This experiment had two factors, the temperature treatment where aphid-infested plants were kept and the length of time plants were kept in that temperature treatment.

We found that there was not a significant interaction between these two factors (Figure 12;  $F(2,124)=1.13$ ,  $p=0.326$ ). Relative aphid fecundity did vary across temperature treatments (Figure 12;  $F(2,124)=17.17$ ,  $p<0.001$ ). However, aphid fecundity did not seem to differ between the continuous (plants that were in the temperature treatments for 72 hours) and replaced (plants that were only exposed to temperature treatments for 24 hours at a time) treatments (Figure 12;  $F(1,124)=0.018$ ,  $p=0.895$ ).



**Figure 12.** Aphid individual fecundity (average  $\pm$  standard error) across the three temperature treatments and the two plant time treatments (continuous and replaced). Fecundity was significantly different between temperature treatments, but not between plant time treatments. The letters a, b, and c indicate significant differences between treatments (Tukey's HSD post hoc analysis ( $p<0.05$ )).

## Discussion

The goal of our experiments was to tease apart direct effects of temperature on pea aphids from possible host plant-mediated indirect effects in a laboratory model system. The first experiment gave a fairly typical baseline where both plants and aphids were exposed to different temperatures (Table 1) and we found that both plant size and aphid fecundity varied with temperature (Figure 8). In the second experiment, plants were grown in different temperatures but then moved to a common environment when aphids were added and then kept in that common environment (Table 1). This should have eliminated direct effects of temperature on the aphids, leaving any observed differences in aphid fecundity to be caused by indirect effects due to the plants previous exposure to different temperatures. While plant size was again different with temperature, aphid fecundity did not vary with treatment (Figure 9). Comparing these two experiments was our first indication of the relative importance of direct effects compared to indirect effects for this type of experiment. The third and fourth experiment explored if these temperature-generated differences in plant size might be important for aphid fecundity. Specifically, they looked for potential effects of plant size compared to other possible plant-mediated effects, with neither finding differences in aphid performance. Our last experiment again exposed aphids to different temperatures and again found differences in performance. However, we did not see any differences in aphid performance when the aphid's host plant was exposed to different temperatures for different periods of time. This again points to the relative importance of direct effects compared to any possible plant-mediated indirect effects for these particular types of experiments in this system.

The baseline experiment (1) results are similar to previous research on the effects of temperature on plant-herbivore systems (Stinner et al., 1974), and on pea aphid fecundity in particular (Bieri et al., 1983; Morgan et al., 2001). Temperature has also previously been shown to also affect plant growth in *Vicia faba* (Catt and Paull, 2017; Confalone et al., 2010, 2011; Yusoff et al., 2013) as well as germination time (Rowland and Gusta, 1977), as we saw in the baseline experiment (1).

In our experimental setting, we thought that differences in plant size might influence pea aphids. Previous research indicates that plant size can influence aphid biology (Dixon, 1985; Guldmond et al., 2003; Leather and Dixon, 1984), especially with cereal aphids (Girma et al., 1990; Hein, 1992; Kieckhefer and Gellner, 1988; Leather and Dixon, 1981; Watt, 1979; Watt and Dixon, 1981). However, this research tends to focus on large differences in plant growth stage such as among the vegetative growth, flowering, and budding phases (Velde et al., 2017). In our case, the plants were all relatively young and still in vegetative growth. Therefore, even though there were clear size differences between them, it may not have been enough to cause a discernable effect on pea aphids. Experiments over much longer time scales, particularly those that use plants across vegetative and reproductive stages might see larger differences in plant size causing indirect effects of temperature on aphids.

Although we failed to see any measured indirect effects of temperature, our system and experimental design might not have allowed us to capture all possible effects. For example, systems where plant phytochemistry is susceptible to temperature (altering plant nutritional quality or plant defense) could produce indirect effects on the herbivores (Kollberg et al., 2015; Puentes et al., 2015). Another possible set of indirect

effects might only occur when temperatures vary while the aphid is feeding on the plant. For example, if plant sap density is rapidly affected by temperature, the moment we change the temperature, the sap density would change too. A promising but challenging experimental design would be to have plants exposed to the three temperature treatments, and aphids encased in a thermal insulation receptacle that still allows it to feed on those plants while exposed to a common temperature.

The temperature treatments we used mimic similar experiments with the pea aphid-fava bean system (Bieri et al., 1983; Campbell and Mackauer, 1975; Stacey and Fellowes, 2002). However, researchers are increasingly investigating temperature by incorporating additional complicating aspects such as fluctuating temperatures or extreme events, and using longer experiments (Bannerman and Roitberg, 2014; Bannerman et al., 2011). These experiments may introduce other indirect effects of temperature, including potential mismatches in the phenology between plants and herbivores (Kharouba et al., 2015), which are generally caused by differences in how much the growth rate of insects and plants respond to temperature (Bale et al., 2002; Berg et al., 2009; Morison and Lawlor, 1999; Veteli et al., 2002). Incorporating fluctuating temperature into temperature experiments could produce different direct and indirect effects since fluctuating temperature affects plants (Arnold et al., 1988; Dale, 1964; Hedhly, 2011; Thompson, 1974; Thompson and Grime, 1983; Thompson et al., 1977), herbivores (Auad et al., 2015; Davis et al., 2006; Mironidis and Savopoulou-Soultani, 2008; Sostak, 2015), and plant-herbivore trophic interactions (O'Connor, 2009).

The reported patterns of aphid performance across treatments were not always consistent among our experiments. Specifically, we saw that in the baseline experiment (1), aphid fecundity at 32C was higher than 24C (Figure 8B), however aphids at 32C in the plant time experiment (5) had lower fecundity at 32C compared to 24C (Figure 12). This was likely due to seemingly minor differences in the exposure period of aphids between the two experiments (first two days in experiment one versus second and third day of exposure in experiment 5) and the decreasing performance of aphids when continually exposed to 32C (see chapter 2: warm experiment). In fact, the first day of exposure in experiment 5 (not used in analysis as it was before any plants were replaced) did show the same pattern as experiment 1 (daily individual fecundity  $\pm$  standard error; 32C:  $10.38 \pm 0.78$  and 24C:  $7.89 \pm 0.77$ ), whereas by day 3 the pattern was reversed (32C:  $4.52 \pm 0.51$  and 24C:  $7.68 \pm 0.55$ ). The aphid density (4 aphids in experiment 1 versus 1 aphid in experiment 5) feeding on the host plant might also play a role in this system, which would also be worth exploring in the future.

In conclusion, for this system there seems to be little evidence of indirect temperature effects on herbivores through their host plants. It is not our intention to state that indirect temperature effects not possible. However, for the methods and study organisms we used (Bieri et al., 1983; Blanford et al., 2002; Morgan et al., 2001; Stacey et al., 2003), indirect effects seem to be overshadowed by the direct effects that temperature has on herbivores. While temperature can influence organisms and their interactions through many different mechanisms, this study suggests there can be large differences in which of these mechanisms is more important. Understanding when these

different possible mechanisms are important will be a crucial next step in accurately predicting how organisms respond to their changing environment.

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## CONCLUSIONS

The main goal of this thesis is to explore the ways that temperature can alter herbivores, as well as their interactions with host plants and natural enemies. The experiments we performed build from the large foundation of information we have regarding pea aphid biology and ecology. Pea aphids are widely used as a model organism for genetic, developmental, evolutionary, ecological, and thermal studies (Brisson and Stern, 2006). However, pea aphids are also important pests on many crops around the world (van Emden and Harrington, 2017). This makes their study useful not only to increase our understanding of ecology and temperature effects, but also to better control and predict their damage to crops.

In the first chapter, we hypothesized that the timing between a heat shock and a host-parasitoid interaction would be important since heat shock effects may influence the insects differently depending if it occurs before, during, or after the host-parasitoid interaction. We showed that the effect of a heat shock is more detrimental for the parasitoid wasps (*Aphidius ervi*) when the heat shock occurred while the wasps were actively foraging on the pea aphids (during). The heat shock had no effect on wasp performance when it happened before or after the host-parasitoid interaction. We thought that the heat shock could have an effect when it occurred after the wasp foraging since the heat shock is affecting aphids that are already stressed by the parasitoid larva inside their body (Jerbi-Elayed et al., 2015). However, this was not the case. It is possible that the time frame we used (heat shock three days after the interaction) was not enough for the wasp eggs to hatch, meaning we heat shocked parasitoid eggs instead of larvae (Martinez et al., 2016). It would be interesting to repeat

this experiment with a different time frame. We could use a later heat shock which would have affected an older developing wasp larva. Another option could be to use a host with a longer lifespan, which would allow us for larger opportunities to implement a similar methodology.

Since the heat shock had a significant negative effect on the wasps when it occurred while the wasps were foraging on the aphids, we wanted to further explore the causes of this effect. We performed a behavioral experiment where we exposed wasps and aphids to a heat shock in a way that we could record their behavior. We saw that pea aphids exposed to a heat shock spent 32% more time moving compared to control aphids, which is similar to other changes in movement from heat shocks seen in this system (Kopco, 2017). This fact alone could make it harder for the wasps to properly forage (Rehman and Powell, 2010). However, it seems likely that the differences we found were influenced by the large effect the heat shock had by inducing signs of heat stupor in the majority of wasps during the behavioral observations.

In conclusion, our results show that timing of discrete abiotic events such as heat shocks is an important factor to take into account. Our results appear to be driven by heat shocks having stronger direct effects on adult parasitoids while foraging than at other times. This could mean that heat shocks only impact a small percentage of parasitoids. A similar experiment with populations instead of individuals would be an interesting next step for this project.

In the second chapter, we compared pea aphid population size under constant and fluctuating temperatures, and we repeated that comparison over a cool (20C) and a warm (28C) temperature range. In the cool experiment we found that constant and

fluctuating temperatures produced populations of similar size. The fluctuating temperature profiles switched from 16C to 24C each day, during the nine-day experiment. However, the population size was the same as the aphids exposed to a constant 20C for nine days. In this case, temperature variation (compared to treatments with no variation) had no effect on the pea aphids, similar to what others have found (Auad et al., 2015; Behrens et al., 1983). When we performed the same experiment at a warmer range, we saw that the fluctuating profile had strong and negative effect on pea aphid population size. The fluctuating temperature switched from 24C to 32C each day. It seems that 32C is highly detrimental to aphid populations when aphids are kept at that temperature constantly, however pea aphids may be able to handle this high temperature if they can recover in milder temperatures, as suggested by others (Ma et al., 2015). One factor that I would like to further test in this chapter is the population structure of the pea aphids under different temperature treatments. In the other chapters we studied individuals (chapter 1) or very small populations over short periods of time (chapter 3). However, in this chapter, populations had 9 days to grow, which could be enough time to allow for changes in the demographic structure of populations.

In conclusion, we found that temperature mean and variation interacted such that fluctuating temperatures were detrimental to aphids only in the warmer range. Climate change scenarios predict both increases in climate warming (increases in average temperature) and climate variability (increases in temperature variation), which suggests that pea aphids will be strongly affected by climate change.

In the third chapter, we focused on the indirect effects temperature can have on herbivores through their host plants by conducting five complementary experiments. In

the first and second experiment we reared plants and aphids in growth chambers at three temperature treatments (16C, 24C, and 32C), however in the first one aphids were exposed to those temperatures, and in the second we took out the plants and infested them with aphids in the same temperature. This should have eliminated direct effects of temperature on the aphids, leaving any observed differences in aphid fecundity to be caused by indirect effects due to the plants' previous exposure to different temperatures. We found large differences in aphid fecundity in the first experiment, however there were no differences in the second. This was our first indication of the relative importance of direct effects compared to indirect effects for this type of experiment. The third and fourth experiment explored if differences in plant size might be important for aphid fecundity. However, we did not find any differences in aphid performance. In the fifth experiment, we modified the time that plants were exposed to different temperatures, while keeping the time that aphids were exposed to those temperatures constant. We did not see any differences in aphid performance when the aphid's host plant was exposed to different temperatures for different periods of time. These results suggest little to no noticeable indirect effects of temperature on pea aphids mediated by their host plants, even when controlling for temperature-caused differences in plant size. One weakness of this set of experiments was its short duration. We measured aphid reproduction over only two days and did not account for possible effects that may have arisen later in life. Another potential factor to address in the future could be that our system and experimental design might not have allowed us to capture all possible effects of temperature on plants, such as changes in plant phytochemistry (Kollberg et al., 2015; Puentes et al., 2015). It is also possible that some

indirect effects might only occur when temperatures vary while the aphids are feeding. An interesting experimental design to further explore the indirect effects of temperature on herbivores would be to expose host plants to the three temperature treatments and then be able to have aphids encased in a thermal insulation receptacle that still allows them to feed on those plants.

In conclusion, for the pea aphid-fava bean system there is no evidence of indirect temperature effects on the pea aphids through their host plants. Our goal is not to generalize this and state that indirect temperature effects are non-existent. However, the indirect effects can be largely overshadowed by the direct effects that temperature has on pea aphids. This is especially relevant for studies that use similar methodologies as ours (Bieri et al., 1983; Blanford et al., 2002; Morgan et al., 2001; Stacey et al., 2003).

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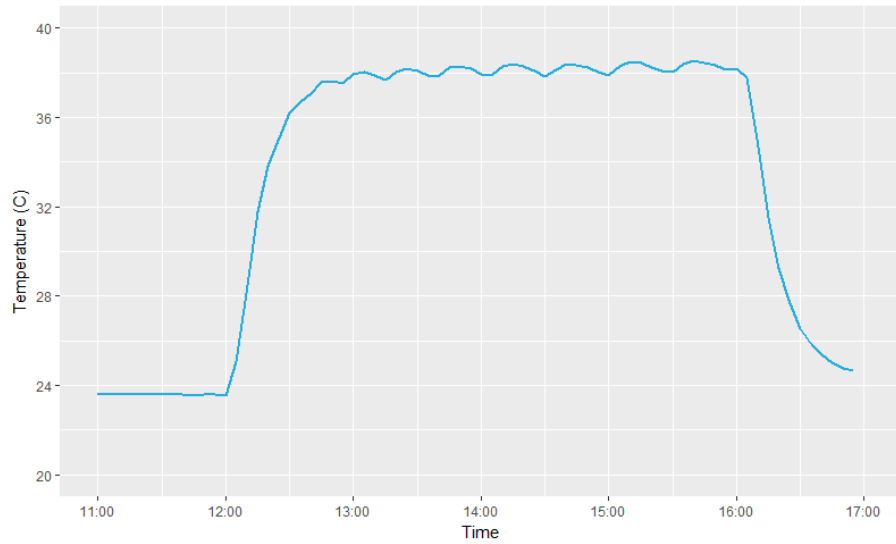
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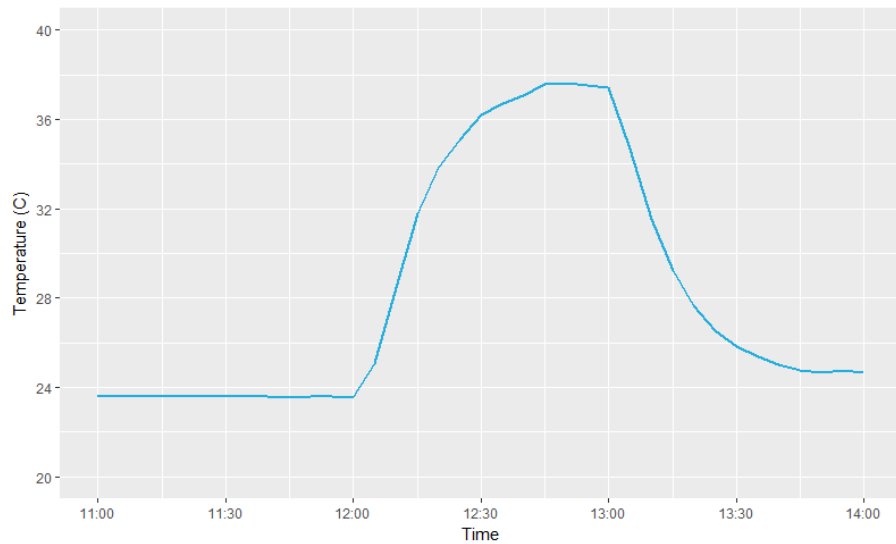
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## APPENDIX



**Figure A1.** Temperature profile of the heat shock experienced by wasps and aphids in experiment 1. Although this temperature is recorded under the cage where the plant is located at, there are likely microclimatic differences between the top and bottom of the plant, or between upper and bottom part of the leaves.



**Figure A2.** Temperature profile of the heat shock experienced by wasps and aphids in experiment 2.

**Table A1.** Behavior categories that wasps showed on experiment 4.

<b>Behavior</b>	<b>Description</b>
Flying	Wasp not in contact with any surface
Hiding	Wasp under the leaf
Still	Wasp not moving, resting in a natural position
Walking	Wasp walking around
Stumbling	Wasp moving its legs but not walking
Foraging	Wasp antenating a pea aphid
Stinging	Wasp attempting to sting or probe a pea aphid
Inactive	Wasp showing a lack of movement of their legs and antennas and wings not in their natural resting position.